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Molecular Characterization of *Aspergillus fumigatus* Allergens

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Abstract

Aspergillus fumigatus (Af) is ubiquitous saprophytic fungus associated with a broad spectrum of diseases in humans. These diseases range from benign colonization of the lung to life threatening diseases such as allergic bronchopulmonary aspergillosis (ABPA) and invasive aspergillosis. Af is the etiologic agent identified in most of the *Aspergillus* related human diseases and is therefore of particular clinical importance. Af induced obstructive airway diseases may be due to transient exposure to fungal spores resulting in a T helper 2 response. The IgE mediated inflammatory reaction could be due to colonization of bronchial airway epithelium by Af. Early and precise diagnosis of *Aspergillus* induced respiratory allergy is essential for preventing irreversible lung damages. The major problems in the diagnosis of A. *fumigatus* induced diseases are due to the lack of standardized and well characterized fungal extracts. The advent of molecular cloning technology and the development of phage surface display technology for cloning genes have facilitated the isolation of more relevant recombinant allergens. Using these techniques, a panel of different Af allergens having distinct IgE binding with various groups of Af sensitized patients have been cloned and characterized. These allergens can be categorized functionally as secreted and cytoplasmic proteins. The distinct IgE binding property of these purified and well characterized recombinant Af allergens may be useful for the differential diagnosis of Af related pulmonary complications.

Key words : *Aspergillus fumigatus*, Allergens, Molecular characterization, Recombinant allergens, Immune responses.

Introduction

Aspergilli are saprophytic fungi widely distributed in nature and because of their universal presence, all individuals are exposed to these organisms. Among the various members of this genus, *Aspergillus fumigatus* (Af) is associated with over 80% of all human syndromes caused by aspergilli. Saprophytic presence to extensive invasion with this organism has been reported with various intermediary conditions depending on susceptibility and immunocompetence of the host¹. Other species that have been associated with aspergillosis include A. *flavus*, A. *nidulans*, A. *niger*, A. *terreus*, A. *oryzae* and A. *ochraceus*(1-5). Allergy or hypersensitivity to *Aspergillus* antigens depends on the mode and frequency of exposures to the organism as well as on the host immune responses.

In this report, we review the recent advances in the field of molecular biology and immunology of *Aspergillus* allergens and how this new information will help in diagnosis and management of allergic aspergillosis.

Aspergillus Induced Allergic Diseases

Aspergillus mediated allergic disorders are allergic asthma, allergic rhinitis, allergic sinusitis, allergic bronchopulmonary aspergillosis, and hypersensitivity pneumonitis. Patients with cystic fibrosis are often reported to have complication of ABPA due to Af colonization in the lung (6). Allergic bronchopulmonary aspergillosis is the result of hypersensitivity to *Aspergillus* allergens in patients with atopic asthma. The immunologic responses in patients can be differentiated into

two categories : first, uncomplicated asthmatic reactions due to transient exposure to fungal spores resulting in a Th2 type response with IgE mediated reactions and eosinophilic inflammation and second, more complex asthmatic reactions due to colonization of the mucus epithelial surface by virulent protease and toxin producing fungi (7). The immune responses to fungal colonization are mainly mediated by exaggerated polyclonal antibody responses against the microorganism and an intense eosinophilic infiltration of the airways (7,8).

Immunodiagnosis of aspergillosis is greatly affected by non-availability of reliable antigen preparations. The complex nature of fungal morphology and metabolism could be the cause for the differences in antigenic profile of conidial, hyphal, and cytoplasmic preparations. The strain variability and diverse culture and extraction conditions may be responsible for batch to batch variation in the allergens. Moreover, sensitivity of serodiagnostic methods is greatly affected by the cross-reactivities among different species of *Aspergillus* as well as between aspergilli and other fungi and bacteria (9-12). The toxins and proteases present in antigen preparations may degrade the allergens and hence, may affect the potency of antigenic preparation.

