[Frontiers in Bioscience 8, s128-139, January 1, 2003]

MOLECULAR BIOLOGY OF *ASPERGILLUS* ALLERGENS

Banani Banerjee, and Viswanath P. Kurup

Allergy-Immunology Division, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226 and Research Service, Zablocki Veterans Affairs Medical Center,5000 West National Avenue, Milwaukee, WI 53295

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1. ABSTRACT

Allergic Bronchopulmonary Aspergillosis (ABPA) is a severe respiratory disease caused by the fungus *Aspergillus fumigatus* (Af). It occurs as secondary complication mostly in patients with atopy and cystic fibrosis. The standardized and well-characterized allergens are essential for the immunodiagnosis of ABPA as well as for understanding the pathophysiology of the disease. Molecular cloning was resorted to obtain purified Af allergens for such studies. Currently, twenty-two recombinant Af allergens have been identified and characterized and several of these can be used as standardized allergens in *in vitro* and *in vivo* diagnosis of ABPA. The knowledge of primary, secondary, and tertiary structures of these allergens may facilitate the identification of immunodominant T and B cell epitopes and may be used to unravel the structure function relationship of these allergens. Such findings may open up novel avenues in the immune modulatory therapy and other effective intervention of the disease.

2. 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 cases of human disease caused by aspergilli. Other species that have been associated with aspergillosis include *A. flavus, A. nidulans, A. niger, A. terreus, A. oryzae, A. umbrosus,* and *A. ochraceous* (1-5). Saprophytic presence to extensive invasion with *Aspergillus* has been reported with various intermediary conditions depending on susceptibility and immuno-competence of the host (2,6). The predominance of Af over other aspergilli in human diseases may be due to the factors such as its ability to grow at human body temperature, small spore size (2 to 3 micrometers) able to reach the lung alveoli, and comparative resistance to oxidative killing (6,7).

There are three main processes involved in the interaction between aspergilli and the lung; colonization, hypersensitivity, and invasion. Colonization is usually a passive process but may lead to immunological tolerance and local production of potentially toxic fungal metabolites. 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. Aspergilloma (fungus ball) develops in pulmonary cavities secondary to other lung diseases, such as histoplasmosis, tuberculosis,

etc. while invasive aspergillosis is usually associated with underlying defects in host defense mechanism (6,7).

In this report, we summarize the recent advances in the field of molecular biology and immunology of *Aspergillus* allergens and how this new information will lead to enhance the knowledge for better diagnosis and management of allergic aspergillosis.

3. CHARACTERISTICS OF ALLERGENS

The induction of allergic response in genetically predisposed individuals involves the cooperation of a number of functional and effector cells in recognizing allergen induced stimulation leading to synthesis of IgE antibodies to *Aspergillus* allergens. A prerequisite for any allergen is the ability to activate Th2 response in affected individuals. It is not known why certain proteins possess allergenicity, although allergens triggering immune responses must be polyvalent and should possess several epitopes to cross-link IgE antibodies on mast-cells leading to mediator release. Interestingly, fewer than 100 molecules of allergenic proteins were reported to interact with less then 15% of the effector cell bound IgE antibodies to induce optimal histamine release from human basophils (8,9). Hence, allergens are more or less constrained in their molecular conformations. The present understanding is that any antigen may be allergenic if it can stimulate the immune system and mast cells, and effectively avoid all Th2 suppressive mechanisms.

4. *ASPERGILLUS* INDUCED ALLERGIC DISEASES

Aspergillus mediated allergic disorders are ABPA, allergic asthma, allergic rhinitis, allergic sinusitis, and hypersensitivity pneumonitis. Patients with cystic fibrosis are often reported to have complication of ABPA due to Af colonization in the lung (10). ABPA is the result of hypersensitivity to *Aspergillus* allergens in patients with atopic asthma. The immunologic responses in patients involving type I, III, and IV hypersensitivity ranges from asthma to fatal destructive lung disease with defined clinical, serological, and pathological features (2). The immune responses of the patients against fungal colonization is mainly mediated by elevated total serum IgE, Af specific IgE and IgG antibodies and eosinophilia (11). 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 are greatly affected by the cross-reactivities among different species of *Aspergillus* as well as between aspergilli and other fungi and bacteria (12-15). The proteases present in antigen preparations may degrade the allergens affecting the potency of antigenic preparation.

