Medical Mycology September 2006, **44**, S261–S267

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Structural aspects and clinical relevance of *Aspergillus fumigatus* antigens/allergens

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Robotics-based high throughput screening of *Aspergillus fumigatus* cDNA libraries displayed on phage surfaces revealed at last 81 different structures able to bind IgE from serum of patients sensitized to this fungus. Among these, species-specific as well as phylogenetically highly conserved structures and such with unknown function have been detected. A subset of cDNAs have been used to produce and characterize the corresponding recombinant allergens which have proven to be useful diagnostic reagents allowing specific detection of *A. fumigatus* sensitization and differential diagnosis of allergic bronchopulmonary aspergillosis. Phylogenetically highly conserved structures like manganese-dependent superoxide dismutase, P_2 acidic ribosomal protein, cyclophilins and thioredoxins induce, beyond sensitization, IgE antibodies able to cross-react with the corresponding homologous self antigens. These reactions, likely to contribute to the exacerbation and perpetuation of allergic bronchopulmonary aspergillosis, can be traced back to shared conformational B-cell epitopes build up from conserved amino acid residues scattered over the surface of the molecules as shown by detailed analyses of the crystal structures.

Keywords *Aspergillus fumigatus*, allergy, diagnosis, recombinant allergens, crystal structures

Introduction

Endemic and opportunistic fungal infections have become an important medical problem, concomitantly with the increasing number of patients undergoing immunosuppressive medical treatment, chemotherapy, and with increases in congenital and acquired immunodeficiency [1–3]. Morbidity and mortality rates associated with fungal species that are normally harmless to immunocompetent hosts (e.g., *Candida*, *Aspergillus*, *Fusarium* and *Trichosporum*) are high and diagnosis and treatment of these infections remain difficult [4]. Fungal infections in humans are associated with a wide variety of diseases, ranging from benign colonization, superficial skin infections, allergy and asthma, to life-threatening diseases like allergic bronchopulmonary aspergillosis (ABPA) and invasive systemic mycoses [5]. Patients at high risk of acquiring invasive fungal mycoses are those with significant reduction in the number or function of granulocytes such as leukaemia patients undergoing chemotherapy [6], patients on long-term treatment with immunosuppressive drugs (e.g., organ transplant patients) [7], or patients with high dosed corticosteroid treatment [8]. In all groups the fatality rate of invasive mycoses is high and the majority of the patients die despite antimycotic treatment [9]. Thus, vaccination with fungal antigens conferring protection against invasive mycoses represents an unmet medical need for patients at risk [10]. It has been shown that acquired immunity against aspergillosis is inducible in animals and that protection is primarily mediated by cellular immune responses involving macrophages and/or CD4+ T cells, but not by antibody transfer from immune mice to naïve mice [11,12]. These observations, together with the absence of an association between deficiencies in antibodies and susceptibility to fungal infections and the presence of

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DOI: 10.1080/13693780600789160

specific antibodies in patients with progressive fungal infections [4] have provided evidence against a protective role of antibodies in fungal infections.

In contrast fungal allergy in general, and to *Aspergillus fumigatus* (*A. fumigatus*) in particular has been shown to be associated with strong humoral responses to fungal antigens [4,5]. Serological assays indicate that fungal allergy is common and skin test surveys suggest that at least 3–10% of the worldwide population is affected by fungal sensitization [13]. The exact prevalence of fungal sensitization has not yet been reliably established since the available reports of skin test reactivity to fungi vary from 3–90%, depending from the study population, the tested species of fungi, and especially from the fungal extract used [14,15]. This huge variation is not astonishing because even commercially available fungal extracts for skin testing show an extremely high variation in their allergen content [16]. Among the moulds involved in allergic diseases, *A. fumigatus* plays a predominant role. The different clinical manifestations related to *A. fumigatus*-associated disease, which usually remain mutually exclusive, can be broadly classified according to their clinical conditions into systemic mycoses (not discussed here), saprophytic colonization and allergic diseases [17]. Saprophytic bronchopulmonary colonization refers to fungal infestation of the airways without evidence for direct tissue damage [17]. As a special case aspergilloma is a saprophytic manifestation of an *Aspergillus* growing in preformed lung cavities of patients with bronchogenic carcinoma, tuberculosis, sarcoidosis or, rarely, ABPA [18]. *A. fumigatus*-related allergic disorders include allergic rhinitis, allergic sinusitis, allergic asthma and ABPA [17,19]. *A. fumigatus* represents the most complex allergenic source described so far and is able to produce more the 80 allergens [20,21] spanning a wide range of molecular structures. An array of these allergens (Table 1) have been cloned, produced as recombinant proteins, and evaluated for their diagnostic performance *in vitro* [22–24] as well as *in vivo* [25–27]. Here we discuss molecular aspects and clinical relevance of these allergens.

