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Recombinant *Aspergillus fumigatus* Allergens: From the Nucleotide Sequences to Clinical Applications

Key Words

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Abstract

Members of the genus *Aspergillus* are opportunistic pathogens for cold- and warm-blooded animals. They are associated with an impressive spectrum of diseases in humans, ranging from benign colonization of the lung to life-threatening diseases such as invasive aspergillosis or allergic bronchopulmonary aspergillosis (ABPA). *Aspergillus fumigatus* is the etiological agent identified in most of the *Aspergillus*-related human diseases and is therefore of particular clinical relevance. A major problem in the diagnosis of *A. fumigatus*-related complications arises from the lack of standardized fungal extracts. The allergenicity of commercial *A. fumigatus* extracts depends on many factors including the strain used, growth conditions, harvesting and extraction procedures hampering the development of diagnostic reagents suitable for the discrimination between *A. fumigatus*-related diseases. Molecular DNA/RNA technologies and biotechnological procedures allow cloning, characterization and production of large amounts of single, highly pure allergens. The development of phage surface display technologies for cloning cDNAs has speeded up the isolation of candidate cDNAs encoding allergens. Screening of an *A. fumigatus* cDNA library displayed on the surface of phage M13 allowed characterization and production of a panel of allergens identified as proteins with known biological function or as allergens without sequence homology to known proteins. They belong to two functional categories: secreted and cytoplasmic proteins. Secreted allergens are recognized by serum IgE of *A. fumigatus*-sensitized individuals with or without ABPA, whereas nonsecreted allergens are exclusively recognized by serum IgE of ABPA patients. The dissection of IgE-mediated immune responses to single *A. fumigatus* allergens allows a discrimination between ABPA and *A. fumigatus* sensitization with high specificity and sensitivity demonstrating the power of recombinant allergens for the differential diagnosis of *A. fumigatus*-related pulmonary complications.

Introduction

Moulds and fungi play a predominant role in our daily life and in the environment where they are ubiquitously distributed and contribute to the decay of organic matter [1]. The

most important industrial applications of fungi are related to fermentation technology for the production of alcohol, improvement of food quality, production of enzymes, immunosuppressive drugs and antibiotics [2]. The systematic classification subdivides fungi into four classes, essentially

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based on how they produce sexual spores: the Phycomycetes, Ascomycetes, Basidiomycetes and Deuteromycetes [3]. The last class includes most of the industrially important strains as well as the majority of the opportunistic fungal pathogens. They grow in mycelial form without production of sexual spores, and are therefore also referred to as fungi imperfecti [4]. Among Deuteromycetes, *Aspergillus* species are considered as the most important pathogens [5]. Although the genus *Aspergillus* includes 132 species and 18 variants thereof [6], only a few are recognized as opportunistic pathogens. *Aspergillus fumigatus* is the etiological agent isolated in about 80% of the *Aspergillus* infections in humans [7–9]. However, opportunistic mycoses such as aspergillosis and candidiasis rarely affect healthy individuals. The type of subject-specific immunodeficiency is believed to account for susceptibility and severity of opportunistic fungal infections more than the virulence of the fungus itself [10]. Recognized risk factors for developing invasive mycoses are represented by both the degree and duration of neutropenia. Recently, two studies showed the importance of polymorphonuclear cells in the phagocytosis of important pathogenic yeasts [11] and in the damage of hyphae of *A. fumigatus* [12]. Beside recent progress achieved in understanding the pathophysiological mechanisms of fungal infections [13] problems related to a reliable diagnosis remain to be solved. Clinically, the most convincing diagnostic evidence for a fungal infection is provided by detection of the fungus by direct microscopic examination, isolation and cultivation. Since *Aspergillus* species are frequent contaminants in cultures, the pathogenic significance of a particular isolate is difficult to evaluate. Moreover, *Aspergillus* can be recovered from the sputum of healthy persons [14], individuals with *Aspergillus* allergy react against allergen exposure without apparent fungal infections and even in the case of patients with pulmonary *Aspergillus* infiltrates, cultivation of the mold is not always successful [15]. Therefore, demonstration of circulating antibodies raised against fungal components is considered as an important criterion for the diagnosis of *Aspergillus*-related diseases [16]. Hallmarks in the diagnosis of a clinical suspicion of *A. fumigatus* sensitization are the radioallergosorbent test (RAST) and prick or intradermal skin tests with fungal extracts, corroborated by various serological methods [16]. The clinical relevance of an IgE-mediated *A. fumigatus* sensitization determined by skin test or by the demonstration of allergen-specific IgE is only reliable, if defined antigens or allergens are applied [16]. Early and precise diagnosis of *Aspergillus*-induced respiratory diseases is essential for patient management differentiating *A. fumigatus* allergy from allergic bronchopulmonary aspergillosis (ABPA) [9]. ABPA may

require treatment with corticosteroids to decelerate progressive impairment of pulmonary functions. The currently available fungal extracts are, however, not satisfactory and need to be improved for dependable results. In this regard, cloning, characterization and production of recombinant allergens from molds can substantially contribute to improve the diagnosis of fungal infections. The availability of pure recombinant allergens will allow determination of allergenic ‘fingerprints’ on individuals suffering from *A. fumigatus*-related complications. The correlation of reactivity patterns of single individuals to the clinical findings may substantially contribute to improve differential diagnosis and treatment of the diseases.

The Clinical Spectrum of Aspergillosis

Aspergillosis is a complex disease with many faces which may cause a wide spectrum of pulmonary complications in humans and animals [5, 8, 9] ranging from benign colonization of the lung to life-threatening complications such as invasive aspergillosis or ABPA. The different diseases related to *A. fumigatus* which usually remain mutually exclusive can be broadly classified according to their clinical conditions into systemic mycoses, saprophytic colonization and allergic diseases.

Systemic Invasive Aspergillosis

Invasive aspergillosis (IA) is considered as an infection affecting immunocompromised subjects [17] suffering from prolonged granulocytopenia [18, 19], leukemia or other hematological malignancies [17]. In particular patients receiving chemotherapy, undergoing total-body irradiation, organ transplantation or prolonged high-dose corticosteroid treatment are prone to IA [17, 20]. Therefore, the increased prevalence of opportunistic mycoses is largely due to advances in medical awareness [15]. Association of IA in patients with acquired immunodeficiency (i.e. AIDS), who would be expected to be at risk for the development of fungal diseases, is still controversial [21]. Because of the unusual predisposition of IA patients, namely the lack of a functionally normal immune system due pathogenic or intentional immunosuppression, there is little or no antibody response elicited to fungal antigens in the early stage of the disease [16]. Since patients develop an immune response to *Aspergillus* antigens only at the terminal stage of the disease, an immunodiagnosis of IA is likely to come too late. Therefore, it is essential to develop rapid assays able to diagnose IA early, like detection of circulating fungal antigens in body fluids [22].

