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Molecule-based diagnosis and allergen immunotherapy

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Summary

Allergen immunotherapy (AIT) represents the only way to modify the natural history of allergic diseases. Unfortunately, AIT is not always followed by a reduction in symptoms. The main reasons for such failure can be inadequate diagnosis and/or the poor treatment. In both cases, an incomplete or insufficient understanding of the component(s) responsible for the IgE sensitization on the one hand, and, on the other hand, the lack of a steady and reliable allergen mixture to be used for the desensitization process, could explain unsuccessful treatment. A more comprehensive IgE reactivity profile evaluation of the patient can be achieved by means of a molecule-based diagnostic approach, in order to distinguish genuine from panallergen-driven antigen recognition. At the same time, a better delineation of AIT products by means of molecular dissection, can allow a stronger correlation between diagnostic findings and immunotherapeutic intervention, thus facilitating the right prescription to the right patient

Introduction

Allergen immunotherapy (AIT) is the only treatment that can change the natural history of allergic disorders (1). For some allergens, an extremely high prevalence of efficacy is observed, such as in the case of Hymenoptera venom desensitization (2), but this is not always the case. Unfortunately, AIT sometimes fails and patients, despite years of expensive treatment, have no measurable benefit. The reasons for such a failure could be bad diagnosis and/or bad treatment.

The lessons learnt from the hundreds of studies performed in the last 15 years is that, in order to properly manage a patient, the appropriate treatment should be decided on and offered only after performing a comprehensive diagnostic workout. Currently, a valid diagnosis in allergic disease requires the demonstration of a genuine sensitization to a given biological source, before suggesting AIT. A recent report demonstrated how the molecular diagnostic approach could result in a marked change in about 50% of the cases of AIT prescription, in comparison to the "classical" diagnostic procedures (3).

On the other hand, a reliable AIT should be, as the name suggests, "allergen-specific", and, clearly, AIT effectiveness relies on the fact that all genuine causative allergen(s) should be equally and constantly represented within the treatment chosen for desensitization (4).

Bad diagnosis

The main purpose for an allergy specialist is undoubtedly to identify an accurate patient IgE reactivity profile. It is widely known that reactivity to a given biological source is

the consequence of IgE recognition of one or more proteins belonging to the allergenic extract used for the diagnosis.

There are at least two types of allergenic molecules accountable for positive extract-based tests. The first group includes molecules found only in a single biological source and not in other allergenic families, such as Phl p 1 from timothy grass (*Phleum pratense*) or Par j 2 from pellitory. Those proteins are considered "genuine molecules" since they can be considered as markers of true sensitization to a given biological source (5). In every single pollen family, as in the case of seasonal airborne allergens, the genuine molecules show marked structural identity. Consequently, the birch molecule Bet v 1 is associated with IgE recognition of virtually all the other molecules belonging to the *Fagales* order, such as alder pollen Aln g 1, beech Fag s 1, chestnut Cas s 1, hazel Cor a 1, hornbeam Car b 1, and oak Que a 1 (6). Similarly, in the case of timothy-grass Phl p 1, all β-expansin molecules belonging to grass pollen, such as *Cynodon dactylon* Cyn d 1 or *Lolium perenne* Lol p 1, are co-recognized (7).

The second group of molecules responsible for a positive extract-based test comprises several phylogenetically conserved proteins sharing homologous epitopes, which are thus involved in crossreactivity between different biological sources (8). These homologous molecules (also called panallergens) could therefore result in misleading interpretation of the allergic test (if it is carried out only with allergenic extracts). Theoretically, a patient could have positive extract-based tests to several allergenic extracts, only in one case due to IgE recognition of both genuine and homologous molecules, whilst, in the other cases, being a result of a panallergen IgE recognition in the absence of a genuine reactivity (Table 1).

Despite the high sequence homology observed among the constituents of each group of panallergens, the use of several representatives of homologous molecules for diagnostic purposes should not to be considered redundant or useless. Further information on epitope recognition, an increase in assay reliability and, possibly, the identification of clinical phenotypes, can be achieved by means of a panel, instead of a single, representative of the panallergen group (8). Furthermore, the molecule that is most frequently recognized within a panallergen group is not always the molecule that reflects the primary sensitizer. Latex Hev b 8 and *Mercurialis annua* Mer a 1, for example, are the best performing profilins for *in vitro* IgE recognition in patients probably primarily sensitized by other profilins (8).