The allergen repertoire of *Aspergillus fumigatus*

All fungal allergen repertoires described so far are quite large, but that of *A. fumigatus* is by far the most complex one [28]. It spans at least 81 different cDNA gene products ranging from 10 to more than 65 kDa in size. The recently sequenced 29.4 megabase *A. fumigatus* genome contains 9926 predicted genes [29,30]

Table 1 Structures cloned as IgE-binding proteins from *Aspergillus fumigatus*

Allergen	Size	CDS[a] (kDa)	Function/sequence similarity	IgE-binding *in vitro* (%)[b]	SPT[c]	Accession no.
Asp f 1	16.9	C	Ribotoxin	66	pos.	S889330
Asp f 2	37.0	C	Fibrinogen binding protein?	ND	NT	U56938
Asp f 3	18.5	C	Peroxisomal protein	88	pos.	U58050
Asp f 4	30.0	P	Unknown	42	pos.	AJ001732
Asp f 5	42.1	C	Metalloprotease	85	pos.	Z30424
Asp f 6	23.0	C	MnSOD	35	pos.	U53561
Asp f 7	11.6	P	Unknown	39	pos.	AJ223315
Asp f 8	11.1	C	P_2 ribosomal protein	10	pos.	AJ224333
Asp f 9	32.3	P	Unknown	66	pos.	AJ223327
Asp f 10	34.4	C	Aspartic protease	18	pos.	X85092
Asp f 11	18.8	C	Cyclophilin	50	pos.	AJ006689
Asp f 12	65.0	P	Heat shock protein	ND	NT	U92465
Asp f 13	34.0	C	Alkaline serine protease	ND	NT	Z11580
Asp f 15	19.5	C	Serine protease?	29	pos.	AJ002026
Asp f 16	43.0	C	Unknown	ND	NT	G3643813
Asp f 17	19.4	P	Unknown	43	pos.	AJ224865
Asp f 18	34.0	C	Vacuolar serine protease	ND	NT	Y13338
Asp f 22w	46.0	C	Enolase	ND	NT	AF284645
Asp f 23	44.0	C	L3 ribosomal protein	ND	NT	AF464911
Asp f 27	18.0	C	Cyclophilin	75	pos.	AJ937743
Asp f 28	12.0	C	Thioredoxin	16	pos.	AJ937744
Asp f 29	12.0	C	Thioredoxin	26	pos.	AJ937745

[a]Coding sequences: C = complete, P = partial.
[b]Incidence among patients sensitized to *A. fumigatus*.
[c]Skin prick test: NT = not tested, pos. = positive.

indicating that only a very small portion to the fungal proteins are able to induce a switch in B cells toward production of specific IgE. The reason for the allergenicity of these specific proteins is unknown. Although the primary structure of an impressive number of different allergens and the three-dimensional structures of an increasing number of allergens have been determined, they have not yet contributed to the understanding of the reasons why some proteins are allergenic and some others are not [31,32]. Notably most of the *A. fumigatus* allergens so far cloned, produced, and evaluated for allergenicity (Table 1) represent phylogenetically conserved proteins widespread in basically every living organism. Among these MnSOD (Asp f 6), P_2 acidic ribosomal protein (Asp f 8), cyclophilins (Asp f 11 and Asp f 27) and thioredoxins (Asp f 28 and Asp f 29) have been shown to belong to families of cross-reactive pan-allergens [33–42]. Interestingly these proteins show cross-reactivity also with their homologous human proteins at both cellular and humoral level. The best investigated structure at this level is human MnSOD shown to bind IgE from patients sensitized to *A. fumigatus* MnSOD *in vitro* [33], to elicit specific skin test reactions *in vivo* [37] and to be potentially involved in the pathogenesis of ABPA and atopic eczema (see below). Beside Asp f 4, Asp f 7 Asp f 9 and Asp f 16 which fail to show any sequence similarity to proteins deposited in the Swissprot database, all but one allergen show extended sequence similarities with known non-fungal proteins (Table 1). The exception is Asp f 1, a potent fungal ribotoxin which was the first fungal allergen cloned [43] and the first recombinant allergen tested in humans at all [44]. Asp f 1 reveals sequence identity to restrictocin, an 18 kDa ribotoxin produced by *Aspergillus restrictus* never described as allergen [45] that is >99% identical to Asp f 1 sequences derived from different clinical isolates of *A. fumigatus* [46,47]. All these proteins are related to ribotoxins such as α-sarcin [48] and mitogillin [49], ribonucleolytic enzymes strictly restricted to the genus *Aspergillus* [50]. Therefore, Asp f 1 can be considered as a species-specific allergen. The species-specificity of allergens seems to be a phenomenon limited to the kingdom of fungi [51], whereas allergens isolated from other allergenic sources like pollen, foods, and mites seems to represent cross-reactive structures [52]. Species-specific allergens represent of course excellent highly specific diagnostic tools (see below) because they allow, in contrast to cross-reactive allergens, tracing back primary sensitization to a clearly defined allergen source. We assume that the 81 different cDNA sequences derived from robotic-based high-throughput screenings of *A. fumigatus* cDNA libraries displayed on phage surface [21] cover almost the complete allergen repertoire of the mould, although we can not exclude that some gene products may not be expressed on phage surface and thus escape our selection strategy. However, the number of different cDNAs identified by phage surface display is clearly superior to the IgE-binding bands detected by Western blot analyses [19] and this relatively low number of structural entities, compared to the 9926 gene products predicted from the *A. fumigatus* genome sequence [29,30], demonstrate that a very limited number of proteins are allergenic. The presence of cross-reactive structures like MnSOD also described as a latex allergen [53], cyclophilin known as a birch pollen allergen [54], thioredoxin described as a prominent food allergen [41], and proteases which play an important role in house dust mite allergy [55] indicate that the whole repertoire of allergenic molecules is highly redundant and, thus, limited to a discrete number of structures.