Identification and Characterization of *Af* Allergens

A number of investigators have attempted to characterize and purify relevant antigens of *Af* using conventional purification methods such as gel filtration, ion exchange chromatography, preparative isoelectric focusing, hydrophobic interaction and affinity chromatography (13-20). However, none of these methods provided sufficient quantities of purified relevant antigens¹. Subsequently, hybridoma technology was introduced to purify relevant *Aspergillus* allergens. Although, still not available commercially for general use, two major allergens of *Af*, *Asp f 1* and *Asp f 2*, have been purified and characterized using this method (21-28).

To circumvent the problems associated with purification and characterization of major allergens, interest has been focused in recent years on molecular biology technology as an effective alternative method for obtaining pure and invariant allergens. The advent of recombinant allergens not only improved diagnosis, but also our knowledge of the amino acid sequences of the proteins and our understanding of the structure function relationship of the allergens.

Allergens from *Af*

A large number of genes have been cloned and characterized from *Af* during recent years. However, the allergenicity of the majority of these recombinant proteins has not been determined. Here we discuss the significance of recombinant *Af* allergens in diagnosis as well as therapy. The list of recombinant *Af* allergens that have been cloned and characterized are shown in table 1.

Table 1.Recombined A . Fumigatus allergens

Aspergillus allergens	Properties	Size (bp)	Mol. wt.kD	IgE reactivity	Gene bank accessio no.	References
<i>Asp f 1</i>	Ribonuclease	678	18	++++	M83781 S39330	21,30,31
<i>Asp f 2</i>	Fibrinogen binding protein	1572	37	++++	U56938	35,36
<i>Asp f 3</i>	Peroxisomal protein	630	21	++++	U20722	38
<i>Asp f 4</i>	-	1080	40	++++	AJ-1732	38
<i>Asp f 5</i>	Metalloprotease	1270	42	++++	Z30424	38
<i>Asp f 6</i>	Manganese superoxide dismutase (MnSOD)	666	26.7	++++	U53561	38, 41
<i>Asp f 7</i>	-	860	10	++	AJ223315	38
<i>Asp f 8</i>	P2 ribosomal protein	870	38	++++	AJ224333	38
<i>Asp f 9</i>	-	920	33.7	++++	AJ223327	38
<i>Asp f 10</i>	Aspartic protease	1000	34	++	X85092	38

Asp f 11*	peptidyl - prolyl isomerase	-	24	-		
Asp f 12	Heat shock protein	1649	65	++	U92465	43
Asp f 15	-	-	-	-	AJ002026	63
Asp f 16	-	1281	43	+++	G3643813	64
Asp f 18	Vacuolar serine proteinase	-	34	++	-	65

Asp f 1

Asp f 1 is a 18 kD major allergen from *Af* belonging to the mitogillin family of cytotoxins (21,29,30). The gene encoding 149 amino acid residues includes an 81 base pair leader sequence, a 52 base pair intron, and a 444 base pair open reading frame. The culture filtrate of *Af* has shown thousand fold more *Asp f 1* than mycelial and spore extracts, suggesting that germination of spores and growth of the fungus are essential for this allergen production. An increased level of *Asp f 1* specific IgE and/or IgG antibodies are found in patients with ABPA, aspergilloma, and cystic fibrosis patients with complication of ABPA. The production of IgG antibodies may have a protective effect to restrict the growth of the fungus in immunocompetent patient. *Asp f 1* specific DNA could be amplified by PCR using genomic DNA from *A. fumigatus* and *A. restrictus*, but not from other *Aspergillus* species (30). The pathogenesis of ABPA may be attributed to the dual role of *Asp f 1*, namely the direct cytotoxicity and to the IgE mediated inflammatory reaction. The recombinant *Asp f 1* (*rAsp f 1/a*) expressed in *E. coli* as a functional molecule elicited immediate skin reaction, however, a ten fold functional difference was reported between the purified native ribotoxin and *rAsp f 1/a* (31).