Saprophytic Bronchopulmonary Colonization

Bronchopulmonary colonization refers to fungal infestation of airways without evidence for direct tissue damage. Some infected individuals may mount an immune response resulting in serum precipitins and immediate or late skin reactions to *Aspergillus* antigens [16]. Saprophytic colonization in its usual uncomplicated form is not an infection in the strict sense. However, the presence of the fungus growing in the tracheobronchial tree may adversely affect the immune responsiveness in a host and interfere with the immunodiagnosis of more severe fungal diseases [16].

Aspergilloma

Aspergilloma also termed mycetoma or fungus ball is a saprophytic manifestation of an *Aspergillus* growing in preformed lung cavities of patients with bronchogenic carcinoma, tuberculosis, sarcoidosis or, rarely, ABPA [23]. Pulmonary aspergillomas can often be diagnosed by their characteristic appearance on roentgenographs [16]. The main immunological response to aspergilloma regards the formation of serum IgG which may interfere with the diagnosis of other *A. fumigatus*-related diseases [24].

Allergic Rhinitis and Allergic Sinusitis

Allergic rhinitis develops as a result of interaction of allergens and IgE antibodies fixed to specific receptors expressed by mast cells on the epithelial surface of the nasal mucosa. *Aspergillus* and a variety of other fungi can act as causative agents for allergic rhinitis [25]. The symptoms of fungus-induced allergic rhinitis are indistinguishable from those caused by other allergens [16]. The diagnosis of allergic rhinitis is based on case history, skin reactivity and RAST positivity to the appropriate allergens [25].

Allergic fungal sinusitis, predominantly occurring in atopic individuals [26], is chronic and affects multiple sinuses without tissue invasion. Several fungi including *Aspergillus* have been implicated in allergic sinusitis. The patients may show skin reactivity, precipitating antibodies and elevated allergen-specific IgG and IgE levels [16, 26], immunological characteristic found also in ABPA [27].

Hypersensitivity Pneumonitis

Hypersensitivity pneumonitis (HP) is related to the exposure of susceptible individuals to different irritants including *Aspergillus* [16, 28]. Sensitization occurs following inhalation of antigens and development of symptoms several hours after exposure to the irritants. Included in this group of occupational disease are farmer's lung, bagassosis and mushroom worker's lung. In the untreated chronic form of HP, irreversible lung damage frequently occurs [28]. The

complexity and cross-reactivity of antigens and the variable immunological responses of patients are the major limitations in the diagnosis of HP [16, 28].

Allergic Asthma

Extrinsic allergic asthma is a chronic disease characterized by increased responsiveness of the tracheobronchial tree to exogenous stimuli, particularly allergen exposure. Several fungi, including *Aspergillus* species, have been implicated in allergic asthma. The symptoms due to mold asthma can be distinguished from those attributable to other allergen-induced asthma by history of exposure, skin test reactivity or in vitro demonstration of *Aspergillus*-specific IgE in patients' sera [29, 30]. The lack of reliable fungal extracts poses a problem in the development of standardized in vivo or in vitro tests to diagnose *A. fumigatus* allergy which needs to be discriminated from ABPA for optimal patient management [31, 32].

Allergic Bronchopulmonary Aspergillosis

ABPA is the most severe allergic pulmonary complication caused by *Aspergillus* species, mainly *A. fumigatus*. ABPA is the result of hypersensitivity to *Aspergillus* antigens mainly in patients suffering from atopic asthma [31–33] or cystic fibrosis (CF) [34]. Although originally considered a rarity [35], ABPA is currently recognized with much greater frequency [36]. The disease has been reported to occur in about 15% of the asthmatic patients sensitized to *A. fumigatus* [37], while in patients with CF the incidence varies from 10 to 35% [34, 38]. ABPA has been described as an immune disease that ranges from asthma to fatal destructive lung disease with defined clinical, serological, radiological and pathological features [31, 39, 40]. Therefore, ABPA should be ruled out in patients at risk which exhibit immediate cutaneous reactivity to *A. fumigatus* [32]. The diagnostic criteria for ABPA are asthma or cystic fibrosis, blood eosinophilia ($>1,000/\text{mm}^3$), immediate cutaneous reactivity and precipitating antibodies to *A. fumigatus* extracts, elevated total serum IgE levels, a history of roentgenographic infiltrates, bronchiectasis, and elevated specific serum IgE and IgG levels to *A. fumigatus* [34, 39, 40–42]. When all criteria are present, the diagnosis can be readily made [41]. However, all of the eight criteria are rarely present at the same time even in classic ABPA patients with central bronchiectasis. With the exception of bronchiectasis and to some extent elevated specific serum IgE and IgG to *A. fumigatus*, the diagnostic criteria are not specific for ABPA [41]. Moreover, pulmonary infiltrates and central bronchiectasis are common in patients with cystic fibrosis also in the absence of *A. fumigatus* sensitization making a

diagnosis of ABPA in these patients even more difficult [34, 42]. Therefore, serological identification of ABPA has a great diagnostic potential [16].

***A. fumigatus* Antigens**

The major problem in the immunodiagnosis of *A. fumigatus*-related diseases is related to the molecular complexity of the fungus. Extracts of *A. fumigatus* are mixtures containing up to 200 different proteins and glycoproteins [29, 43] and low molecular weight compounds [44, 45]. The fungus has been reported to be able to produce more than 40 IgE-binding components [29] generating complex immunopatterns when extracts are assayed with sera from allergic individuals in Western blots [46, 47]. Sera from different patients recognize highly variable patterns of IgE-binding proteins [29, 47] depending on the sensitization of the subject, extract used and stage of the disease [47]. Problems encountered in the standardization of allergenic extracts from *A. fumigatus* are due to different patterns of allergens produced by different clinical isolates [48], batch to batch variations [49], time-dependent liberation of IgE-binding components during the fungal growth [50], instability of the extract due to protease content [51] and culture conditions used to grow the fungus [52]. These problems have been recognized and efforts to standardize the production of fungal extracts [53, 54] as well as to isolate single components with biochemical methods [55, 56] have been undertaken. However, even biochemically purified allergens may be contaminated with traces of IgE-binding components of similar biochemical properties as demonstrated for one of the major allergens of *A. fumigatus*, Asp f 1 [57]. The clinical relevance of such contaminants is difficult to assess, however, in view of the extremely low amounts of allergen required to elicit a positive type 1 skin reaction which are in the 10^{-12} gram range [58]; the presence or lack of such contaminants in the skin test solution may strongly influence the outcome. Even if conditions of growth, strain used, harvesting and allergen extraction procedures are kept constant, considerable batch to batch variation occurs [59]. Molecular biology offers the possibility to produce large quantities of perfectly standardized single highly pure allergens which may be used to reconstitute the complex IgE binding pattern of extracts by mixing single components, thus generating standardized diagnostic reagents.