Cross-Reacting Carbohydrate Determinants (CCD) IgE reactivity should also be ruled out, since all vegetable (pollen and latex allergens) and Hymenoptera extracts can be weakly recognized by specific IgEs in the patient vs. CCD, thus representing a possible cause of false *in vitro* IgE detection (9, 10). CCD IgE reactivity could affect only native allergens, since recombinant allergens that are not glycosylated are not involved in CCD recognition.

Single reactivity to a single biological source by means of extract-based tests could cause one to consider the molecule-based approach as being of no value in a particular case, and therefore being irrelevant to the AIT decision process. However, this is not always true. In some cases, as in cypress or pellitory pollen mono-sensitization, a further molecular approach might be considered unnecessary; however, in other situations, such as reactivity to house dust mite or grass(es), precise identification of the molecule(s) involved in the positive extract-based tests is mandatory. House dust mite-positive results may occur in patients who are Der p 1- or Der p 2-negative, but positive for other molecules (Der p 7, Der p 9, etc.); these are situations where an AIT approach would be questionable. Similarly, Phl p 1 mono-reactive subjects constitute a different model when compared to a "grass-molecule polysensitized" patient, who concurrently recognizes Phl p 1, Ph p 2, Phl p 4, Ph p 5, Phl p 6, and Phl p 11 (11).

Another important point to underline is the false self-reliance that the most recent diagnostic tools could give to the allergy specialist. It is important to begin "thinking molecular", but it is also important to be aware that the amount of molecules that can be tested constitutes about 4% of all the molecules currently known (Table 1). A negative allergen molecule-based test (including panallergens) should lead to careful consideration of the possibility that some relevant component, still unavailable, could be responsible for a positive extract-based test (unless there is a contamination causing a false-positive response to the allergen extract (12)). This is quite a common situation, as already mentioned, in the case of a house dust mite reaction in a patient who is negative for Der p (and/or Der f) 1, Der p 2, or Der p 10.

Bad treatment

Sublingual or subcutaneous administration of the allergens involved in clinical symptoms, in order to induce a tolerogenic response, is the main rationale in AIT (1,13).

The immunological mechanisms underlying modification

Table 1 - Molecular allergens relevant for AIT. A practical list.

