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In-vitro allergy diagnostics

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Abstract:In vitro allergy diagnostics is rapidly advancing. This is primarily due to the development of component-based diagnostic tools. The availability of allergen components now allows a more precise and patient-tailored diagnostics, which has implications for therapeutic strategies including decision about specific immunotherapy. Furthermore, differential diagnostics of food intolerances and food allergies is also advancing because of this novel test. Another area of advancement is cellular diagnostics, which is primarily based on basophile activation tests. These recent developments will be discussed in this article.

Keywords: allergy diagnostics; basophile activation tests; cellular diagnostics; component-based diagnostics; food allergies; food intolerances.

Molecular, component-based allergy diagnostics

Allergic diseases are on the rise. They manifest themselves at the interfaces of the organism to the environment, thus:

- Skin (eczema, atopic eczema)
- Upper respiratory tract (allergic rhinitis, "hay fever"),
- Lower respiratory tract (asthma),
- Gastrointestinal tract (food allergies).

The focus of allergy diagnostics continues to be the measurement of allergen-specific IgE-antibodies. These antibodies are produced on the basis of a complex gene-environment interaction. Allergic diseases, as complex, chronic inflammatory diseases (such as other chronic inflammatory diseases as well), have a genetic component. This involves multigenic diseases in which polymorphisms are detected in a large number of genes. The affected genes encode primarily molecules of the immune system, structural elements of the organs concerned, barrier functions, etc. Each disease, and in turn each patient (inter-individual heterogeneity), exhibits its own genetic pattern.

Based on the genetics, this requires exposure to certain environmental factors to bring the disease to clinically manifest itself. The Western, industrialized lifestyle seems to be of particular significance. Factors in this environment include nutrition, exposure to microbes ("hygiene hypothesis"), stress factors and others. As a result of this complex gene-environment interaction, the patient's immune system is dysregulated insofar as exposure to harmless environmental antigens triggers and perpetuates a chronic inflammatory immune response, at the center of which are so-called Th2-T cells that regulate, via their cytokine production, the effector phase of allergic inflammation (Figure 1). A significant consequence is the production of (allergen-specific) IgE antibodies.

Of great importance is the distinction between so-called *sensitization* and a *clinically relevant allergy*. Sensitization is the development of a specific immune response and IgE production to an allergen. It is the responsibility of the clinician to examine to what extent the proven IgE antibodies are responsible for the clinical responses of patients. This usually requires further diagnostic measures, such as an allergen provocation on the end organ, elimination diets in cases of suspected food allergy, etc. *In no case will a diagnosis of allergy be made on the sole evidence of specific IgE!*

For the detection of specific IgE antibodies, there are different test procedures available. Essentially, today these test methods differ in whether the allergens in the test system occur as carrier-bound or in liquid form. There are commercial test systems for both approaches.

Furthermore, a distinction must be made between tests with single allergens and tests with allergen mixtures. As for single allergens, one must then differentiate further for native allergens used in the test system. Generally, these involve protein mixtures that are more or less

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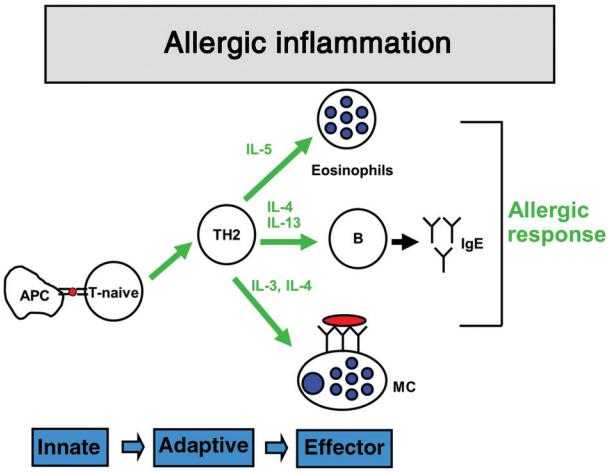

Figure 1: Regulation of the allergic inflammatory response and effector response via the formation of TH2-T-cells, which in turn control eosinophilia, mast cell activation and IgE production as part of the inflammatory response.

well characterized and purified, and the use of individual components of these allergens. Such components may be purified (in which case, they also contain the carbohydrate portion), or they are produced in recombinant form (without carbohydrate).

In the era of *molecular allergology,* researchers in the past two decades have succeeded in largely characterizing the allergens at the molecular and genetic level (at least concerning the most important allergens). This has made clear that individual allergens, such as birch, hazelnut, peanut, mite or grass pollen, each represent allergen mixtures. They are composed of a variable number of protein components. These components have a greater or lesser significance for triggering allergic reactions.