5. 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 (16-22). However, none of these methods easily provided sufficient quantities of purified relevant antigens (21,23). Subsequently, hybridoma technology was introduced to purify relevant *Aspergillus* allergens (24-28). Although, still not available commercially for general use, a number of major allergens of Af have been purified and characterized using this method (24-31).

To circumvent the problems associated with the conventional purification and characterization of major allergens, interest in recent years has been focused on molecular biology technology as an effective alternative method for obtaining pure allergens. The use 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 allergens.

6. ALLERGEN NOMENCLATURE

As the number of identified allergens increases continuously, specific designations need to be assigned to well characterized allergens according to the accepted taxonomic nomenclature. The International Union of Immunologic Societies through the Subcommittee for Allergen Nomenclature and Standardization presented a unified designation system for purified allergens and their individual components detected within complex allergen extracts (32-34). The first three letters of the genus and the first letter of the species are used to indicate the organism from which the allergens are derived. An Arabic numeral is assigned according to the order of their identification, and the allergens are further labeled by the strain number. The Roman numerals are now used for gene designation and Arabic numerals for protein designation. The biological name of the various well-characterized Af allergens are listed in Table 1.

7. MOLECULAR CLONING OF AF ALLERGENS

Almost a decade ago, researchers began to isolate and characterize complementary DNAs (cDNA) encoding allergens from various sources in order to determine which proteins induce IgE response in human (35). Indeed striking sequence similarities between allergens and known proteins were found; the major allergen Asp f 1 from Af showed more than 99% sequence homology to restrictocin, a ribotoxin from *A. restrictus* (36). A sequence homology of more than 77% was present between heat shock protein allergen from Af and Hsp 90 family of proteins present in other organisms (7). Another *Aspergillus* allergen identified as manganese superoxide dismutase (MnSOD) exhibited 67.2% sequence homology to the corresponding human enzyme (38,39). Among other allergenic proteins from different sources, the white face hornet venom allergen Dol m 5 and major pollen allergen Bet v1 showed sequence homology to different classes of plant pathogenesis related proteins (40,41). The major house

Table 1. Major Allergens of Aspergillus

Allergen [1]	kD	Nature of Allergen	Binding of Patients' IgE	Sequence Data	Accession Number
Asp f 1	17	Ribotoxin	83 [2]	C	MB3781
Asp f 2	37	Fibrinogen binding	90	C	S39330
Asp f 3	18	Peoxisomal protein	94	C	U56938
Asp f 4	30	-	78	C	U20722
Asp f 5	42	Metalloproteinase	93	C	AJ001732
Asp f 6	23	Mn superoxide dismutase	56	C	Z30424
Asp f 7	12	-	46	C	U53561
Asp f 8	11	Ribosomal protein-P2	-	C	AJ223315
Asp f 9	34	-	89	C	AJ224333
Asp f 10	34	Aspartic proteinase	28	C	AJ223327
Asp f 11	24	Peptidyl-prolyl isomerase	-		X85092
Asp f 12	47	Heat shock protein-90	-		U92465
Asp fl 13	34	Alkaline serine protease	-		
Asp o 13	34	Alkaline serine protease	-		
Asp f 13	34	Alkaline serine protease	-	C	
Asp n 14	105	Beta xyloxidase	4		
Asp f 15	16	-	-	C	AJ002026
Asp f 16	43	-	70	C	
Asp f 17	-	-	-	C	g3643813
Asp f 18	34	Vacuolar serine proteinase	-		AJ224865
Asp n 18	34	Vacuolar serine proteinase	-		
Asp f 22		Enolase			AF284645

[1] Allergens are designated according to the taxonomic name of their sources as follows: the first three letters of the genus name, space, the first letter of the species name, space, and an Arabic numeral. The number has been assigned to the allergens in the order of their identification and the same number has been assigned to designate homologous allergens from the related species.
[2] % of patients' serum IgE showing binding to the allergen.

dust mite allergen Der p 1 contained sequence motifs of a protease, Bet v 2, the birch pollen allergen was identified as the actin binding protein profilin (42,43). A common structural or functional feature has yet been found among characterized allergens, which would allow the prediction of whether a certain protein possesses an intrinsic property to behave as allergen (44-51).

