

Cross-reactivity among fungal allergens: a clinically relevant phenomenon?

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Summary

Atopic patients suffering from allergic asthma, allergic rhinitis, or atopic eczema often have detectable levels of serum IgE antibodies to fungi. Although the association between fungal sensitisation and different forms of allergic diseases, including allergic asthma and life-threatening allergic bronchopulmonary aspergillosis, is well established, the clinical relevance of cross-reactivity among different fungal species remains largely unknown. Recent progress in molecular cloning of fungal allergens and the availability of more than 40 completely sequenced fungal genomes facilitates characterisation, cloning, and production of highly pure recombinant allergens, identification of homologous and orthologous allergens widespread among the fungal kingdom, *in silico* prediction, and experimental *in vitro* and *in vivo* verification of cross-reactivity between homologous pan-allergens. These studies indicate that cross-reactivity is an important component of fungal sensitisation.

Key words: Fungi, allergens, allergy, cross-reactivity, structure, clinical relevance.

Introduction

Although the fungal kingdom spans over 100 000 fungal species, only a few hundred are opportunistic pathogens, and less than hundred are reported to be involved in mycoses.¹ Around 80 mould genera have been shown to be able to induce allergen-specific IgE in predisposed individuals,² and cDNAs encoding allergens have been isolated from 23 fungal genera.³ It is estimated that about 10% of the general population have IgE antibodies to common inhalant moulds,² and about half of these individuals might develop, at some time, allergic symptoms as a consequence of fungal exposure.⁴ The most common diseases associated with fungi are allergic asthma,⁵ allergic rhinitis,⁶ allergic sinusitis,⁷ atopic eczema,⁸ and although rarely, allergic bronchopulmonary mycoses.⁹ With some exceptions like workplace exposure^{10,11} and allergic

bronchopulmonary aspergillosis (ABPA¹²) which are well documented, the contribution of fungal sensitisation to the severity of allergic reactions and asthma largely remains to be investigated. The problems have already started with the definition of the prevalence of fungal sensitisation. Results of skin test surveys suggest that a least 3–10% of the worldwide population is affected by fungal sensitisation.¹³ However, the exact prevalence of fungal sensitisation has not yet been determined because of the huge variations obtained using different fungal extracts.¹⁴ Moreover, the medical effect of mould exposure is a highly controversial issue¹⁵ as demonstrated by the many comments to a recently published position paper.¹⁶ Dissected at molecular level, the IgE-mediated disorders can be traced back to discrete molecules, the so called allergens. In contrast to clinical practice where an 'allergen' is often referred to as a single allergenic source or an extract thereof, for example birch pollen or cat dander extract, for a molecular biologist an allergen is defined as a single protein, encoded by a single mRNA, able to induce a switch to IgE production in B cells. It is important to distinguish between allergens and IgE-binding proteins, because not all IgE-binding proteins have to necessarily

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possess the capacity to induce allergic symptoms.¹⁷ However, all IgE-binding proteins displaying at least two identical or different B cell epitopes have the potential to induce symptoms through cross-linking of specific IgE immobilized on the high affinity receptor for IgE on effector cells¹⁸ (Fig. 1). Many of these proteins show cross-reactivity, a complex phenomenon complicating the diagnosis of fungal sensitisation.

Fungal sensitisation in clinical practice

Sensitisation to moulds and fungi in clinical practice is diagnosed either by the determination of allergen-specific IgE against fungal extracts¹⁹ or by skin tests with commercially available solutions.²⁰ A first screening is usually performed *in vitro* with fungal extract mixtures containing the most frequent species known to induce allergic reactions e.g. *Penicillium notatum*, *Cladosporium herbarum*, *Aspergillus fumigatus*, *Alternaria alternata*, and *Candida albicans*. Patients showing specific IgE antibodies against the mixture might be submitted to a confirmatory skin test with the single extracts to define the exact source of sensitisation. However, one of the major problems in diagnosing sensitisation to fungi is that the patient in the normal case cannot provide the allergologist with useful information about the clinical history which still represents the most important criterion for the diagnosis of allergy. The reason is that these patients are rarely aware to be exposed to fungi in contrast to individuals suffering from other forms of

allergy like pollinosis or insect sting allergy, which, in most cases, are able to correlate symptoms with exposure. Therefore most of the patients are tested for fungal sensitisation if they suffer from allergic diseases of unknown aetiology assumed to be associated with fungal exposure. The most important group of candidates are patients suffering from asthma where sensitisation to *A. fumigatus* should be ruled out. Asthmatic patients sensitised to *A. fumigatus* are at risk for the development of ABPA which, if untreated, will result in end-stage lung fibrosis.²¹ Beside asthma, rhinitis,²² sinusitis,⁷ and hypersensitivity pneumonitis²³ of unknown aetiology are indications justifying an extended screening for fungal sensitisation.