Cloning Allergens from *A. fumigatus*: The Filamentous Phage Approach

A wide variety of cloning techniques have been successfully used to isolate complementary DNAs (cDNA) encoding allergens and during the past few years an impressive number of primary structures of allergenic proteins have been elucidated [for reviews see 60–63]. Starting from cDNA [60, 64], the fastest method to clone a gene is amplification of the desired DNA fragment by the polymerase chain reaction (PCR) [65]. PCR has been successfully used to clone allergens and allergen isoforms [57, 66, 67]. However, a strong limitation of the PCR technology lies in the requirement of sequence information, at least from the N-terminal sequence of the protein, in order to be able to design primers for the PCR amplification reaction. N-terminal sequence determination is not expected to be easily achievable for gene products of rare mRNA species expressed at a low level. The most frequently used strategy to clone allergens consists of the construction of cDNA expression libraries which can be screened with IgE-containing sera from allergic patients or with antibodies raised against purified allergens [68, 69]. However, libraries constructed in bacteriophage lambda vectors as well as plasmid systems [64, 68, 69] require immobilization to solid phase supports for screening. The immobilization of a library hampers selective enrichment of clones expressing proteins of interest. Moreover, large numbers of recombinant clones have to be screened to find products encoded by rare mRNA species, a strong limitation for the screening of complex allergenic systems such as ragweed [70] or *A. fumigatus* [29]. A rational approach to clone allergens and to clone cDNAs at all would be to selectively enrich clones expressing products of interest from cDNA expression libraries. A prerequisite to achieve this goal is the physical linkage between gene product and its cDNA, a requirement not fulfilled by most available cloning systems. Filamentous phages are ideal for the construction of such cloning systems because they fulfill the basic requirement of linking expressed cDNA products displayed on the phage surface to the genetic information required for their production [71, 72] (fig. 1A). Phage displaying a protein, and therefore carrying the encoding cDNA, may be readily isolated from large libraries by successive rounds of phage growth and selection on surfaces coated with the ligand (fig. 1B). The gene III (gIII) coat protein located at the tip of the filamentous phage M13 is responsible for the attachment of phage to the bacterial F pilus [72] and has been used to display peptide epitopes [73], proteins [74] and immunoglobulin fragments [75, 76] on the surface of phage. Phages expressing fusion proteins of

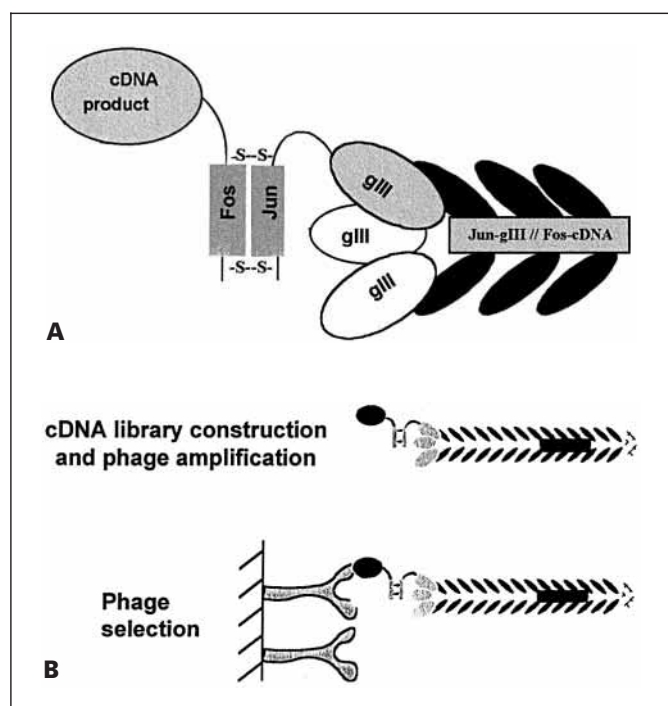


Fig. 1. **A** Schematic representation of the physical linkage between cDNA products displayed on phage surface and the genetic information required for their production achieved by Jun/Fos heterodimerization. **B** Principle of selective enrichment for phage displaying a protein with affinity to a ligand coated on solid phase surfaces (biopanning).

interest were isolated from large display libraries by selection with a variety of solid phase immobilized ligands [77]. In spite of rapid progress achieved in disparate fields ranging from epitope screening [78] to the construction of catalytic antibodies [79] and semisynthetic random mutagenized Fab libraries [80], display systems allowing selection of genes from cDNA libraries were not available because translational stop codons present at the 3'-end of nontranslated regions of eukaryotic mRNA [81] prevent construction of fusion proteins N-terminal to the gIII gene product, while fusions to the membrane-bound C-terminus would hamper the incorporation of the fusion protein into the phage coat [82]. To covalently link expressed cDNA products to the phage surface, a modified gIII product, obtained by fusing the DNA encoding the *jun* leucine zipper [83] N-terminal to the viral coat protein, was expressed from a *LacZ* promoter and secreted into the periplasmic space of *Escherichia coli* by a *pel* B leader peptide, thereby being structurally incorporated into phage particles during infection with a helper phage [84–87]. Using a second *LacZ* promoter of the phagemid, the *fos* leucine zipper [83] coex-

Table 1. Typical enrichment of phage from a cDNA phage surface display library by biopanning

Round of panning	Phage input ^a	Phage output ^b	Enrichment factor ^c	IgE-specific phage/phage tested ^d
1	1.8×10^{11}	4.5×10^3	5.6×10^{-6}	0/10
2	8.6×10^{10}	2.4×10^4	2.5×10^{-5}	0/10
3	9.3×10^{10}	4.6×10^4	3.8×10^{-5}	0/10
4	6.5×10^{10}	1.1×10^5	1.9×10^{-4}	1/10
5	1.8×10^{11}	3.8×10^6	2.3×10^{-3}	8/10
6	2.1×10^{11}	8.4×10^6	4.0×10^{-3}	10/10
7	9.4×10^{10}	5.8×10^7	6.2×10^{-2}	10/10

Phages displaying cDNA products from an *A. fumigatus* expression library were applied to a single well of a microtiter plate coated with human serum IgE [85, 86]. After adsorption and extensive washing adherent phages were eluted and used to infect *E. coli* for a further round of phage growth and selection.

^a Number of phages applied to a single well of a microtiter plate.

^b Number of phages eluted from the well after washing.

^c Percentage yield of phages from each round of panning [% yield = number of phages eluted $\times$ 100]/(number of phages applied)].