Structural aspects of fungal allergens

X-ray crystallography is the method of choice to exactly characterize surface structures of allergens which are relevant for antibody binding [31,36,37]. The list of known allergen crystal structures with coordinates deposited in the Protein Data Bank (PDB, http://www.rcsb.org/pdb/) report about 40 structures. The best investigated allergenic source in structural terms is *Phleum pratenses* where 5 structures have been solved, followed by profilins [31]. However, only three crystal structures derived from fungal allergens, ribotoxin (Asp f 1[56]), MnSOD (Asp f 6 [36]) from *A. fumigatus* and a Xylanase from *Paecilomyces varioti* [57] have been reported so far. Recently the crystal structures of a cyclophilin of *A. fumigatus* (Asp f 11 [39]) and *Malassezia sympodialis* (Mala s 6 [40]), as well as the crystal structure of thioredoxin of *Malassezia sympodialis* (Mala s 13 [42]), have been determined at high resolution. With exception of *A. fumigatus* cyclophilin which reveals dimerization by 3D domain swapping and represents one of the first proteins with a swapped central domain [39], these structures are *per se* not new. This statement is not restricted to fungal allergens but apply to the solved allergen crystal structures in general [58]. The structural shape of Asp f 1 belongs to the class of small basic ribosome inactivating proteins with typical structural features common to the superfamily of ribonucleases [59]. MnSOD, cyclophilin, and thioredoxin represent phylogenetically highly conserved proteins and, as a consequence thereof, display very similar backbones. Although the number of solved allergen structures is

rapidly increasing, no characteristic structural or functional features of allergens have been detected so far, that would allow the prediction of the allergenicity of a protein. In contrast, elucidation of the 3D structure of allergens has essentially contributed to our understanding of cross-reactivity among homologous structures derived from phlyogenetically distant allergenic sources [36,40,60]. Based on the coordinates of a known homologous crystal structure (Table 2) it is easy to model a structure starting from the known amino acid sequence of an allergen as shown for example for enolase [61] and MnSOD [37], widespread allergens among the genus of fungi. However the primary structure of a protein contains all the clues for its tertiary structure, prediction of the three-dimensional structure of a new protein is not (yet) possible and allergenicity, e.g., the capacity to induce IgE antibody production and to activate mast cell sensitization is mostly determined by factors other than the primary sequence [32,58].