Molecular cloning of allergens comprise the isolation of messenger RNA (mRNA) from the *Aspergillus* strains and the reverse transcription of the mRNA into cDNA. The allergen encoding genes were identified by southern hybridization using radiolabeled oligonucleotides or by antibody screening of cDNA libraries with sera from allergic patients. The amino acid sequences of the cDNA encoded protein can be deduced from the nucleotide sequences of the cDNA. To date the majority of Af allergens have been cloned using the phage expression vector lambda gt11 and lambda ZAP II. Recently, investigators have used cloning and sequencing based on polymerase chain reaction, although this method requires knowledge of the partial sequences of the protein for the construction of oligonucleotide primers. On the other hand, using advanced PCR-RACE (rapid amplification of cDNA ends) method, full-length cDNA with complete 5' and 3' ends could be generated from rare transcripts at fewer than 30 copies per cell. This method is also useful for generating large cDNA transcripts of at least 9 Kb in length. Of late, filamentous phage, M13 has been used to express the Af and other fungal proteins from cDNA library on phage surface and screened for allergens binding to IgE antibody of allergic patients (52,53). Molecular cloning and sequencing of relevant *Aspergillus* genes encoding antigens and allergens reported in recent years are listed in Table 1. The application of molecular biology technologies for allergen characterization involves the expression and cloning of biologically active recombinant allergen that has structural and functional properties comparable to its natural counterpart. The detailed characterization of the biochemical and immunochemical properties of recombinant allergens is necessary before they be considered for diagnosis and therapy.

Among different expression vectors in use for recombinant allergens, pET has been the most favored prokaryotic expression system. It is a tightly controlled T7 polymerase based gene expression in *E. coli*. The expression of the target protein is induced by a T7 RNA polymerase in the host *E. coli* (54,55). The majority of Af allergens have been cloned in high expression prokaryotic system to obtain the recombinant purified proteins for serodiagnosis and skin prick test. The IgE antibody binding of rAsp f 1 and rAsp f 2 purified by pET system are comparable to the native proteins suggesting that the IgE antibody binding is directed mainly to the protein part of the molecule and post translational modification is not essential for obtaining the proper conformation for antibody epitope interaction.

Pichia expression is an eukaryotic system designed for high level expression of recombinant protein in the yeast *Pichia pastoris*. The target gene under alcohol oxidase promoter can express as much as 12 mg/liter of recombinant protein. Recently, Asp f 2 has been cloned

and overexpressed in glycosylated form in *Pichia pastoris* (56). The new method of displaying functional cDNAs or other gene products on the surface of filamentous phage M13 has been developed by exploiting the high affinity interaction of the Jun and Fos leucine zippers (52,57). The pJuFo cloning vector was designed to display cDNA library from Af on the surface of filamentous phage and selective isolation of surface expressed allergens was carried out by screening IgE antibody binding with sera from Af sensitized patients. The phage carrying specific gene products on their surface via leucine zippers could be selectively enriched by 10^4 to 10^6 over nonspecific phage using antibody based screening procedures (52). The enzyme MnSOD, a major allergen from Af, has been cloned and characterized using phage display method (38,39). In a recent study, a robotic based high throughput screening of clones selected for IgE-binding capacity from phage surface displayed cDNA libraries of Af allowed rapid characterization of 81 different IgE binding peptides (58).

8. ALLERGENS FROM AF

In recent years, a large number of genes have been cloned and recombinant proteins obtained from Af. However, the allergenicity of the majority of these recombinant proteins have not been determined. The following are the major Af allergens expressed by molecular cloning.