Fungal mono- and polysensitisation

Epidemiological studies investigating the distribution of mono- and polysensitisation to fungi are rare and somehow arbitrary because they depend on the quality of the extracts used to assess patients' reactivity.¹³ However, there is a large consensus that the majority of mould-sensitised patients (>75%) reacts only to one species, whereas sensitisation to *Alternaria* is the most common one, and mainly because of the sensitisation against the species-specific major allergen Alt a 1.¹³ Interestingly, monosensitisation to *A. fumigatus*, *C. herbarum*, *P. notatum*, and *Saccharomyces cerevisiae* is very rare (<1%), indicating a highly cross-reactive potential of these species. The most extensive skin prick

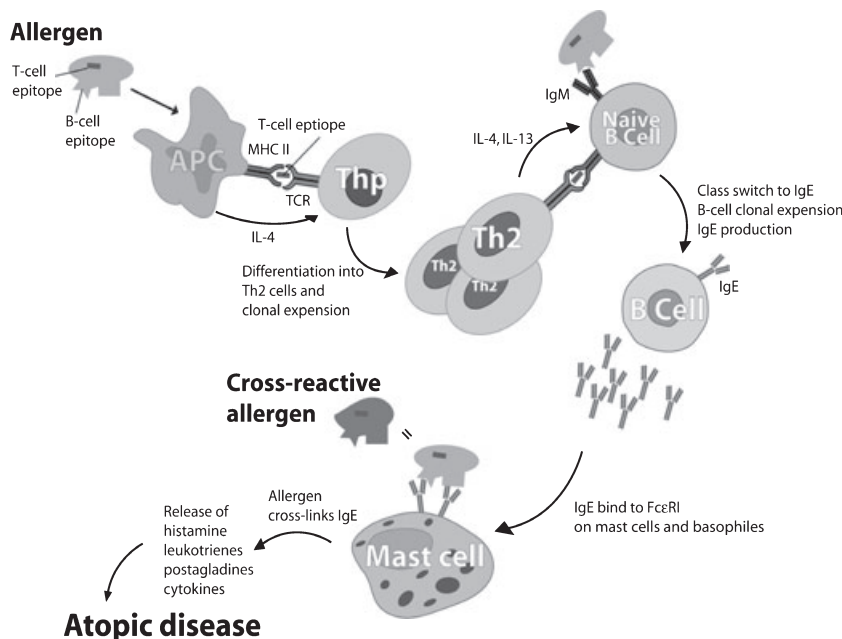


Figure 1 The type I hypersensitivity reaction and cross-reactivity. Exposure of atopic individuals to environmental allergens leads to a cascade of events ending in the production of allergen-specific IgE antibodies. They are captured by Fcε-high affinity receptors on effector cells and are responsible for the sensitisation of allergic individuals. Re-exposure either to the same environmental allergen or to homologous molecules sharing B cell epitopes with the environmental allergen induces cross-linking of the IgE antibodies on the surface of effector cells, degranulation, and as a consequence thereof to allergic symptoms.

test (SPT) study performed with fungal extracts involving 621 sensitised subjects¹³ suggest that most of them (>77%) were monosensitised either to *A. alternata*, *C. albicans* or *Trichophyton mentagrophytes*. Only four, three and one of the tested individuals were monosensitised to *C. herbarum*, *A. fumigatus*, and *P. notatum* respectively. In contrast, around 23% of the subjects showed reactivity to two or more extracts with prevalence for *Aspergillus* and *Cladosporium* exceeding 80%, whereas the prevalence for *Penicillium* was around 50%. This might indicate a higher potential for cross-reactivity of these species compared with *Alternaria*, *Candida*, and *Trichophyton*, which show a high tendency towards monosensitisation probably related to species-specific allergens.