^d Single colonies from plates used to titre phagemids were grown in liquid culture, phage-induced and purified. Phages coated directly to microtiter plates were tested for IgE binding capacity by an IgE-specific ELISA [84, 90]. IgE-specific phage represents phage able to bind serum IgE/number of phages tested.

pressed as N-terminal fusion peptide to cDNA gene products, was secreted into the periplasmic space of *E. coli* [82]. The incorporation of the gIII-Jun fusion into phage particles provides a covalent link for random recombinant cDNA products decorated with the Fos leucine zipper by Jun-Fos heterodimerization [84]. The developed phagemid pJuFo [84–87] (fig. 1A) allows expression and display of cDNA libraries on phage surface and application of the powerful screening technology based on biopanning [77, 82] (fig. 1B). For the isolation of allergens the use of capture antibodies has proved to be very effective because monoclonal antibodies raised against the constant C_e domains of IgE do not interact with the antibody binding site and because the coated anti-IgE selectively immobilize IgE antibodies without denaturation [88]. Therefore, after washing away antibodies of other isotypes together with all other serum components, a specific surface able to adsorb phage displaying IgE binding proteins is obtained [85–87]. The application of pJuFo to display and select cDNAs from an *A. fumigatus* library [85, 86] yielded a variety of phage able to bind IgE from sera of *A. fumigatus*-sensitized patients (table 1).

Table 2. Physiochemical and biochemical characteristics of the allergens

	Allergens ^a							
	rAsp f 1	rAsp f 3	rAsp f 4	rAsp f 5	rAsp f 6	rAsp f 7	rAsp f 9	rAsp f 10
GenBank accession No.	S339330	U58050	NA	Z34024	U53561	NA	NA	X85092
Molecular weight ^b , kD	16.86	18.45	30.04	42.06	23.03	11.63	33.7	34.4
Amino acid residues ^b	149	168	286	388	207	112	302	325
Isoelectric point ^b	9.60	5.34	4.45	4.9	8.02	4.43	4.91	3.95
Biochemical function	Ribotoxin	PMP	NS	MEP	MnSOD	NS	NS	PEP
Production yield ^c , mg/l	150	38	26	45	220	65	120	75
Purity ^d , %	>95	>95	>95	>95	>95	>95	>95	>95
Reference No.	57, 90	93	86	86, 95	86	86	86	86, 102

NA = Not available; PMP = peroxisomal membrane protein; NS = allergens without sequence similarity to known proteins; MEP = metalloprotease; MnSOD = manganese superoxide dismutase.

^a Nomenclature officially approved [62].

^b Values calculated from the deduced amino acid sequences without (His)₆ tag.

^c Quantified after Ni²⁺-NTA affinity chromatography purification.

^d Estimated from lack of contaminating bands in SDS-PAGE and Western blots.

Compared with the screening of λ -libraries, the screening procedure for cDNA libraries displayed on phage surfaces has manifold advantages. The most important one results from the fact that the phage library is kept in liquid phase, where only phages with affinity to the ligand are retained on the surface after washing [82, 87]. Phages are biological systems able to autoreplicate in a suitable host and therefore, a desorbed phage can be used to infect *E. coli* in order to amplify phages with affinity for the ligand [82]. Successive rounds of phage growth and selection allow enrichment of phage-displaying proteins with affinity for the ligand (table 1). After selection, aliquots of 10^{10} to 10^{13} colony-forming units of each candidate phage can be readily analyzed by SDS-PAGE and by Western blots [84, 85]. The pJuFo phagemid represents an universally applicable system useful for rapid isolation of cDNAs by gene product-ligand interaction [77, 87] able to enrich specific phages present in a library at a frequency as low as one phage in a population of 1×10^7 [84].

Analysis of cDNA Inserts Encoding Allergens

The amino acid sequences deduced from the nucleotide sequences of inserts harbored by a phage displaying IgE-binding proteins from *A. fumigatus* can be subdivided into two classes: those with homology to described proteins and others without any homology to sequences deposited in the gene banks (table 2).

An insert of 690-bp codes for Asp f 1, one of the major allergens of *A. fumigatus*. The allergen first described as an 18-kD allergen has been cloned, sequenced and produced as recombinant protein in *E. coli* [57]. Asp f 1 is identical to restrictocin cloned from *Aspergillus restrictus* [89] and >99% identical to the Asp f 1 sequences derived from different clinical isolates of *A. fumigatus* [90, 91]. All these proteins are related to ribotoxins of the mitogillin family [55] produced by members of the genus *Aspergillus*. Ribotoxins inhibit translation by cleaving a conserved region of the 28 S rRNA and are potent inhibitors of eukaryotic protein synthesis [92].

The allergen Asp f 3, a major allergen of the fungus, is encoded by an insert of 617 bp. The deduced amino acid sequence of this cDNA predicts a protein of 168 amino acids with a molecular mass of 18.5 kD which shows 36% identity and 58% similarity with the amino acid sequences of two peroxisomal proteins from *Candida boidinii* reported as PMPA and PMPB [93, 94]. Western blots and inhibition experiments with sera of individuals sensitized to Asp f 3 showed that rAsp f 3 and the two *Candida* proteins share common IgE-binding epitopes [93].

A clone containing an insert of 751 bp revealed strong homology with nucleotide sequences encoding manganese superoxide dismutases (MnSOD). The deduced amino acid sequence of this cDNA was homologous to MnSOD, showing the highest sequence identity of 48–52% to the human, fruit fly, gum tree, yeast, *E. coli* and *Mycobacterium leprae* enzymes [95, 96]. MnSOD is one of three enzyme forms

that catalyze the dismutation of superoxide radicals and are considered as the first line of defense against the toxicity of oxygen-related radicals postulated to underlie the pathogenesis of various inflammatory diseases [97]. Multiple sequence alignments show that the *A. fumigatus* MnSOD (rAsp f 6) shares high homology with the human MnSOD (51.8% identity, 67.2% similarity). Notably, both recombinant *A. fumigatus* and human MnSOD induce proliferation in peripheral blood mononuclear cells of *A. fumigatus* allergics with specific IgE to *A. fumigatus* MnSOD. Moreover, the two recombinant MnSODs elicited Type I skin reactions in individuals sensitized to the fungal enzyme providing evidence for autoreactivity to human MnSOD in allergic patients sensitized to the environmental *A. fumigatus* allergen [95].

Two allergens, rAsp f 5 and rAsp f 10, belong to the family of secreted proteases claimed to be allergens [98] and virulence factors of *A. fumigatus* [99]. However, the first cloned protease from this fungus, a secreted alkaline protease (ALP) [100], does not elicit IgE antibodies in individuals sensitized to *A. fumigatus* [101]. The fungus secretes an aspartic protease (PEP) [102] a serine (ALP) [100] and a metalloprotease (MEP) [103]. The genes encoding ALP and MEP were cloned and disrupted in wild type *A. fumigatus* strains. The *alp*, *mep* mutant strain was completely devoid of proteolytic activity in vitro but showed no difference in the mortality curves of infected mice compared to wild type strains [103]. Therefore the role of secreted proteases in virulence of *A. fumigatus* is questionable [99]. Both, PEP and MEP have not been described as allergens. However, phages displaying these two proteases enriched from an *A. fumigatus* cDNA library using serum IgE of *A. fumigatus*-sensitized individuals were shown to exhibit IgE binding properties (see below).