8.1. Asp f 1

Asp f 1 is a 18 kD major allergen from Af belonging to the mitogillin family of cytotoxins (24,36,59,60). The gene encoding 149 aa residues includes an 81 base pair leader sequence, a 52 base pair intron, and a 444 bp 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 (61). The production of IgG antibodies may have a protective effect to restrict the growth of the fungus in immunocompetent patients. 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 (36). A comparison of the amino acid sequence of Asp f 1 with mitogillin from *A. restrictus* revealed more than 99% sequence similarity. A single amino acid in Asp f 1 molecule, aspartic acid (Asp) at position 115, is substituted by asparagine (Asn) in mitogillin. Some of 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 f1/a (61).

8.2. Asp f 2

The gene encoding the second major allergen Asp f 2 is a 310 amino acid protein with a 42 aa long signal peptide, a 268 amino acid open reading frame, two introns of 83 and 52 base pair each respectively and four possible glycosylation sites (26,62). 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 a fibrinogen binding protein and pH regulatory protein from *Candida albicans* (63,64). 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 (62-66). The 37 kD Af culture filtrate protein, purified using monoclonal antibody affinity column exhibited N-terminal sequence homology to recombinant Asp f 2 (26). Both the native and recombinant Asp f 2 have significantly elevated IgE binding with sera from patients with ABPA, specifically with sera from patients with central bronchiectasis (62). 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 (67). Although the N-terminal amino acid residues of both the proteins exhibit significant sequence homology, gp 55 has 11 more amino acids upstream of native Asp f 2 near the N-terminal region. 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 suggests its role in the latter stage of the disease and probably in the irreversible lung damages detected in such patients.

8.3. Asp f 3

The allergen Asp f 3, is encoded by an insert of 607 bp. The deduced amino acid sequence of this cDNA encoded a protein of 18.5 kD and exhibited 36% identity and 58% similarity with the amino acid sequences of two peroxisomal membrane proteins from *Candida boidinii* reported as PMPA and PMPB (68).

8.4. Asp f 4

The biochemical function of Asp f 4 remains unknown. The primary experiments on localization of the protein using monoclonal antibody raised against Asp f 4 indicate that Asp f 4 is not secreted by the fungus. Recent reports suggest that Asp f 4 binds to IgE antibody of sera from *Aspergillus* sensitized patients. On the other hand, recombinant Asp f 5 belongs to a family of secreted proteases, possesses allergenicity and also claimed to be a virulence factor of Af (69).

8.5. 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 expressed (39). The identified clone reported to have 51.1% identity and 67.2% homology to the corresponding human enzyme. These two

recombinant proteins were both recognized by IgE antibodies from patients allergic to *Aspergillus* and elicited immediate type 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. In a recent study, MnSODs from humans, *Aspergillus*, 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 (38).

8.6. Other Recombinant Allergens

Asp f 7 is a minor allergen of Af reacts with less than 50% of sera from *Aspergillus* sensitized patients. The biochemical function and cellular localization of Asp f 7 are unknown. Asp f 9, reported to a be major Af allergen exhibits extensive sequence homology with a recently reported allergen Asp f 16 (70). The amino acid sequence homology between Asp f 9 and Asp f 16 indicates that both allergens are originated from the same gene by differential gene splicing and rearrangement. Asp f 10, a minor allergen of Af represents a secreted aspartic protease produced by the fungus only when grown in the acidic medium. This protease has been reported from infiltrates of fatal cases of invasive aspergillosis indicating that it can be produced and secreted in the lung tissues of infected patients (69).

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 (37). 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. The immune responses to Hsp 70 and Hsp 90 family of proteins were reported to stimulate protective immunity against malaria in monkeys (71). However, no such information are available with the Af Hsp.