Asp f 2

The gene encoding the second major allergen *Asp f 2* is a protein of 310 amino acid residues with a 42 amino acid long signal peptide, a 268 amino acids open reading frame, two introns of 83 and 52 base pairs each respectively and four possible glycosylation sites. The Gene bank sequence similarity searches revealed significant sequence homology of *Asp f 2* to a protein from *A. nidulans*, *Asp nd 1* and to fibrinogen binding protein and pH regulatory protein from *Candida albicans*. The sequence homology between *Asp f 2* and fibrinogen binding protein may explain its involvement in epithelial damage of host extracellular matrix that leads to the inflammatory reactions at the mucosal surfaces or at the site of fungal colonization (32-36). The 37 kD *Af* culture filtrate protein, purified using monoclonal antibody affinity column exhibited N-terminal sequence homology to recombinant *Asp f 2*. Both the native and recombinant *Asp f 2* have significantly high IgE binding with sera from patients with ABPA, specifically with sera from patients with central bronchiectasis (36). Another glycoprotein (gp55), a 55 kD allergen purified from water soluble mycelial extract of *Af* exhibited complete N-terminal amino acid sequence homology to *Asp f 2* (37). The immunochemical characterization of *Asp f 2* and gp 55 as well as the amino acid sequence data suggested that various antigens/allergens of *Af* would have a common peptide part and the differences in their molecular mass and reactivity with ABPA and other aspergillosis sera could be due to differential glycosylation and proteolytic processing of the antigens and the strain variability of antigen preparations. Moreover, the elevated level of *Asp f 2* specific IgE in patients with ABPA-CB suggesting its role in the latter stage of the disease and probably in the irreversible lung damages in patients with ABPA.

Asp f 3

The third allergen 18.45 kD *Asp f 3* has shown extensive sequence homology with peroxisomal membrane proteins (PMPS) from two yeasts; *Candida albicans* and *Malassezia furfur*. In a recent study, recombinant PMPS from *Candida albicans* and *Af* exhibited common IgE binding epitopes in *Af* sensitized allergic patients (38,39).

Asp f 4

The gene encoding 286 amino acid residues for *Asp f 4* expressed a protein of 30.04 kD. The biochemical function of *Asp f 4* is still not defined properly, however, preliminary experiments using *Asp f 4* specific monoclonal antibody indicated intracellular nature of the protein. The characteristic feature of *Asp f 4* is its distinct IgE binding with sera from patients with ABPA (38,40).

Asp f 6

Another allergen Asp f 6, a 26.7 kD protein identified as the enzyme manganese superoxide dismutase (MnSOD) has been cloned and isolated from a cDNA library displayed on the surface of filamentous phage (41). The identified clone reported to have 51.1% identity and 67.2% homology to the corresponding human enzyme. Both human and *Af* MnSOD coding sequences were expressed in *E. coli* as histidine tagged fusion proteins and purified by Ni²⁺-chelate affinity chromatography. Both the recombinant proteins were recognized by IgE antibodies from patients allergic to *Af* and also elicited immediate (Type I) allergic skin reaction in those individuals. Moreover, both recombinant human and *Af* MnSODs induced proliferation in peripheral blood mononuclear cells of *Af* allergic patients who were positive for skin prick test and IgE antibody to MnSOD. This implies the existence of *in vitro* and *in vivo* humoral and cell mediated autoimmune reactivity to human MnSOD in patients allergic to *Af*. Although it is still not clear whether this autoimmune reaction is caused by molecular mimicry between conserved T and B cell epitopes present on the fungal enzyme and structurally related human enzyme or through sensitization to fungal MnSOD. In a recent study, MnSODs from *Af*, man, yeast, *Drosophila melanogaster*, and *E. coli* showed about 50% identity and 70% similarity in the primary structures. All these recombinant MnSODs have been able to bind IgE in sera from individuals sensitized to the *Af* MnSOD (42).

Asp f 12 (Hsp 90)

The 65 kD fusion protein (*Asp f* 12) cloned and expressed from the cDNA library of *Af* belongs to the heat shock protein 90 (Hsp 90) family (43). This fusion protein was recognized by IgE and IgG antibodies from patients with ABPA. Because of the extraordinary conservation of the Hsps among phylogenetically distant organisms, and to their potential immunogenicity, these Hsps are considered to be important for stress response during inflammation. In a recent study, *Candida albicans* Hsp 90 and its breakdown products are recognized as immunodominant antigens and seroconversion to these antigens are reported to be associated with recovery from systemic candidiasis (44). However, no such information is available with regard to *Af* Hsp.