Allergen	Genuine Molecule(s)	Panallergen(s)
Moulds	***Alternaria alternata*** **Alt a 1 (Acidic Glycoprotein),** Alt a 3 (Heat Shock Proteins), Alt a 4 (Disulfide Isomerase), Alt a 8 (Mannitol Dehydrogenases), Alt a 10 (Aldehyde Dehydrogenase), Alt a 12 (Acidic Ribosomal Protein P1), Alt a 13 (Glutathione-S-transferases)	***Alternaria alternata*** **Alt a 6 (Enolase)** Alt a 5 (Ribosomal Protein P2) Alt a 7 (YCP4 protein) Alt a 8 (Mannitol Dehydrogenases)
	Aspergillus fumigatus **Asp f 1 (Mitogillin family), Asp f 2, Asp f 3 (Peroxisomal Protein), Asp f 4,** Asp f 5 (Metallo Protease), Asp f 7, Asp f 9, Asp f 10 (Aspartate Protease), Asp f 11 (Peptidyl-prolyl isomerase), Asp f 12 (HPS), Asp f 13 (Serine Protease), Asp f 15, Asp f 16, Asp f 17, Asp f 23 (L3 Ribosomal Protein), Asp f 27 (Cyclophilin), Asp f 28 (Thioredoxin), Asp f 29 (Thioredoxin), Asp f 34 (PhiA Cell Wall Protein)	***Aspergillus fumigatus*** **Asp f 6 (Mn Superoxide Dismutase)** Asp f 8 (Ribosomal Protein P2) Asp f 18 (Vacuolar Serine Protease) Asp f 22 (Enolase)
	Cladosporium herbarum Cla h 2, **Cla h 8 (Mannitol Dehydrogenase),** Cla h 10 (Aldehyde Dehydrogenase), Cla h 12 (Acid Ribosomal Protein P1)	***Cladosporium herbarum*** Cla h 5 (Ribosomal Protein P2) Cla h 7 (YCP4 Protein) Cla h 6 (Enolase) **Cla h 8 (Mannitol Dehydrogenase)** Cla h 9 (Vacuolar Serine Protease)
House dust mite	***Dermatophagoides pteronyssinus*** **Der p 1 (Cysteine Protease), Der p 2 (NPC2 family),** Der p 3 (Trypsin), Der p 4 (α-Amylase), Der p 5, Der p 6 (Chymotrypsin), Der p 7, Der p 8 (Glutathione S-Transferase), Der p 9 (Collagenolytic Serin Protease), Der p 14 (Apolipophorin), Der p 15 (Chitinase-like Protein), Der p 18 (Chitin-Binding Protein), Der p 21, Der p 23 (Peritrophin-like Protein Domain)	***Dermatophagoides pteronyssinus*** **Der p 10 (Tropomyosins)** Der p 11 (Paramyosin) Der p 20 (Arginine Kinase)
	Dermatophagoides farinae **Der f 1 (Cysteine Protease), Der f 2 (NPC2 family),** Der f 3 (Trypsin), Der f 6 (Chymotrypsin), Der f 7, Der f 13 (Fatty Acid Binding Protein), Der f 14 (Apolipophorin), Der f 15 (Chitinase), Der f 16 (Gelsolin/villin), Der f 17 (Calcium Binding Protein), Der f 18 (Chitin-Binding Protein), Der f 22, Der f 24 (Ubiquinol-Cytochrome C Reductase Binding Protein Homologue)	***Dermatophagoides farinae*** Der f 10 (Tropomyosins) Der f 11 (Paramyosin) Der f 20 (Arginine Kinase)
	Euroglyphus maynei Eur m 1 (Cysteine Protease), **Eur m 2 (NPC2 family),** Eur m 3 (Trypsin), Eur m 4 (α-Amylase), Eur m 14 (Apolipophorin)	
	Lepidoglyphus destructor **Lep d 2 (NPC2 family**), Lep d 5, Lep d 7, Lep d 13 (Fatty Acid Binding Protein)	***Lepidoglyphus destructor*** Lep d 10 (Tropomyosin)
	Blomia tropicalis Blo t 1 (Cysteine protease), Blo t 2, Blo t 3 (Trypsin), Blo t 4 (α-Amylase), **Blo t 5**, Blo t 6 (Chymotrypsin), Blo t 12, Blo t 13 (Fatty Acid Binding Protein), Blo t 19 (Anti-Microbial Peptide Homologue), Blo t 21	***Blomia tropicalis*** Blo t 10 (Tropomyosin), Blo t 11 (Paramyosin)

(continued)

Table 1 (continued) - Molecular allergens relevant for AIT. A practical list.