We are talking about *major allergens* if at least 50% of the affected patients respond to this component (e.g. Bet v 1). The other group consists of *minor allergens.* Minor allergens are components in which less than 15% of patients exhibit a clinical response. Nevertheless, such a clinical response may be quite violent in nature (e.g. Bet v 2).

Many of these individual components have been shown to have phylogenetic relationships among each other. Such components that can be allocated to a protein family and can be detected at various allergen sources with high homology are then responsible for clinically relevant *cross-reactions.* On the other hand there are also allergen-specific components. If a sensitization is detected here, it is a reaction specific to this particular allergen.

Table 1 summarizes important and clinically relevant protein families, which will be discussed in detail by way of selected examples [1, 2].

Table 1: Major and clinically relevant protein families.

Profilins
PR-10
Lipid transfer protein
Storage proteins
Tropomyosins
Parvalbumins

Profilins

Profilins are widespread in the plant kingdom. Sensitization is encountered frequently, but very rarely with clinical relevance. Prominent examples are the birch pollen allergen Bet v 2 or the latex allergen Hev b 8 [1].

Storage proteins

Storage proteins are at the other end of the spectrum of clinical relevance. These are major allergens in leguminous plants, seeds, and other plants. They often make up the largest protein share of these allergens. They are heat-stable and therefore also reactive even when cooked. Furthermore, they are often resistant to enzymatic degradation, and even low exposure levels trigger reactions. The most common and most important group are 2S albumins.

From this it is already clear that the storage proteins can be divided into several subfamilies, namely 2S, 7S and 11S globulins. Table 2 summarizes the current state of knowledge and distribution for various allergens:

An important example of clinical relevance is the *peanut* [3–9]. The peanut is made up of a large number of components that each belong to different protein families. Ara h 1, Ara h 2, Ara h 3, Ara h 4 and Ara h 6 are storage proteins.

Which diagnostic procedure has established itself in recent times?

First, it has become clear that the detection of IgE reactivity of storage proteins is of high diagnostic importance. In particular, the component Ara h 2 bears mentioning in this context. The isolated positivity at Ara h 8 (AMPR10 protein) is also of interest. This seems, when looked at in isolation, to be associated with tolerance to peanuts. A diagnostic algorithm has been proposed by Dang et al. [3].

In another study, over 200 children have been evaluated, half of whom tested positive for peanut allergy in double-blind, placebo-controlled food challenge tests, while the other half was peanut-tolerant. The best

Table 2: Key storage proteins.

	2S albumine	7S globulins	11S globulins
Peanut (Arachis hypogaea)	Ara h 2 Ara h 6	Ara h 1	Ara h 3 Ara h 4
Soy (Glycine max)		Gly m 5	Gly m 6
Hazelnut (Corylus avellana)	Cor a 14	Cor a 11	Cor a 9
Walnut (Juglans regia)	Jug r 1	Jug r 2	Jug r 4
Brazil nut (Bertolletia excelsa)	Ber e 1		
Buckwheat (Fagopyrum esculentum)	Fag e 2		
Sesame (Sesamum Indicum)	Ses i 1		

diagnostic sensitivity and specificity were obtained with the following diagnostic algorithm:

- First, subjects are tested with a peanut extract. When the IgE for the peanut extract is greater than 15 kU/L, then
- A test with recombinant Ara h 2 is done. If this is positive, the diagnosis of peanut allergy can be made without the need for a challenge test. This diagnostic algorithm – at least in this study – does not require 2/3 of the necessary challenge tests.

Soy allergy

Another important, clinically-relevant example of the relevance of storage proteins is soy allergy [10, 11]. The components Gly m 4, Gly m 5 and Gly m 6 play important roles here, with Gly m 5 and Gly m 6 being storage proteins. Gly m 4-sensitization can be found mainly in the context of cross-reactivity between pollen and soy. This can be attributed to cross-reactivity with Bet v 1 (a PR10 protein).

Gly m 5 and Gly m 6 have a high positive predictive value for the diagnosis of soy allergy.

From this, the following mapping for the clinical significance of soy allergen components has now emerged (Figure 2):

- Gly m 5 and Gly m 6: In particular, severe reactions in the gastrointestinal tract, as well as urticaria, angioedema and anaphylaxis,
- Gly m 4 as a PR10 protein as part of the oral allergy syndrome in cross-reactions between pollen and food,
- Gly m 1 and Gly m 2: Possible significance in bronchial asthma as an inhalant allergen.