Other recombinant allergens

Other recombinant *Af* allergens identified and characterized using phage display method as listed in table 1 are proteins in the molecular weight range of 10 to 50 kD and have different enzymatic activities. These allergens exhibit extensive sequence homology with known proteins such as proteases from human and other mammals. The reported metalloprotease (*Asp f* 5) and aspartic protease (*Asp f* 9) having allergenicity may also damage the airway epithelium and trigger allergic responses in patients.

Recombinant Allergens for Diagnosis of Allergic Aspergillosis

Fungal extracts contain a mixture of allergenic and non-allergenic components and are difficult to standardize. Total allergen extracts prepared from natural sources may sometimes lack certain components either because of poor extraction conditions or due to enzymatic degradation during isolation and purification of antigen preparations. Hence, the currently used *in vitro* and *in vivo* diagnostic methods could be improved by using recombinant *Aspergillus* allergens. In view of the recent advances in the molecular biology techniques, it is conceivable that a complete panel of recombinant allergens will be soon available for the diagnosis of allergic aspergillosis.

Among the recombinant *Af* allergens, *rAsp f* 1 has been used extensively for diagnosis of *Af* mediated diseases such as ABPA and other respiratory diseases with complication of ABPA (45,46). *Asp f* 1 showed specific IgE binding with sera from more than 75% ABPA patients without any significant binding with normal controls (45). Recently immuno CAP carrying immobilized *rAsp f* 1 (Pharmacia CAP system) and allergen specific direct ELISA were compared for IgE binding in various subject groups (47,48). The results of these two detection systems correlated closely ($r = 0.997$) and were in perfect agreement with skin test results. Being a potent cytotoxin, extreme caution should be exercised in using *Asp f* 1 for *in vivo* skin testing. As amino acid residue, histidine, at position 136 is attributed to the cytotoxicity of *Asp f* 1, appropriate chemical modification can be made to render the protein non-cytotoxic while still retaining antigenicity. The recombinant allergen *Asp f* 2 reported to exhibit specific IgE binding with more than 90% of sera from ABPA patients without significant cross-reactivity with other aspergillosis and normal control subjects^{23,36}. In a recent study, four recombinant *Af* allergens *Asp f* 1, *Asp f* 3, *Asp f* 4, and *Asp f* 6 were evaluated for differential diagnosis of ABPA and other *Af* mediated diseases⁴⁰. The results indicated that the allergens *Asp f* 1 and *Asp f* 3 could recognize IgE antibodies from both *Af* sensitized individuals with or without ABPA. In contrast,

recombinant *Asp f 4* and *Asp f 6* were recognized only by sera from patients with ABPA. In a study using five different recombinant *Af* allergens and three groups of subjects, we have demonstrated a significant difference in IgE binding of sera from *Af* sensitized patients with the purified allergens (Table 2).

Table 2. Recombinant *Af* allergen specific IgE antibodies in serum of patients with ABPA, *Af* skin prick test positive asthma and healthy controls

Subjects	Mean IgE antibody (*)				
	<i>Asp f1</i>	<i>Asp f2</i>	<i>Asp f3</i>	<i>Asp f4</i>	<i>Asp f6</i>
ABPA	1.277 ± 0.976	0.871 ± 0.986	0.631 ± 0.876	0.547 ± 0.685	0.262 ± 0.415
Allergic asthma	0.113 ± 0.066	0.064 ± 0.063	0.056 ± 0.053	0.087 ± 0.146	0.126 ± 0.269
Normals	0.191 ± 0.065	0.115 ± 0.116	0.082 ± 0.168	0.064 ± 0.123	0.059 ± 0.121

*Ten subjects from each group were included in the study and ELISA values were expressed in OD at 490nm (mean ± SD).