Allergen	Genuine Molecule(s)	Panallergen(s)
Animal dander	**Cat (*Felis domesticus*)** **Fel d 1 (Uteroglobin),** Fel d 3 (Cystatin), Fel d 5w (IgA), Fel d 6w (IgM), Fel d 7 (von Ebner Gland Protein), Fel d 8 (Latherin-like Protein)	**Cat (*Felis domesticus*)** **Fel d 2 (Serum Albumins)** **Fel d 4 (Lipocalin)**
	Dog (*Canis familiaris*) **Can f 1 (Lipocalin), Can f 2 (Lipocalin)**, Can f 4 (Lipocalin), **Can f 5 (Arginine esterase)**	**Dog (*Canis familiaris*)** **Can f 2 (Lipocalin)** **Can f 3 (Serum Albumins)** Can f 6 (Lipocalin)
	Horse (*Equus caballus*) **Equ c 1 (Lipocalin),** Equ c 2 (Lipocalin), Equ c 4 (Latherin)	**Horse (*Equus caballus*)** **Equ c 3 (Serum Albumins)**
Hymenoptera Venoms	**Honey bee *(Apis mellifera)*** **Api m 1 (Phospholipase A2),** Api m 3 (Acid phosphatase), **Api m 4 (Melittin),** Api m 5 (Dipeptidylpeptidase IV), Api m 6, Api m 7 (CUB Serine protease), Api m 8 (Carboxylesterase), Api m 9 (Serine Carboxypeptidase), Api m 10 (Icarapin Variant 2, Carbohydrate-rich Protein), Api m 11 (Major Royal Jelly Protein), Api m 12 (Vitellogenin)	**Honey bee (*Apis mellifera)*** Api m 2 (Hyaluronidase) Api m 5 (Dipeptidylpeptidases IV)
	Common Wasp (*Vespula vulgaris*) **Ves v 1 (Phospholipase A1B), Ves v 5 (Antigen 5),** Ves v 6 (Vitellogenin)	**Common Wasp (*Vespula vulgaris)*** Ves v 2 (Hyaluronidase) Ves v 3 (Dipeptidylpeptidases IV)
	Mediterranean Paper Wasp (*Polistes dominulus*) Pol d 1 (Phospholipase A1), Pol d 4 (Serin-Protease), **Pol d 5 (Antigen 5)**	**Paper Wasp (*Polistes annularis)*** Pol a 2 (Hyaluronidase)
		CCD MUXF3 from Bromelain
Latex	**Para rubber tree *(Hevea brasiliensis)*** **Hev b 1 (Rubber Elongation Factor),** Hev b 2 (-1,3-Glucanase), **Hev b 3 (Small Rubber Particle Protein),** Hev b 4 (Lecithinase Homologue), **Hev b 5, Hev b 6 (Hevein Precursor),** Hev b 7 (Patatin-like Protein), **Hev b 9 (Enolase),** Hev b 10 (Mn Superoxide Dismutase), **Hev b 11 (Class I Chitinase),** Hev b 12 (nsLTP), Hev b 13 (Esterase), Hev b 14 (Hevamine)	**Para rubber tree (*Hevea brasiliensis*)** **Hev b 6 (Hevein Precursor), Hev b 8 (Profilin), Hev b 9 (Enolase),** Hev b 10 (Mn Superoxide Dismutase), **Hev b 11 (Class I Chitinase),** Hev b 12 (nsLTP)
		CCD MUXF3 from Bromelain
Pollens	**Weed pollen** **Ragweed (*Ambrosia artemisiifolia*)** **Amb a 1 (Pectate lyase),** Amb a 3 (Plastocyanine), Amb a 4 (Defensin-like Protein), Amb a 5, Amb a 7 (Plastocyanin)	**Weed pollen** **Ragweed (*Ambrosia artemisiifolia*)** Amb a 6 (nsLTP), Amb a 8 (Profilin), Amb a 9 (Polcalcin), Amb a 10 (Polcalcin-like Protein [4 EF-hand])
	Mugwort (*Artemisia vulgaris*) **Art v 1 (Defensin-like Protein), Art v 3 (nsLTP),** Art v 2 (PR-1), Art v 6 (Pectate Lyase)	**Mugwort (*Artemisia vulgaris*)** **Art v 3 (nsLTP),** Art v 4 (Profilin), Art v 5 (Polcalcin)
	Pellitory *(Parietaria judaica)* Par j 1 (Phospholipid Transfer Protein), **Par j 2 (Phospholipid Transfer Protein)**	**Pellitory (*Parietaria judaica*)** Par j 3 (Profilin) and Par j 4 (Polcalcin)

(continued)

Table 1 (continued) - Molecular allergens relevant for AIT. A practical list.