Hazelnut allergy

Progress has also been made in the diagnosis of hazelnut allergy [12, 13]. The components Cor a 9 and Cor a 14 are storage proteins and are closely associated with severe reactions, as well as genuine primary hazelnut allergy. By contrast, the Cor a 1 component, which is a PR10 protein, is closely related to the birch pollen allergen Bet v 1 and thus of importance to the oral allergy syndrome (cross-reactivity between pollen and food). Sensitization to Cor a 8 is rarely observed.

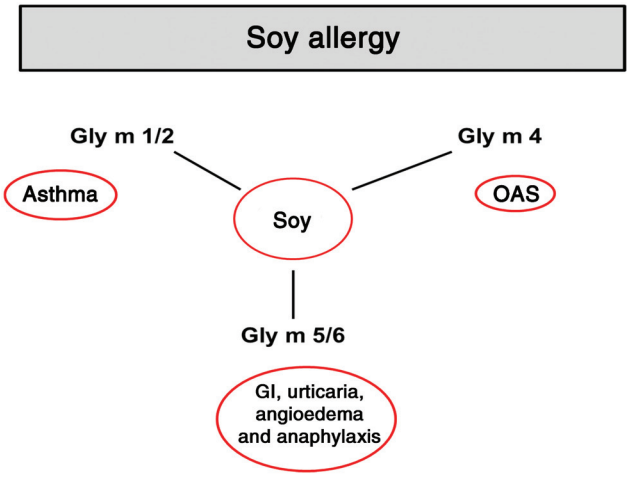

Figure 2: Role of the components Gly m 4, Gly m 5 and Gly m 6 in connection with diagnosing soy allergy.

The PR10 protein family (pathogenesis-related proteins)

PR10 proteins are also very common in nature. Unlike storage proteins, they are heat-sensitive, so they are generally tolerated even when cooked or baked. These types of sensitization are frequently associated with the oral allergy syndrome, that is, with mild local reactions, as are commonly found in fruit and vegetable pollen syndromes, especially in central and northern Europe. Figure 3 summarizes this again in a table.

Protein family: Pathogenesis-related Protein family 10 (PR-10)	
Birch (Bet v 1) Peanut (Ara h 8) Soy (Gly m 4) Hazelnut (Cora 1) Apple (Mal d 1) Kiwi (Act d 8) Peach (Prup 1) Carrot (Dau c 1)	• Heat-sensitive • Tolerance when cooked • Oral allergy syndrome (OAS) • Fruits/vegetables in Northern Europe

Figure 3: Important characteristics and examples of the PR10 protein family.

The problem of cross-reactive carbohydrate determinants (CCD)

Allergens and allergen components in the native state are usually glycosylated proteins. Short sugar chains attach to these proteins. Such short sugar chains often contain only one IgE-binding epitope. In a patient who forms IgE antibodies against these CCD determinants, these IgE antibodies do bind in the in-vitro-assay system, but in the biological reality, there is no cross-linking on the surface of IgE-receptor-bearing effector cells (e.g. mast cells and basophils), and thus no activation and mediator release from those cells either. Therefore, clinical relevance is extremely low, and clinical symptoms with such IgE positivity are very rare.

Examples of this are bromelain, which occurs in pineapples and other fruits, for example, and horseradish peroxidase. Commercial test systems are available today to detect IgE antibodies against CCD determinants.

The diagnosis of CCD IgE is significant, for example, in the case of *bee and wasp venom allergy* [14–18]. Here, two different diagnostic algorithms can be distinguished:

Initial testing with native bee and wasp venom extract. If the test of one of the two extracts is positive, one may assume specific sensitization. If, however, both tests are positive, the question arises whether the patient has a true double sensitization to bees and wasps, or whether this is a non-specific reaction (to CCD epitopes). For the proteins contained in the native toxins have precisely such CCD-relevant sugar side chains. In this case, the patient is tested for CCD IgE in a second step in order to differentiate between cross-reactivity (non-specific sensitization) and genuine cross-sensitization.

Alternatively, today it is also possible to start the test with recombinant allergens that do not have these sugar side chains. The appropriate diagnostic algorithm has been shown.

It must also be noted in this context that extract tests do not identify all allergy sufferers, particularly in the case of wasp allergy. This has been demonstrated in a recent publication. Only once recombinant Ves v 5 had been added was it possible to differentiate the population further that had tested negative in the extract test. The reason for this is likely that the component Ves v 5 occurs only in very low concentrations in the natural extract. Therefore, the question arises whether the natural extract can be spiked with recombinant Ves v 5.