Cross-reactivity between fungal extracts

In contrast to polysensitisation, which is easy to demonstrate by simple determination of the RAST values of sera against different allergen extracts, cross-reactivity can only be shown by inhibition experiments determining the degree to which an extract in fluid phase inhibits IgE-binding to a different solid-phase coated extract.¹⁴ RAST, ELISA, and immunoblot inhibition techniques in homologous and heterologous inhibition experiments with different fungal extracts have been used to indicate cross-reactivity between different fungal species.^{24–27} Unfortunately, extensive systematic studies investigating cross-reactivity of fungal extracts are lacking. However, inhibition techniques based on extracts do not provide any information about the molecular nature of the allergens involved. Still unclear is the role of post translational modifications of

the allergens like glycosylation in cross-reactivity.²⁸ The so called cross-reactive carbohydrate determinants (CCDs), which are structurally based on asparagine-linked carbohydrate moieties, are able to elicit anti-glycan-specific IgE responses in atopic individuals. Although CCD-specific IgE is clearly detectable by serum-based diagnostic procedures *in vitro*,^{29,30} the clinical relevance of a positive serum test to CCDs is highly questionable (see below).

Cross-reactivity at molecular level

The rapid progress in cloning, expression and characterisation of (fungal) allergens,^{31,32} down to the determination of three dimensional structures by X-ray crystallography,^{33–38} allows an experimental assessment of cross-reactivity at molecular level. Behind pollens with 184 and mites with 164 reported single allergens, fungi with 100 allergens represent the third group of organisms from where the largest number of allergens has been cloned, produced and characterised (<http://www.allergen.org>). They cover structurally and functionally heterogeneous protein families including enzymes, backbone and cell wall proteins, as well as proteins with unknown functions (Table 1). From a phylogenetical point of view, they can be subdivided into two classes: species-specific, and cross-reactive allergens. The major allergens of *A. fumigatus*, *A. alternata*, *Coprinus comatus*, and *Malassezia sympodialis* (Asp f 1,³⁹ Alt a 1,⁴⁰ Cop c 1,⁴¹ and Mala s 1,³⁸ respectively) represent species-specific proteins, which are so far not found in any other genera as shown by extensive comparative genomics based on 22 fully sequenced fungal genomes.⁴² However, except these and

Table 1 Prominent cross-reactive fungal allergen structures

Protein family	MW range (kDa)	Species (allergen)	References
MnSOD's	22–25	<i>Aspergillus fumigatus</i> (Asp f 6), <i>Malassezia sympodialis</i> (Mala s 11)	34,43,52,77,78,85
Cyclophilins	16–20	<i>A. fumigatus</i> (Asp f 11, Asp f 27), <i>M. sympodialis</i> (Mala s 6), <i>Psilocybe cubensis</i> (Psi c 2)	35,36,44,86
Thioredoxins	10–12	<i>A. fumigatus</i> (Asp f 28, Asp f 29), <i>Coprinus comatus</i> (Cop c 2), <i>M. sympodialis</i> (Mala s 13)	37,45,80
Serin proteases	28–35	<i>P. citrinum</i> (Pen c2), <i>Penicillium notatum</i> (Pen c 13)	48,81
Enolases	45–48	<i>Alternaria alternata</i> (Alt a 6), <i>A. fumigatus</i> (Asp f 22), <i>Cladosporium herbarum</i> (Cla h 6), <i>Curvularia lunata</i> (Cur l 3), <i>P. citrinum</i> (Pen c 22)	46,72,82
P ₁ ribosomal proteins	11–12	<i>A. alternata</i> (Alt a 12), <i>C. herbarum</i> (Cla h 12), <i>Penicillium brevicompactum</i> (Pen b 26)	72,83
P ₂ ribosomal proteins	11–12	<i>A. alternata</i> (Alt a 5), <i>C. herbarum</i> (Cla h 5), <i>A. fumigatus</i> (Asp f 8), <i>F. culmorum</i> (Fus c 1)	49,72
Heat shock proteins	80–90	<i>A. alternata</i> (Alt a 3), <i>A. fumigatus</i> (Asp f 12), <i>M. sympodialis</i> (Mala s 10)	72,84,85
Peroxisomal proteins	17–19	<i>A. fumigatus</i> (Asp f 3), <i>Malassezia furfur</i> (Mala f 3, Mala f 4), <i>P. citrinum</i> (Pen c 3)	8,50,87