Allergen	Genuine Molecule(s)	Panallergen(s)
Pollens	**Russian thistle or saltwort (*Salsola kali*)** **Sal k 1 (Pectin Methylesterase),** Sal k 2 (Protein Kinase Homologue), Sal k 3 (Cobalamin Independent Methionine Synthase), Sal k 5 (Ole e 1-like Protein)	**Russian thistle or saltwort (*Salsola kali*)** Sal k 4 (Profilin)
	English Plantain (*Plantago lanceolata*) **Pla l 1 (Ole e 1-related Protein)**	
	Grass pollen	**Grass pollen**
	Timothy (*Phleum pratense*) **Phl p 1 (β-Expansin), Phl p 2, Phl p 4 (Berberine Bridge Enzyme), Phl p 5, Phl p 6, Phl p 11 (Ole e 1-related Protein),** Phl p 13 (Polygalacturonase)	**Timothy (*Phleum pratense*)** **Phl p 7 (Polcalcin), Phl p 12 (Profilin)**
	Bermuda grass (*Cynodon dactylon*) **Cyn d 1 (β -Expansin),** Cyn d 15, Cyn d 22w (Enolase), Cyn d 23, Cyn d 24 (PR-1)	**Bermuda grass (*Cynodon dactylon*)** Cyn d 7 (Polcalcin), Cyn d 12 (Profilin)
	English Ryegrass (Lolium perenne) Lol p 1 (β-Expansin), Lol p 2, Lol p 3, Lol p 4, Lol p 5, Lol p 11 (Ole e 1-related Protein)	
	Tree pollen	**Tree pollen**
	Birch (*Betula verrucosa*) **Bet v 1 (PR-10), Bet v 6 (Isoflavone reductase),** Bet v 7 (Cyclophilin)	**Birch (*Betula verrucosa*)** **Bet v 1 (PR-10), Bet v 2 (Profilin),** Bet v 3 (Polcalcin-like Protein [4 EF-hand]), **Bet v 4 (Polcalcin)**
	Black alder *(Alnus glutinosa)* **Aln g 1 (PR-10)**	**Black alder (*Alnus glutinosa*)** **Aln g 1 (PR-10), Aln g 4 (Polcalcin)**
	Hazelnut tree (*Corylus avellana*) **Cor a 1 (PR-10),** Cor a 10 (Luminal Binding Protein)	**Hazelnut tree (*Corylus avellana*)** Cor a 1 (PR-10), Cor a 2 (Profilin)
	Olive *(Olea europea)* **Ole e 1 (Common Olive Group 5),** Ole e 4, Ole e 5 (Superoxide Dismutase [Cu-Zn]), Ole e 6, **Ole e 7 (nsLTP), Ole e 9 (1,3-β Glucanase),** Ole e 10 (X8 Domain Containing Protein), Ole e 11 (Pectin Methylesterase)	**Olive (*Olea europea*)** Ole e 2 (Profilin), Ole e 3 (Polcalcin), **Ole e 7 (nsLTP),** Ole e 8 (Polcalcin-like Protein [4 EF-hands])
	Japanese cedar (*Cryptomeria japonica*) **Cry j 1 (Pectate Lyases),** Cry j 2 (Polygalacturonase) **Cypress (Cupressus arizonica)** **Cup a 1 (Pectate Lyases)**	
	Plane tree *(Platanus acerifolia)* **Pla a 1 (Putative Invertase Inhibitor), Pla a 2 (Polygalacturonase), Pla a 3 (nsLTP)**	**Plane tree (*Platanus acerifolia*)** **Pla a 3 (nsLTP)** **CCD MUXF3 from bromelain**

Notes
Molecular allergens in bold characters are currently available for specific IgE testing. The other molecules belonging to the respective biological source, but not available for IgE testing, are expressed (in grey) according to the **WHO-IUIS** Allergen Nomenclature (http://www.allergen.org/).

(continued)

Table 1 (continued) – Molecular allergens relevant for AIT. A practical list.

Moulds
Alt a 1 is recognized in about 80–100% of patients (19). IgE reactivity to Asp f 2, Asp f 4, and Asp f 6 is associated with allergic bronchopulmonary aspergillosis (20), whilst Asp f 1 and/or Asp f 3 sensitization seem to be related to allergic asthma development (21).

House dust mites
About 5% of the patients reactive to house dust mite show reactivity to tropomyosins, which are responsible for the cross-reactivity with arthropods, cephalopods, crustaceans, insects (cockroach), molluscs, and nematodes (*Anisakis simplex*) (7).