This plays a role in certain allergies to red meat. It has been shown that after tick bites IgE antibodies to alpha-GAL (galactose-alpha-1,3-galactose) can be detected. Alpha-GAL is found in the gastrointestinal tract of the tick. But alpha-GAL is also part of gelatin (from bovine collagen). Patients who are allergic to gelatin generally react also to red meat if the gelatin allergy is due to alpha-GAL. This connection seems increasingly to be of clinical importance [19, 20].

The protein family of tropomyosins

Tropomyosins are actin-binding proteins occurring primarily in muscle cells. There is cross-reactivity between dust mites, crustaceans and shellfish (Figure 4).

This is of particular relevance because a subset of patients with dust mite allergy also react with IgE to the component Der p 10. Der p 10 is the tropomyosin of the mite, and responsible for often severe clinical reactions to shellfish and crustaceans in dust mite allergy sufferers.

Protein family: tropomyosin	
Dust mite (Der p 10) Anisakis (Ani s 3) Shrimp (Pen a 1)	Actin-binding proteins in the muscle cell Cross-reactivity between • Crustaceans and shellfish • Dust mites • Cockroaches • Nematodes

Figure 4: The protein family of tropomyosins – properties and occurrence.

Animal allergens

Sensitization and allergies to animals occur with increasing frequency [21]. Prominent examples include cat allergy (caused by the secretoglobin Fel d 1 from cat saliva), as well as horse and dog allergies. Allergies to mice (and rats) are significant in people exposed for professional reasons (e.g. animal caretakers, etc.). In this case, it is often lipocalins that are responsible for the respective reactivities.

The clinical relevance of such sensitization is well known especially in patients with asthma. This has to do with the fact that animal allergens are often inhaled. A recent study looked at school children with severe asthma. It showed that children with multi-sensitization to animal proteins suffer more severe forms of the condition than children who are oligo-sensitized or not sensitized at all to animal hair (Table 3).

Food intolerance – food allergy

Clinical reactions to food are a common clinical problem. Various pathogenetic mechanisms and principles can be causally responsible in this context. Current differential-diagnostic algorithms distinguish between immunological and non-immunological reactions. Non-immunological reactions may be enzyme defects in the lactase gene, which lead to lactose intolerance.

Immunological reactions may be allergic or non-allergic in origin. Non-allergic reactions include autoimmune reactions, such as celiac disease with gluten sensitization.

Wheat components also play a key role in this context [22, 23]. Recently, a new syndrome has been described in this field – wheat dependent exercise induced anaphylaxis (WDEIA), that is, the wheat-dependent, exercise-induced anaphylaxis syndrome. This is found in patients who develop anaphylaxis in the case of a temporal proximity between the consumption of wheat-containing foods and physical activity. Only in this temporal context is omega-5 gliadin IgE reactivity clinically relevant. As to how to explain this pathogenetically, this still remains open and is the subject of further studies. However, the WDEIA syndrome appears to be on the rise in our latitudes. It is based on an IgE reactivity to omega-5 gliadin (Tri a 19).

In contrast, IgE reactivity to Tri a 27 and 28 is significant in flour dust inhalation, such as with "baker's asthma", an occupational disease.

More information about the details of the biochemistry, pathophysiology and clinical relevance of different allergen components – including rare allergens – was recently published in a consensus paper, under the auspices of the World Allergy Association (WAO). It is available as an Open Access Document.

From this, one can develop a "Top 10" list of the most important component-based allergens associated with a severe clinical progression and high risk potential.

Table 3: Pet allergens.

Lipocalins	Can g 1,2	(Dog)
	Fel d 4	(Cat)
	Equ c 1	(Horse)
	Mus m 1	(Mouse)
Secretoglobins	Fel d 1	(Cat)
Kallikreins	Can g 5	(Dog)

The allergy-protein array

An allergen microchip that probes the most extensive spectrum of allergen components is in increasing use. This represents the arrival of protein-array diagnostics in everyday laboratory medicine. In its most current version, 112 component of 51 different allergen sources are applied to this array. Each of these components is analyzed in triplicate in order to optimize the quality of the results produced. There are also positive controls and standards. The result is reported in semiquantitative form, using ISU international standardized units (ISU). However, these units are not identical to the International Units, calibrated against a WHO standard, which are routinely used in single-component analysis today. But it has been shown that for many – but not for all by far – components, there is a close correlation between ISU and IU. Deviations can be found especially in the low concentration range of specific IgE antibodies. Discrepant results are most frequent with sensitization ≤ 1 IU specific IgE.