perhaps some other few exceptions, the majority of the fungal allergens represent cross-reactive structures covering different protein families. The most important basic structures showing extended cross-reactivity derive from phylogenetically highly conserved proteins like MnSOD,^{34,43} cyclophilin,^{35,44} thioredoxin,^{36,45} enolase,⁴⁶ heat shock proteins,⁴⁷ proteases,⁴⁸ ribosomal⁴⁹ and peroxisomal proteins.⁵⁰ Cross-reactivity requires similarity in the three dimensional structure, not only in the strictly architectural sense, but also in the physicochemical properties such as similar charge distribution, hydrogen-bonding potential and hydrophobicity patterns.⁵¹ The availability of homologous recombinant allergens allows an exact determination of cross-reactivity between single molecules by ELISA inhibition experiments between a solid-phase coated allergen and homologous proteins added in fluid phase. Moreover, pure recombinant allergens can be used to estimate their contribution to the allergenicity of an extract by RAST inhibition assays.⁵² More than 80 fungal genomes have been fully sequenced or are currently being sequenced,⁵³ providing a formidable tool to search whether allergen orthologues are widely spread among the fungal kingdom. In this regard, it is important to note that cross-reactive proteins belong to similar protein fold families, whereas not all proteins belonging to the same protein fold family are cross-reactive. As algorithms for the prediction of the fold family for a novel protein are very efficient,⁵⁴ potentially cross-reactive proteins can easily be identified. Clearly, homology to a known allergen alone, although necessary, is not sufficient to prove that the proteins are cross-reactive. However, surveys of allergens with assessed cross-reactivity suggest that cross-reactivity commonly occurs in proteins having >50% sequence identity⁵⁵ and the sequences retrievable from completed genomes allow fast cloning and production of putative orthologue allergens to assess their allergenic and cross-reactive potential.⁴²

The clinical relevance of fungal cross-reactivity

As mentioned before, allergens can be either glycosylated or not, raising the question of the clinical relevance of CCDs. The first link between protein glycosylation and IgE was established more than 30 years ago with the demonstration that serum IgE from allergic patients cross-reacts with food and insect venom allergens. The observed cross-reactivity is abolishable by periodate treatment, which oxidises the glycan structures.⁵⁶ Although the harsh treatment of glycoproteins with periodate might also destroy peptide

epitopes, the findings were corroborated by later experiments clearly demonstrating that N-glycans can bind IgE as brilliantly summarised in a recent review.²⁸ However, IgE-binding determined *in vitro* does not *per se* necessarily correlate with clinical relevance because many individuals with allergen-specific IgE in serum do not develop allergy symptoms.⁵⁷ Many recent studies which investigated the discrepancies between *in vitro* IgE determinations and *in vivo* tests as well as between a single glycosylated allergen and the deglycosylated form thereof showed a very limited clinical relevance of CCDs, if any.^{58–60} In contrast, it is clear that anti-CCD IgE lead to false positive results in *in vitro* testing when natural allergen extracts are used.^{61,62}

Unfortunately, the few studies investigating cross-reactivity between fungal extracts are mostly inconclusive. A study about the cross-reactivity between *C. albicans* extract and extracts obtained from other pathogenic *Candida* species and airborne yeast species by dot blot analysis and IgE-binding in ImmunoCAPs showed an extended cross-reactivity.⁶³ In inhibition experiments, it was shown that *C. albicans* mannoproteins, but not proteins without glycosylation, strongly inhibited IgE-binding to all yeast species, suggesting that cross-reactivity is because of the protein glycosylation. These results confirmed earlier observations delivered by affinity chromatography fractionations of *Pityrosporum ovale* and *C. albicans* extracts on Con A columns showing that most of the IgE-binding capacity of the crude extracts bound to the column and could be eluted with mannoside.⁶⁴ Cross-reactivity was also shown by immunoblot analysis of *Penicillium* extracts separated by SDS-PAGE indicating cross-reactivity to a 33 kDa component present in different *Penicillium* species, although the chemical nature of the compound was not identified.⁶⁵ These studies show that carbohydrates are unlikely to be of clinical relevance. Accordingly, a skin prick test study involving *Basidiomycete* and *Deuteromycete* extracts showed that, although shared allergenic determinants were present, *in vivo* cross-reactivity between the species was minimal. In contrast radioimmunosorbent test (RAST) inhibition was significant between some phylogenetically distant fungal species indicating IgE-binding components without clinical relevance.⁶⁶ This result is likely to be explained by the presence of CCD in the extracts which are able to bind IgE *in vitro*, but hardly induce wheal and flare reactions in skin tests.⁶⁷ Obviously cross-reactive CCD determinants obscure the clinical relevance of cross-reactivity assessments between different extracts performed *in vitro*. This might be one of the major reasons for

the lack of comparative skin test studies between fungal extracts.