Clinical aspects of (recombinant) *A. fumigatus* allergens

In spite of many commercially available allergen extracts, only a few standardized extracts recognized by the FDA are currently available [62] and fungal extracts are not included. The diagnosis of allergy is based on clinical history, skin test reactivity to the offending allergen and *in vitro* determination of allergen-specific IgE-antibodies in serum [63]. However, the results of both, *in vivo* and *in vitro* diagnostic procedures strongly depend on the allergen preparation used [63]. One of the major problems related to the diagnosis of fungal allergy derive from the low quality of commercially available extracts [16]. Thus the accuracy of skin tests and IgE determinations in serum can be compromised if conducted with different protocols or with allergen extracts having insufficient quality control. Considering the technological advancement in production, purification, quantification, and quality control, recombinant allergens, producible as highly pure reagents satisfying the high standards for human use, can offer a standardized approach for diagnosis and treatment of allergic diseases [63]. Single recombinant allergens can also be used as vaccines for allergen-specific immunotherapy as recently demonstrated [64,65]. However, the success of such a treatment will depend on a patient-specific component-resolved diagnosis of the molecular structures to which a given individual is sensitized in order to avoid new sensitizations to additional allergens present in the extracts used for therapy [66]. In view of the complexity of most fungal allergen repertoires recombinant allergen-based immunotherapeutic approaches of fungal allergy appears as a hazardous task. In contrast, recombinant *A. fumigatus* allergens have proven to be extremely useful for diagnostic applications. As reviewed elsewhere [26] allergy diagnosis based on recombinant allergens by skin prick tests is, in contrast to those based on extracts, always consistent with the results obtained *in vitro* avoiding false positive or false negative results. This might be largely due to the fact that the extracts used to produce ImmunoCAPs, the standard *in vitro* procedure used in most clinics, are not available as skin test reagents whereas the same batch of a recombinant allergen can be used for both, *in vitro* and in *vivo tests*. For a reliable diagnosis of allergic conditions based on recombinant allergens, however, the whole allergen panel contained in a given allergenic source should be available and, with rare exceptions like birch pollen or honey bee venom [67,68], we are far from this final goal. The most important contributions of recombinant allergens to our understanding of aspergillosis derives from the demonstration of the presence of ABPA-specific allergens [22,23] and from the demonstration that some phylogenetically conserved structures induces autoreactivity to self antigens in this long lasting chronic allergic disease [33–35,69]. Although at the beginning the presence of ABPA-specific allergens was only descriptive [19], recent investigations based on the expression of MnSOD during the germination of conidiae offer a rational explanation for the earlier observations [70]. Obviously immune reactions are elicited only against antigens seen by our immune system. *A. fumigatus* as an

Table 2 Modelled fungal allergen structures

Allergen	Size (kDa)	Basic structure	Coordinates*	Reference
Cla h 6 (*C. herbarum*)	47.5	Enolase	1ONE	[72]
NTF 2 (*C. herbarum*)	14.2	Nuclear transport factor 2	1OUN	[73]
NTF 2 (*A. alternata*)	13.7	Nuclear transport factor 2	1OUN	[73]
MnSOD (*S. cerevisiae*)	22.9	MnSOD	1ABM	[37]

*PDB-coordinates of the structure used for modelling.

environmental allergenic source is able to induce sensitization in predisposed individuals through exposure to inhaled conidiae normally eliminated by the mucociliar system and by alveolar macrophages [71]. In ABPA, however, the fungus is able to colonize its environment by the formation of hyphae found in the lung of ABPA patients, but not in patients suffering from *A. fumigatus* sensitization [70]. The demonstration that Asp f 6, the fungal MnSOD, is specifically expressed only in hyphae explain why an IgE response to Asp f 6 occurs in ABPA patients but not in *A. fumigatus* sensitized individual. MnSOD [33] together with acidic ribosomal P_2 protein [34], cyclophilin [38], and thioredoxin [42] also induce strong humoral and cellular immune responses against the corresponding self antigens. The IgE-mediated autoreactivity in patients suffering from ABPA most likely contributes to exacerbation and perpetuation of the disease [35] and has been clearly traced back to molecular mimicry between conserved B-cell epitopes deriving from conserved amino acid residues scattered over the whole surface of the molecules [36,39,40,42]. Thus cloning, characterization, production and clinical evaluation of *A. fumigatus* allergens have strongly contributed to a substantial improvement of the diagnosis of ABPA and to a deeper understanding of the pathophysiological mechanisms underlying the disease.

Outlook

Large scale serological studies showed that specific IgE responses to at least four *A. fumigatus* allergens (Asp f 2, 4, 6, and 8) are highly specific for sera of patients suffering from ABPA. These disease-specific allergens allow a serological discrimination between ABPA and *A. fumigatus*-sensitization with 100% specificity and 90% sensitivity in asthmatic patients. Such discrimination is not possible using conventional fungal extracts, highlighting the potential of recombinant allergens for diagnostic purposes. However, to reconstruct the whole allergenicity of the fungal extract, more of the IgE-binding cDNA sequences identified by phage surface display need to be cloned and used to produce and clinically evaluate the encoded recombinant allergens. Many partial *A. fumigatus* cDNA allergen sequences derived from high throughput screening experiments are known. The recently published *A. fumigatus* genome sequence represents a formidable instrument to speed up complete cloning of the whole allergen repertoire of the fungus. Together with the rapidly growing knowledge about 3D structures a complete allergen repertoire will allow us to gain deep knowledge about the role of each single structure in the pathophysiology of ABPA and, perhaps, to develop a panel of allergens for the immunotherapy of the disease.

Acknowledgements

Work supported by the Swiss National Science Foundation Grants No. 31-63382.00/2 and 31-63382.00/3 and by the OPO-Foundation, Zürich.

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