In contrast to the cDNAs encoding the allergens described which can clearly be assigned to proteins with known function, the nucleotide sequence of other cDNAs do not share significant homology to any known protein and the gene products are only characterized by the function for which they were selected: IgE binding (table 2).

Production and Characterization of Recombinant Allergens

For investigations of biochemical properties and for diagnostic applications, large amounts of recombinant allergens are needed. Expression of recombinant proteins in *E. coli* can be obtained at low level (0.3–3% total cellular protein) with vectors able to direct the produced protein into

the periplasmic space of the host or at high level (up to 40% total cellular protein) with vectors leading to the accumulation of insoluble inclusion body protein [reviewed in 104]. Recently described vectors, constructed for the production of proteins tagged with six adjacent histidine residues, allow a high level expression and one step purification of recombinant proteins by Ni²⁺ affinity chromatography [105] and were used to produce all *A. fumigatus* allergens (table 2). From our experience, the short [His]₆ tag does not interact with sera of allergic individuals. Therefore, [His]₆ allergens can be used in a straightforward way for in vitro and in vivo applications. However, for each single allergen an in vitro refolding protocol needs to be established [106] which may be uncomplicated as in the case of Asp f 1 [57] or Asp f 6 [95] or extremely laborious as in the case of bee venom phospholipase A₂ (PLA₂) [58, 107].

A major concern with regard to the use of recombinant allergens for the diagnosis of allergic diseases is whether they are functionally comparable to their native counterparts. Antibodies of human sources mainly recognize conformational epitopes [108] and require therefore a correct three-dimensional structure to interact with the antigen. Unfortunately, simple and direct assay systems to assess the correct folding of recombinant proteins are not available. However, a wide number of allergens belong to the class of enzymes which requires a correct three-dimensional structure in order to reconstitute an adequate spatial orientation of the residues involved in the active center needed for the catalytic activity [109]. Therefore, recombinant proteins able to exert a predicted enzymatic reaction can be assumed to have a native-like three-dimensional conformation [58, 107]. *A. fumigatus* allergens [57, 93, 95] and several other allergens showing IgE-binding properties comparable to those of native allergens are well expressed in *E. coli* [60, 61]. The rapid progress in industrial production and handling of recombinant proteins for clinical use [110] will allow the production of large amounts of allergens with a high batch-to-batch consistency satisfying the quality requirements of medicinal products manufactured by recombinant DNA technology.

Use of Recombinant *A. fumigatus* Allergens for in vitro Diagnostics

Currently, the diagnosis of sensitization to *A. fumigatus* is based on clinical history and on extracts prepared from fungal cultures. The reliability of the diagnosis largely depends on the quality of the extract used, which is directly influenced by several factors including the raw material

used [48], protease content and storage conditions [51]. Even standardized extracts are heterogeneous mixtures of proteins [29, 43] subjected to batch variation with unknown allergen content. Therefore, in vitro or in vivo tests using commercial extracts from different sources may show some degree of discrepancy [111]. The use of recombinant allergens could provide useful tools to characterize the differential sensitization pattern of individuals in relation to the disease. Therefore, all allergens described have been evaluated in vitro for their predictive diagnostic value. The binding of IgE antibodies to recombinant allergens was assessed by a direct solid-phase ELISA [57, 112].

For a large scale evaluation of the in vitro diagnostic value of recombinant *A. fumigatus* allergens sera from 54 patients suffering from ABPA and from 35 allergic asthmatics with *A. fumigatus* sensitization but without ABPA were selected. All patients had asthma and met the guidelines for the diagnosis and management of asthma [113]. As negative controls, sera from 10 allergic asthmatics without *A. fumigatus* sensitization and 10 healthy individuals without history of atopy were investigated. In contrast to sera from sensitized individuals, the serum samples of the 20 control individuals showed allergen-specific IgE antibody values below the background value for all recombinant allergens [114].

The serological data obtained reflect the results from Western blot analyses with fungal extracts which demonstrate that sensitized subjects recognize a variable pattern of *A. fumigatus* allergens [29, 46, 47]. However, the availability of single, highly pure recombinant allergens allows a more detailed dissection of the immune response to *A. fumigatus* allergens in allergic asthmatics and in patients suffering from ABPA. rAsp f 1 and rAsp f 3 can be regarded as major allergens of the fungus [93, 112]. From the total of 89 sera analyzed an overall sensitization of 69 and 76% for rAsp f 1 and rAsp f 3, respectively, was detected. Comparison of specific IgE antibody levels for both allergens between patients with ABPA and *A. fumigatus*-allergic subjects revealed significant differences ($p < 0.001$), the ABPA patients having higher IgE levels for both allergens. However, the strong overlap between the values of the two groups does not allow to differentiate ABPA from *A. fumigatus* allergy by serological determination of specific IgE raised against Asp f 1 and Asp f 3 [114]. Important for an overall diagnosis of allergy to *A. fumigatus* is the fact that $>97\%$ of the sera investigated showed relevant levels of specific IgE against rAsp f 1 or rAsp f 3. Due to the study design devoted to detect allergens specific for an immune response in ABPA patients, this group is over represented compared to the group of allergics. Allergy to *A. fumigatus* has been estimated to be in the range of 30% among the asthmatics [115]. In

contrast, the incidence of ABPA is estimated to reach values around 15% among the allergic asthmatics sensitized to *A. fumigatus* [37]. The rAsp f 1- and rAsp f 3-based diagnosis reach, if only the 35 sera of the allergic asthmatics sensitized to *A. fumigatus* are considered, a sensitivity of 70%. The sensitivity of the diagnosis among the *A. fumigatus* allergic asthmatics is dramatically increased if the results of the serology obtained with rAsp f 5 are further included. The allergen is recognized by 26 of the 35 sera from the allergic asthmatics and by 50 of the 54 sera from patients with ABPA. From the combination of the IgE-specific serology with rAsp f 1, rAsp f 3 and rAsp f 5, 34 out of 35 sera from allergic asthmatics sensitized to *A. fumigatus* can be identified as positive to at least one of the allergens, resulting in a diagnostic sensitivity in the range of 97%.

rAsp f 7 and rAsp f 10 represent minor allergens which are recognized only by 35 and 16, respectively, of the total of 89 sera tested. According to the currently used terminology, allergens are designated either as major or minor depending on whether more or less than 50% of the patients' sera tested contains the respective allergen-specific IgE [62]. rAsp f 7 and rAsp f 10 do not contribute significantly to the improvement of the diagnosis of a sensitization to *A. fumigatus* as shown in table 3. The biochemical function and the cellular localization of Asp f 7 are unknown. In contrast, Asp f 10 corresponds to a secreted aspartic proteinase (PEP) produced by the fungus only in acid media [102]. However, PEP was shown to be present in fungal infiltrates in a fatal case of invasive aspergillosis indicating that the protease can be produced and secreted in the lung tissue of infected patients [102].