Nevertheless, this protein array helps especially patients with difficult histories or diverse sensitization (so-called "polysensitized patients"), and in other special cases of allergological clarification. Using such a multi-parameter analysis yields ever-new insights into the ontogenesis of allergic sensitization in patients. Thus, several studies have shown that IgE antibodies against certain individual components can be detected some years before the onset of a clinically manifest allergy. An Italian study as part of a retrospective analysis has demonstrated that grass pollen allergy sufferers exhibit, in particular, sensitization to Phl p 1, Phl p

2 and Phl p 4 years before the onset of clinical symptoms. This is consistent with another study whose data already cover 18 life years (also a retrospective analysis). This study has identified IgE reactivity before the onset of disease especially in the case of pollen, animal hair, peanut, soy and fish.

Cellular tests

Cellular tests play an increasing role in (extended) allergy diagnostics. They are used for patients with discrepant results between case history, skin test and specific IgE diagnostics, as well as for patients with special issues, such as drug intolerance and drug allergies. But cellular test systems also constitute an effective alternative when it comes to exotic allergens that are not available in the regular panel of in-vitro diagnostics.

From a historical perspective, histamine measurement and/or histamine release following stimulation of effector cells was the first step towards routine cellular testing. However, the measurement of histamine or its degradation products poses an analytical challenge, due to low concentration levels and rapid metabolization.

Leukotriene-release assays are another step forward. The principle of these tests is based on a two-stage system. First, patient cells are incubated with the appropriate allergens or drugs. This is then followed by the measurement of the mediator by means of (manual and complicated) ELISA technology.

Flow cytometric tests now offer an alternative [24–39].

The focus of interest is on basophils. Basophils express the high-affinity IgE receptor (as mast cells do), and can thus be put to mediator degranulation via IgE-mediated cross-linking. At the same time, these cells can be put to degranulation and mediator release also via non-IgE-mediated mechanisms (C5a binding, FNLP-mediated activation, etc.). Thus, basophils behave, at least with regard to these aspects, very similarly to mast cells, as important effectors of allergic reactions. Today, it is the patient's whole blood (50 µL per test is sufficient) that is used. Further enrichment of the cells is not necessary, since the basophils are then identified using flow cytometry by way of corresponding markers on the surface. Here, the cells can be identified via the expression of CD123 (IL-3 receptor), as well as the characteristic positioning in the forward and side-scatter. Furthermore, antibodies against CCR3 instead of CD123 can also be used.

The expression of CD63 is measured to determine activation. Physiologically, CD63 is localized intracellularly, and is expressed only after activation/degranulation on the surface. In most cases, the CD63 expression correlates with the release of histamine [24]. Apart from a small basal CD63 expression, one sometimes also finds a bimodal curve following activation. This indicates that two populations of mast cells are present in the peripheral blood of this patient, that is, cells that react particularly strongly and have a very high CD63 expression, and a second population with weak reactivity. However, this is only of secondary importance for the clinical interpretation of the test result, because these tests serve above all to examine whether the relevant allergen/antigen leads to cell activation at all.

To bring basophil activation in full swing, it requires the additional exposure to IL-3. Commercial assays, therefore, include IL-3. But IL-3 also causes the upregulation of CD69 and of CD203c. In other words, if the patient already has elevated expressions of CD69 and/or CD203 on his/her basophils, this indicates previous in-vivo exposure to IL-3 (commonly associated with a prior mast-cell/basophil activation).

The basophil activation test (BAT) is used in many ways, both in research and routine laboratory testing [25–38].

An important practical point is the question of drug allergies/intolerance. Clinical routine practice, thus, distinguishes three main groups of drugs (Figure 4):

– Beta-lactam antibiotics

These are the only drugs in which IgE antibodies can often be detected. For discrepant results between skin test, IgE and patient history, one would then run a BAT as part of a second step.

– Neuromuscular blocking agents used in anesthesia

Generally, no IgE antibodies are found. The skin test with the corresponding drugs is the test of choice in diagnostics. If there are again discrepant findings between skin test, history and clinical exposure, a BAT is run as a second step.

– Nonsteroidal anti-inflammatory drugs (NSAID)

Only the skin and challenge tests are significant here; IgE diagnostics are negative and often unsuccessful, as is the BAT.

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