Cell Mediated Immune Responses to *Af* Allergens

Lymphocyte infiltration and granuloma formation in the lung clearly demonstrate the presence of cell mediated immune responses in *Af* sensitized patients (49). The *Af* antigen induced proliferative responses of lymphocytes from ABPA patients correlated well with disease activity (50). Analysis of phenotypic expression clearly demonstrated a significant increase in T helper cells without any effect on the suppressor cell levels in patients. Antibody dependent, cell mediated cytotoxicity has also been reported for both ABPA and aspergilloma. In order to study the involvement of various cytokines and activated T cells in the regulation of IgE synthesis and eosinophil activation and differentiation, *in vitro* immune responses of patients' PBMCs should be evaluated using purified allergens from *Af*. In a recent study, fractionated Con A non-binding *Af* proteins demonstrated significant lymphoproliferative responses in patients (51). The *Asp f 1* specific T cell lines established for ABPA have been characterized as CD4+ Th2 like in their cytokine synthesis pattern (52). In addition, *Asp f 1* specific T cell clones have been reported to be IL-4 producing CD4+ T cells of Th2 phenotypes.

The majority of these *Asp f 1* specific T-cell clones isolated from peripheral blood of ABPA responded to two peptide fragments of *Asp f 1* representing amino acid residues 46-65 and 106-125 and restricted by HLA-DR2 and HLA-DR5 alleles. However, *Asp f 1* peptides differentially inducing CD4+ Th2 and CD4+ Th1 cells in mice have recently been reported in a mouse model (52,53). Hence, attention needs to be focused on the delineation of the functional regions of *Af* allergens with respect to their ability to stimulate T-cell proliferation *in vitro*. Recently we have investigated proliferative responses of PBMCs from ABPA patients against six recombinant *Af* allergens (Table 3).

Table 3. Proliferative response of PBMCs from ABPA patients to the recombinant *Af* allergens

Patients	Antigen 5µg/ml	Average CPM	SI
1	<i>Asp f1</i>	43,910	9.4
	<i>Asp f2</i>	66,603	14.3
	<i>Asp f3</i>	50,879	10.9
	<i>Asp f4</i>	121,676	26.1
	<i>Asp f6</i>	13,986	3.0
	<i>Asp f12</i>	56,116	14.2
	No antigen	4,659	-
2	<i>Asp f1</i>	2,746	2.1
	<i>Asp f2</i>	23,162	17.9
	<i>Asp f3</i>	20,940	16.2
	<i>Asp f4</i>	26,002	20.1
	<i>Asp f6</i>	2,281	1.8
	<i>Asp f12</i>	24,899	19.2
	No antigen	1,296	-
3	<i>Asp f1</i>	12,083	7.2
	<i>Asp f2</i>	33,124	19.8
	<i>Asp f3</i>	19,633	11.7
	<i>Asp f4</i>	39,940	23.9
	<i>Asp f6</i>	16,655	10.0
	<i>Asp f12</i>	38,056	22.7
	No antigen	1,673	-

Average CPM is the mean of three individual counts. Stimulation indices (SI= Counts per minute in cvulture with antigen/counts per minute in culture with medium alone) are shown for each recombinant allergen.

A high stimulation index with an average count of more than 20,000 cpm could be seen with majority of the *Af* allergens indicating their involvement in the cell mediated immune responses in patients.

Recombinant *Af* Allergens in Immunotherapy

Allergen specific therapy of allergic aspergillosis appears feasible in the future because of the availability of an increasing number of functional recombinant *Aspergillus* allergens. The precise determination of the patient's sensitization pattern (allergy profile/allergogram) and selection of a few model recombinant allergens that bind to specific IgE may be the new approach for safer immunotherapy (54,55). Allergen specific therapy may aim to the prophylaxis of atopy, the induction of tolerance or to the modification of ongoing immune reaction (56-60). Such attempts may be based on the application of T-cell epitopes as multiple peptides, pure recombinant allergens or as IgE binding haptens. More recent approaches such as modification of allergens by manipulating allergen encoding cDNAs (i.e. site directed mutagenesis) or gene immunotherapy using cDNA encoding relevant allergens (naked DNA therapy) may also find application in immune modulation and immune intervention of allergic aspergillosis (61,62).

Despite the increasing knowledge of the molecular biologic properties of *Aspergillus* allergens (63), our understanding of the immunopathogenesis and pathophysiology of the disease is very much limited. Taking into account the complexity of fungal antigen extract, selection of allergens for routine diagnosis may be carried out after multi-center studies with a panel of purified and standardized recombinant allergens. The future application of these allergens also needs further clinical evaluation of the recombinant allergens, modified allergens and their derivatives as well as immunodominant epitopes that can selectively induce anergy in patients

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