A different picture derives from investigation using single recombinant allergens. As *Escherichia coli*, the standard bacterium used for the production of recombinant proteins⁶⁸ is unable to perform most of the post translational modifications, recombinant allergens produced in *E. coli* are not glycosylated. This allows a direct comparison between biochemically purified natural allergens with their recombinant counterpart and, in the case of homologous allergens, a more precise definition of cross-reactivity at molecular level. One of the first glycosylated allergens cloned and expressed in *E. coli* was Api m 1 (phospholipase A₂ [PLA₂]), the major allergen of bee.⁶⁹ It was demonstrated that the diagnostic specificity *in vitro* as well as *in vivo* obtained by the recombinant protein was increased compared with the sensitivity obtained with bee venom or purified natural PLA₂.⁷⁰ Because PLA₂ is glycosylated at a single site⁷¹ the CCD determinant can not induce cross-linking and therefore lacks any clinical relevance as it is the case for many other glycosylated allergens.²⁸

However, the majority of the fungal allergens are intracellular proteins and therefore not glycosylated.⁷² This allows direct comparison of the cross-reactivity between homologous molecules originating from different fungal species without interferences deriving from CCD modifications. MnSODs,^{34,43,52} cyclophilins,^{35,36,44} enolases,⁴⁶ ribosomal proteins,^{49,73} and thioredoxins^{37,45} are typical pan-allergen families related to phylogenetically highly conserved protein structures. All these proteins have been shown to be highly cross-reactive *in vitro* as assessed by inhibition experiments, as well as *in vivo* as shown by their ability to induce positive skin test reactions.⁷⁴ A positive skin test demonstrates that a recombinant allergen is able to induce cross-linking of receptor-bound allergen-specific IgE on effector cells and thereby strongly indicates its clinical relevance. The best evidence for clinical relevance of fungal allergens is their ability to induce eczematous reactions on healthy skin areas of patients suffering from atopic eczema in atopy patch tests.^{8,52,74–76} Of particular interest in this respect is the ability of phylogenetically highly conserved human self-antigens with structural homology to environmental allergens to induce T cell proliferation and skin test reactivity in patients sensitised to the corresponding environmental allergens.^{49,52,77,78} These observations represent most probably the strongest evidence for an important role of cross-reactivity in perpetuating the symptoms of chronic long-lasting allergic diseases.^{79,80} Comparisons of the solved crystal structures of the environmental allergens

Asp f 6 (MnSOD³⁴), Mala s 6 (cyclophilin³⁵) and Mala s 13 (thioredoxin³⁷) with the solved structures of human MnSOD, cyclophilin B, and thioredoxin provide a rational explanation for the observed cross-reactivity at humoral level. Pair-wise superposition of the solved structures allows a detailed analysis of the molecular surfaces of the proteins to identify spatially conserved identical, surface exposed amino acid residues which obviously are the only amino acids able to contribute to cross-reactivity. In spite of the fact that the molecules share around 50% sequence identity, the structural analysis showed that most of the conserved amino acids are located in the backbone of the proteins. In all cases only a minority of the identical residues are exposed to the solvent and, therefore, likely to be accessible for antigen-antibody interactions. These amino acids are scattered over the linear gene sequence in both, environmental and human primary structures, and spatially cluster to patches forming putative conformational B cell epitopes potentially involved in IgE-mediated cross- and autoreactivity.^{35–37}

However, these self-antigens are stress-inducible intracellular proteins and as such normally not available for antigen-antibody interactions. As demonstrated by atopy patch tests with human MnSOD, the enzyme is able to induce eczematous reactions if externally applied to the skin. A role of autoreactivity in the pathogenesis of allergic diseases requires, however, release of the self-antigens at the site of inflammation, likely to occur as a consequence of tissue damage resulting from the inflammatory process.^{49,52,78–80} IgE-mediated reactions to self-antigens are assumed to be important in perpetuating the symptoms in chronic allergic diseases like atopic eczema or ABPA also in the absence of exposure to environmental allergens highlighting the clinical relevance of cross-reactivity.

Conclusions

Fungal sensitisation can lead to severe allergic complications often neglected in clinical practice. A clear link between fungal sensitisation, atopic eczema, asthma, and ABPA has been demonstrated in several studies. However, the clinical relevance of cross-reactivity among different fungal species is still an open question. Clearly, cross-reactive carbohydrate determinants, earlier assumed to be important in fungal sensitisation, are unlikely to be of clinical relevance but might account for a large number of false positive diagnoses in serological determinations.²⁸ Recent investigations using highly pure recombinant allergens demonstrate that cross-reactivity between homologous fungal proteins can play

a decisive role in the exacerbation of allergic complications related to fungal sensitisation, especially in atopic eczema⁵² and ABPA.⁴⁹

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