The immune response to rAsp f 9 gives an intermediate picture compared to the results obtained with rAsp f 1, rAsp f 3 and rAsp f 5 which are recognized at high frequency by the sera of both *A. fumigatus*-allergic asthmatics and ABPA patients, and the immune response raised against rAsp f 4 and rAsp f 6 which occurs prevalently in patients suffering from ABPA (see below). The data demonstrate that the majority of the sera from ABPA patients tested (89%) had detectable levels of rAsp f 9-specific IgE. However, 11 out of 35 sera from allergic asthmatics sensitized to *A. fumigatus* also showed significant amounts of rAsp f 9-specific IgE (table 3). According to the definition [62] rAsp f 9 is a major allergen since 66% out of 89 sera tested score positive. In contrast, if only the 35 sera of the allergic asthmatics sensitized to *A. fumigatus* are considered, rAsp f 9 should clearly be classified as a minor allergen in view of the low number of positive scores reaching 31% of the sera tested. This example shows the ambiguity and the limitation of the definition. However, the sequence of

Table 3. Sensitization of asthmatic patients with ABPA or *A. fumigatus* allergy to different recombinant *A. fumigatus* allergens

Subjects groups	rAsp f 1	rAsp f 3	rAsp f 4	rAsp f 5	rAsp f 6	rAsp f 7	rAsp f 9	rAsp f 10
ABPA (n = 54)	173±510 45 (83)	412±454 51 (94)	47±67 42 (78)	54±67 50 (93)	54±110 30 (56)	37±63 25 (46)	51 63 48 (89)	15±35 15 (28)
Allergics (n = 35)	29±53 16 (46)	110±238 17 (49)	2±1 0 (0)	34±58 26 (74)	2±1 0 (0)	5±6 10 (29)	14±36 11 (31)	2±1 1 (3)
ABPA and allergics (n = 89)	116±404 61 (69)	293±410 68 (76)	29±56 42 (47)	46±64 76 (85)	33±89 30 (34)	25±52 35 (39)	36±57 59 (66)	10±27 16 (18)
Healthy controls (n = 20)	<5 0 (0)	<5 0 (0)	<5 0 (0)	<5 0 (0)	<5 0 (0)	<5 0 (0)	<5 0 (0)	<5 0 (0)

For each group of subjects the upper row of figures represents the mean value of IgE binding to the allergen ± SD (ELISA units/ml), the lower row the number (percentage in parentheses) of individuals sensitized to the allergen. Responses to the ABPA-specific allergens are in bold print.

Asp f 9 does not show any homology to known proteins and the question of whether Asp f 9 is secreted or intracellular localized remains open.

The serological investigations concerning Asp f 4 and Asp f 6 show a completely different picture compared to the serological outcome obtained with the other *A. fumigatus* allergens (table 3). Specific IgE against these allergens was not detectable in the 35 sera from allergic asthmatics sensitized to the fungus. In contrast, the 54 sera from ABPA patients recognized rAsp f 4 and rAsp f 6 with a frequency of 78 and 56%, respectively, whereas 49 sera recognized at least one of these two allergens (table 3). Therefore, serological diagnosis of ABPA with the two allergens has a specificity of 100% and reaches a sensitivity of >90% [114]. The MnSOD (rAsp f 6) represents a strictly intracellular enzyme. The biochemical function of Asp f 4 remains unknown, however, preliminary experiments devoted to localizing the protein with monoclonal antibodies raised against Asp f 4 indicate that the protein is not secreted by the fungus. Therefore, both proteins are unlikely to be present in free form as aeroallergens explaining the lack of specific IgE raised against these allergens in allergic asthmatics sensitized to *A. fumigatus*. In contrast, patients suffering from ABPA have or had the fungus growing in the lung [31, 116] and as a result of fungal damage related to cellular defense mechanisms [12, 15] become also exposed to nonsecreted proteins. Therefore, patients suffering from ABPA are also able to mount an immune response to intracellular *A. fumigatus* proteins [114].

Different Reactivity to Recombinant *A. fumigatus* Allergens in Patients with Atopic Dermatitis Sensitized to *A. fumigatus*

As pointed out in the introduction *A. fumigatus* affects mainly individuals with asthma or CF. However, patients with atopic dermatitis (AD, neurodermitis costitutionalis atopica) can be sensitized to *A. fumigatus* in the absence of asthma [117]. The individuals suffering from AD and *A. fumigatus* sensitization but without asthma showed unexpected serological responses to rAsp f 1. It has been reported that Asp f 1 elicits antibody responses in 60–85% of the subjects sensitized to the fungus [56, 112]. The results of rAsp f 1-specific serology in AD patients were compared with those obtained from a group of *A. fumigatus*-allergic asthmatics without ABPA and from healthy control individuals. All patients with AD as well as the *A. fumigatus*-allergic asthmatics showed a RAST class 2 to *A. fumigatus*. The rAsp f 1-specific IgE determination was clearly positive in 6 out of 10 allergic asthmatics with *A. fumigatus* sensitization, as expected from the incidence of Asp f 1 sensitization reported [56, 112]. According to the selection criteria which were, with exception of the absence of asthma, comparable to the criteria used to select the *A. fumigatus*-sensitized subjects with asthma and to the reported incidence of rAsp f 1 sensitization among *A. fumigatus* allergics, a high frequency of IgE-positive sera among the individuals suffering from AD was presumed. None of the 15 sera from this group of individuals showed detectable amounts of rAsp f 1-specific IgE [117]. The results of the serological investigations extended to all available recombinant *A. fumigatus* allergens are not conclusive. In fact, specific IgE

against rAsp f 3, 4, 5, 7, 9 and 10 was not detectable in any of the sera. The lack of an IgE response to this panel of allergens in patients with AD remains to be explained [117].