Animal dander
Elevated IgE levels to Fel d 1 are associated with augmented risk for asthma in cat-allergic children (22) as well as concurrent sensitization towards multiple animal-derived allergens, such as uteroglobin (Fel d 1), lipocalins (Can f 1, 2, Equ c 1, Fel d 4, and Mus m 1), and kallikrein (Can f 5) (23). Fel d 4 has high sequence identity to Equ c 1 (24). IgE cross-reactivity has been demonstrated between Can f 1 and Can f 2 and between Equ c 1, Mus m 1, and Fel d 4 (25). A high degree of sequence homology among serum albumins from different animals has been demonstrated (26).

Hymenoptera venoms
Most Hymenoptera venom allergens have CCDs, and double IgE positivity to *Apis mellifera* and *Vespula* spp. is often caused by CCD recognition (27). Hyaluronidases (Api m 2 and Ves v 2) are the most important molecules showing CCD, whilst a "true" cross-reactivity by means of recognition of hyaluronidase peptides is less common. Only the detection of recombinant Api m 1, Ves v 1, and Ves v 5 venom allergens can be considered evidence for a genuine sensitization, where an AIT would be indicated (28).

Latex
Profilin (Hev b 8) (29) and/or Hev b 12 (30) reactivity are not associated with latex allergy. Hev b 6 (hevein) is the most important sensitizer and is involved in the latex–fruit syndrome (latex–avocado–banana–chestnut–kiwi) (31,32).

Pollen
The major allergens of *Fagales* pollen can be considered a genuine marker of sensitization and as a panallergen (PR-10, pathogenesis-related proteins group 10) due to its presence in several plant-derived foods (33). Profilin, a small (12–15 kDa) actin-binding protein structurally involved in the cytoskeletal structure in all eukaryotic organisms (34), and polcalcin, a calcium-binding protein present in nearly all plant species (35), are the most important cross-reacting pollen panallergens. Increased risk for adverse reaction during AIT has been suggested in the presence of reactivity to Ole e 7 or Ole e 9 (5).

of the allergenic response after AIT are only partially understood (14), but the first prerequisite to obtain a reliable desensitization is that the molecule(s) responsible for the IgE production is (are) actually present in the allergenic extract used for the treatment, and, hopefully, at the same concentration throughout the treatment. As during antibiotic treatment, the same amount of drug is administered to the patient throughout the treatment period; hence, one could require AIT manufacturers to provide a product where, not only all the genuine molecule(s) are represented in a comparable amount, but where also a steady relationship between the different components is guaranteed.

As shown in a number of papers, the protein content of such products may vary across the various commercial products, and increased heterogeneity, for example in the respective concentrations of Phl p 1 and Phl p 5, is observed when comparing products (15). Consequently, the commercial products used for AIT are not all equal and, sometimes, in a given product, some important differences can be recorded by comparing one batch to another. A possible response to all these concerns could be the preparation of recombinant formulations for AIT, where ideally and hypothetically all the different relevant molecules are equally represented. Unfortunately, AIT based on recombinant molecules has not yet demonstrated a clear advantage compared to traditional allergen extracts (16,17). Furthermore, their preparation would require individual testing and registration for each molecule isolated (for instance, for grasses only, theoretically more than 5 distinct products

should be registered), thus representing a considerable financial burden for manufacturers (18). As a result, the availability of recombinant AIT for patient-tailored immunotherapy seems to be a distant scenario. On the other hand, an accurate description of molecules present (or absent) in each commercial product, and the certification of concentration stability for each single molecular component seems to be a more realistic goal for manufacturers.

Conclusions

Beginning at diagnosis, the molecular-based approach better supports the specialist in patient management. Component-resolved diagnosis (CRD) adds pivotal information about risk, specificity, and cross-reactivity, enabling the selection of patients for AIT. Furthermore, through CRD, it is possible to explain symptoms due to cross-reactivity and to distinguish exactly the symptoms elicited by cross-reactions from those caused by genuine sensitization. Thus, allergenic molecule-based tools currently available can help in reducing the diagnostic mistakes of the past. However, this improved diagnostic approach should be supported by an equal molecule-driven development of the products designed for AIT (in terms of the identity and quantity of the allergen molecules present in the product), as it currently remains the only area that can be modified to ensure the natural evolution of treatment for allergic diseases.

Conflicts of interest

Enrico Scala has no conflicts of interest that are directly relevant to the content of paper.

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