Serological Discrimination between Sensitization to *A. fumigatus* and ABPA in Patients with CF

The ability of *A. fumigatus* to colonize the respiratory tract of patients with CF is well recognized [118] and found in up to 57% of the cases [42]. Sensitization to *A. fumigatus* is frequent in CF patients as demonstrated by immediate skin reactivity to *A. fumigatus* extracts in nearly 60% of the patients with CF [111, 119, 120]. The prevalence of ABPA among patients with CF sensitized to *A. fumigatus* has been reported at rates varying between 0.5 and 11% [34, 119, 120]. The high variability in the incidence of ABPA among patients with CF reported reflects the difficulties encountered in the clinical evaluation of the patients. In a longitudinal study of sensitization to *A. fumigatus* in patients with CF, 42% of the patients without ABPA were skin test-positive to *A. fumigatus*, 42% were precipitin positive, 60 and 68% had specific IgE and IgG to *A. fumigatus*, respectively. In parallel, a spontaneous reduction in immune parameters of *A. fumigatus* sensitization in patients with CF, including 11% of patients losing skin reactivity, 54% losing serum precipitins, 53 and 45% losing *A. fumigatus*-specific IgE and IgG, respectively, and a marked decrease in total serum IgE were observed [119]. These findings emphasize the high variability of serological parameters in patients with CF. A diagnosis of ABPA in asthmatic patients can be made based on clinical and serological criteria according to the eight criteria proposed by Rosenberg et al. [39] and Patterson et al. [40]. Many of these features, such as sensitization to *A. fumigatus*, pulmonary infiltrates, elevated eosinophils and total serum IgE levels, episodes of bronchial obstruction and central bronchiectasis, may occur in CF in the absence of ABPA [119, 121]. Central bronchiectasis, the sine quo criterion for the diagnosis of ABPA in patients with asthma [41], has been reported to be detectable not only in 71% of the CF patients suffering from ABPA but also in 71% of CF patients without ABPA irrespective of whether they had asthma [34, 199]. On the other hand, sputum cultures for *Aspergillus* have been shown to be positive in 80% of the CF patients with ABPA but also in 50% of CF patients without ABPA [38, 42].

In a first study the diagnostic value of rAsp f 1 for serology [120] and skin testing [111] in groups of CF patients with or without *A. fumigatus* sensitization and in a small

number of subjects additionally suffering from ABPA was investigated. The serological assays revealed a 10-, 5- and 4-fold increase in rAsp f 1-specific IgE, IgG1 and IgG4 antibodies, respectively, in CF patients with ABPA compared to CF patients with or without sensitization to *A. fumigatus*. The overlapping serological data between sensitized patients with or without ABPA does not allow yet a differential diagnosis of ABPA based on rAsp f 1-specific serology [111, 122]. The results of a skin prick test study with rAsp f 1 in patients with CF sensitized to *A. fumigatus* and in control CF subject showed that only patients with detectable rAsp f 1-specific IgE in serum reacted to rAsp f 1 skin challenges [111]. In contrast to discrepancies found between the skin test results obtained with different *A. fumigatus* extracts, no discrepancy in the reliability of repeated skin challenges with rAsp f 1 was observed [111]. This emphasizes the need for standardized fungal extracts for routine tests in the clinical practice. The availability of a panel of recombinant *A. fumigatus* allergens [85, 86], two of them highly specific for ABPA, prompted us to test the possibility of a differential diagnosis of ABPA based on serology among CF patients [122]. The study included 37 patients with routine assessment for CF and allergy [111, 120]. 15 were diagnosed as having ABPA according to the clinical and immunological criteria proposed by Laufer et al. [34] and Nelson et al. [42]. 12 patients belonged to the group with documented sensitization to *A. fumigatus* and 10 were assigned to the CF control group based on the absence of sensitization *A. fumigatus* [111, 120]. Allergen-specific IgE levels were determined for the allergens rAsp f 1, rAsp f 3, rAsp f 4 and rAsp f 6, in the serum of each individual. The results of the serological investigation (table 4) show that the majority of the CF individuals sensitized to *A. fumigatus* analyzed are sensitized to rAsp f 1 and rAsp f 3 (85 and 100%, respectively) and 85% to both allergens. Therefore, rAsp f 1 and rAsp f 3 are sufficient to diagnose a sensitization to *A. fumigatus* in all investigated sera from CF patients. Relevant serum IgE levels against rAsp f 4 and rAsp f 6 were detectable only in sera of CF patients with a clinical diagnosis of ABPA (table 4). In terms of sensitivity rAsp f 4-specific IgE levels exceeding the cutoff value were detected in 13 of 15 patient sera, whereas relevant levels of rAsp f 6-specific IgE were detected in 10 of 15 patient sera analyzed. If we consider elevated allergen-specific IgE levels raised towards rAsp f 6 or rAsp f 4 sufficient to indicate an ABPA, all patients were covered by the serological diagnosis, whereas 8 patients out of 15 were sensitized to both rAsp f 4 and rAsp f 6.

Toward an Automated Serology Based on Recombinant Allergens

Recombinant allergens may contribute to a significant improvement of the tools for diagnosis of allergic sensitization [60–63] provided that they become available in a pure, fully antigenic form suitable for routine assessments. The Pharmacia CAP System is calibrated against the WHO standard for IgE, and allows quantitative expression of total or allergen-specific IgE [123]. Therefore, this system was chosen to evaluate the performance of rAsp f 1 [124] as well as rAsp f 3, rAsp f 4 and rAsp f 6 (unpubl. results) in a fully automated assay for the detection of allergen-specific serum IgE. ImmunoCAPs were produced and used for the titration of specific serum IgE antibodies according to the manufacturer's instructions [124]. Results were expressed quantitatively in kU_A/l of allergen-specific IgE and compared with the results of IgE binding to the allergen in a standard direct solid phase ELISA expressed as arbitrary ELISA units per milliliter (EU/ml) [112]. The comparison of the results obtained for rAsp f 1 [124] and for the other recombinant allergens obtained with the two allergen-specific IgE detection systems showed a close correlation ($r > 0.98$) confirming that the recombinant allergens can be used as immobilized proteins in the Pharmacia CAP System without a relevant loss of allergenicity [124].

Diagnostic Value of Recombinant *A. fumigatus* Allergens for in vivo Tests

In clinical practice, a diagnosis of specific allergy is usually based on the clinical history, supplemented by diagnostic tests used to confirm or exclude the presence of specific IgE antibodies (i.e. sensitization). For pollinosis and allergy to animals a clinical history and a confirmatory skin test or in vitro determination of specific IgE raised against the relevant allergen extract are usually sufficient to support the diagnosis [125]. In contrast, individuals may not be able to help in setting up a clinical history of fungal exposure because the allergen source cannot be easily identified by the patient. However, the final demonstration that a preparation acts as allergen in vivo is its ability to elicit Type 1 skin reactions in allergic individuals and has to be demonstrated specially for recombinant proteins which may be incorrectly folded [106]. So far, a few recombinant allergens including PLA₂ from honeybee venom [58, 126], pollen allergens [127] and mites [128] have been evaluated in clinical studies. The first recombinant allergen demonstrated to be useful for skin testing, rAsp f 1 [57], has been evaluated in clin-

Table 4. Serological investigations of patients with CF with or without sensitization to *A. fumigatus* extracts

Sub- jects	Age years	Sex	Total IgE kU/l	RAST	Specific IgE (relative ELISA units/ml) to			
					rAsp f 1	rAsp f 3	rAsp f 4	rAsp f 6
CF patients sensitized to <i>A. fumigatus</i> with ABPA								
1	7	m	3,804	4	747	4,027	405	733
2	12	m	866	ND	1,037	3,511	524	60
3	16	m	ND	4	1,439	749	422	381
4	17	m	5,423	4	173	658	53	100
5	16	m	ND	3	195	145	29	55
6	28	m	576	4	208	287	18	346
7	28	m	2,126	4	122	340	17	23
8	15	f	1,017	4	45	178	13	30
9	11	f	2,398	4	1,224	265	76	1
10	12	f	5,226	4	233	867	35	5
11	22	f	1,532	4	65	502	44	5
12	16	m	945	4	402	1,057	27	5
13	14	m	ND	ND	81	128	43	3
14	19	m	928	3	48	183	4	97
15	27	f	491	4	115	1,529	5	118
CF patients sensitized to <i>A. fumigatus</i> without ABPA								
1	12	m	435	4	63	453	2	5
2	30	f	554	4	45	21	2	5
3	24	f	323	4	2	1,176	3	1
5	24	m	166	3	5	32	3	0
6	14	f	146	3	7	117	1	3
7	21	m	304	4	145	121	4	1
8	33	m	372	3	91	437	3	0
9	16	f	103	3	3	131	3	0
10	29	f	270	3	21	88	2	0
11	28	m	47	2	87	124	4	2
12	31	m	409	3	167	128	0	2
CF controls without sensitization to <i>A. fumigatus</i>								
1	20	f	56	0	2	9	5	2
2	30	m	115	0	3	7	2	4
3	30	m	ND	0	5	3	2	0
4	26	f	ND	0	1	1	0	0
5	31	m	40	0	4	3	2	0
6	32	m	59	0	6	4	3	2
7	30	m	22	0	5	3	2	1
8	36	f	ND	0	4	3	0	1
9	24	f	ND	0	1	4	3	0
10	33	m	27	0	2	1	0	0

Positive responses to ABPA-specific allergens are in bold print.
ND = Not determined.

ical studies enrolling allergic asthmatics with or without ABPA [112], patients with CF sensitized to *A. fumigatus* [111] and individuals suffering from AD sensitized to *A. fumigatus* [117]. The experience accumulated in skin tests involving 380 individuals suffering from various *A. fumiga-*

tus-related diseases and 40 healthy controls allows us to draw the following firm conclusions.

(1) Only individuals with serum levels of rAsp f 1-specific IgE >5 EU/ml scored positively in skin test to rAsp f 1 and had rAsp f 1 ImmunoCAP values >0.35 kU_A/l. In contrast, healthy controls and *A. fumigatus*-sensitized individuals with rAsp f 1-specific IgE values <0.35 kU_A/l in ImmunoCAP or below the cutoff value of 5 EU/ml in ELISA were consistently non reactive to intradermal rAsp f 1 challenges. Therefore, no discrepancy between serology and skin test outcome was observed so far [111, 112].

(2) The overall positive response to rAsp f 1 skin test among the individuals sensitized to *A. fumigatus* extracts enrolled in the studies is in the range of 70% in agreement with earlier reports showing that the Asp f 1 allergen elicits IgE antibody responses in 70–85% of the subjects tested [56, 112].

(3) The magnitude of the wheal surface and the amount of rAsp f 1-specific IgE correlate fairly well ($r = 0.87$) [112].

(4) rAsp f 1 did not induce false-positive or unexpected skin reactions in allergic patients or healthy control subjects.

The results obtained from skin testing with rAsp f 1 are comparable to those obtained in vitro with ELISA or ImmunoCAP determinations, demonstrating that this recombinant allergen can be used for a reliable diagnosis of *A. fumigatus* sensitization. In contrast, discrepancies between in vitro and in vivo findings have been reported for several allergen mixtures [111, 125]. These differences may be explained in part by the fact that the extracts used for skin tests and for in vitro diagnosis, commercially supplied by different sources, are unlikely to be identical. The use of *A. fumigatus* extracts from different commercial sources may result in patient-specific discrepancies in reactivity due to differences on the allergen panel present in different extracts [111, 125] and to the specific sensitization of each single individual to a varying allergen pattern [46, 47].

A major point of interest with regard to the large scale diagnostic use of recombinant allergens is whether they will be able to allow a substitution of in vivo provocation tests with less invasive in vitro IgE detection systems. The results obtained so far by comparison of serology and in vivo testing with rAsp f 1 [111, 112, 117, 120] and recombinant PLA₂ from honeybee venom [58, 126] are promising and can be extended to additional recombinant *A. fumigatus* allergens. These clinical studies involving groups of *A. fumigatus*-sensitized asthmatics with or without ABPA and healthy control subjects and the allergens rAsp f 3, 4 and 6 confirmed the results of the serological evaluations discussed in a previous section as well as the absolute specificity of rAsp f 4

and rAsp f 6 for patients suffering from ABPA. From these studies it can be concluded that recombinant *A. fumigatus* allergens (1) are able to elicit Type 1 skin reactions in sensitized individuals and therefore of in vivo relevance, (2) are reliable in diagnosing sensitization to *A. fumigatus* due to the lack of discrepancies between serology and skin test results [111, 112], (3) allow a substitution of skin challenges by serological methods, (4) are suitable for the development of a fully automated serology [124], and (5) allow a discrimination between ABPA and *A. fumigatus* sensitization [114, 122].

However, the potential applications of recombinant *A. fumigatus* allergens are far broader and can be extended to other *A. fumigatus*-related diseases. The availability of the recombinant allergens described allow the selection of sera from patients suffering from aspergillosis lacking immune responses to the already cloned allergens. These sera can be subsequently used to screen the *A. fumigatus* phage surface display library in order to isolate phage displaying antigens/allergens specific for the different diseases. The powerful screening procedure based on biopanning [72, 76, 85, 86], together with a rational selection of sera, allow the isolation of the relevant proteins in a reasonable time and, perhaps, a complete substitution of *A. fumigatus* extracts with recombinant allergen preparations.

Conclusions and Perspectives

Our understanding of the molecular nature of proteins involved in aspergillosis has increased dramatically during the last few years. The rapid progress is mainly due to the development of new cDNA cloning systems based on phage surface display technology which allow selective enrichment of phage coding for products of interest by product-ligand interactions. The consequent application of this technique to a phage surface display library generated using cDNA of the allergenic organism *A. fumigatus* facilitated selective enrichment of phage-displaying IgE-binding proteins of the mold. Sequencing of the cDNA inserts allowed straightforward identification and characterization of a panel of allergens of *A. fumigatus*. They include secreted and intracellular enzymes as well as proteins without sequence similarity to any known proteins. Serological analyses and skin test studies showed that these allergens can be subdivided into two distinct classes: secreted allergens recognized by sera of individuals sensitized to *A. fumigatus* and cytoplasmic proteins recognized exclusively by sera of ABPA patients. They allow a discrimination between ABPA and *A. fumigatus* sensitization never reached with other ser-

ological methods. Cloning, characterization and evaluation of further antigens/allergens of *A. fumigatus* may allow the generation of patient-specific reactivity patterns against single allergens and to correlate the reactivity to different diseases related to the fungus.

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