Another allergen Asp f 13, an alkaline serine protease with a molecular weight of 33 kD and a PI of 6.2 has been identified and purified (72). The amino acid sequence comparison has shown 42-49% identity of Asp f 13 with the Pen c 1 allergen from *P. citrinum*. A 34 KD vacuolar serine proteinase from Af (Asp f 18) was characterized as a major allergen by immunoblotting and N-terminal amino acid sequencing. The results from immunoblot inhibition showed IgE cross-reactivity among several protease allergens of *A. fumigatus, P. notatum* and *P. oxalicum* (73). Recently, the N-terminal amino acid sequencing, immunoblotting , and IgE binding of 8 most prevalent *Penicillium* and *Aspergillus* species (*P. citrinum, P. notatum, P. oxalicum, P. brevicompactum, A. fumigatus, A. flavus, A. oryzae* and *A. niger*) were examined (74). The results indicate that alkaline and/or vacuolar serine proteinases are the major allergens in these fungal species. By two-dimensional immunoblotting and N-terminal amino acid sequencing, a 47 kDa functional protein, enolase from Af was identified (75). The corresponding cDNA clone for Af enolase (Asp f 22) encodes a protein of 438 amino acids. The Af enolase showed 94% identity with enolase from *P. citrinum*. Another allergen from Af has been cloned and characterized as protein disulfide isomerase (PDI) (76). The PDI superfamily of proteins may play an important role in the protein folding mechanisms of the fungus for its interaction with the host. Other recombinant Af allergens identified and characterized using phage display method listed in Table 1 are proteins in the molecular weight range of 10 to 50 kD and have different enzymatic activities.

9. *ASPERGILLUS* PROTEINS INVOLVED IN PATHOPHYSIOLOGY

Recently, a number of Af genes encoding proteins with various functional and structural properties have been cloned, sequenced, and characterized. This includes various *Aspergillus* proteases such as 42 kD elastinolytic metalloproteinase, 33 kD elastinolytic serine proteases from Af and *A. flavus*, 39 kD aspartic proteinase, and 32 kD extracellular alkaline proteinase from Af cloned and expressed in *E. coli* (77-82). The Af proteases play a role in the fungal penetration of the host barriers by catalyzing the hydrolysis of the major structural proteins of the basement membrane of lungs such as elastin, collagen, and laminin. Although these secreted proteases have not been reported to elicit an IgE response in patients with allergic aspergillosis, they undoubtedly play a role in the colonization and tissue invasion and may further facilitate the entry of *Aspergillus* antigens and/or allergens into submucosal tissues and exacerbate an existing allergic response in sensitized patients (83).

A hydrophobin rodlets gene (HYP 1) of Af, which encodes an outer-coat conidial protein has been cloned and sequenced (84,85). This 159 amino acid protein showed 85% homology to rodA gene of *A. nidulans*. These regularly arranged rodlets proteins are insoluble and may be responsible for conidial hydrophobicity of *Aspergillus* species (86,87). The extreme resistance of rodlets to chemical degradation suggest their role in resistance of Af to host phagocytic defenses.

A cDNA clone of Af representing an 85 kD protein has been cloned recently in the yeast *Pichia pastoris* (88). The expressed glycoprotein is antigenic and possesses the characteristic properties of class IV dipeptidyl peptidases (DPPIV). This DPPIV from Af displayed significant sequence homology to the surface differentiation marker CD26 present on T-lymphocytes in mammals (89). The peptidase activity of DPPIV and its binding to collagen may help the fungus in colonizing the proteinaceous matrix of the lung (90).

10. 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, Asp f 1 has been used extensively for diagnosis of Af mediated diseases such as ABPA and other respiratory diseases with complication of ABPA (91,92). Asp f 1 showed specific IgE binding with sera from more than 75% ABPA patients without any significant binding with Af positive allergic asthma and normal controls (91). Recently immunoCAP carrying immobilized rAsp f 1 (Pharmacia CAP system) and allergen specific direct ELISA were compared for IgE binding in various subject groups (93,94). 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. The histidine at position 136 is attributable to the toxicity of Asp f 1. Hence, appropriate chemical modification can be made to render the protein non-cytotoxic while still retaining its 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 (26,62). Recombinant Asp f 6 and Asp f 12 have also shown significant *in vivo* and *in vitro* IgE antibody binding and have shown considerable promise as standardizable allergens. The purified recombinant allergens of Af used in immunological testing falls into two categories, namely secreted and cytoplasmic proteins (69). Secreted allergens such as Asp f 1 and Asp f 3 are recognized by serum IgE of Af sensitized individuals with or without ABPA, whereas non secreted proteins such as Asp f 2, Asp f 4 and Asp f 6 are exclusively recognized by serum IgE of ABPA patients. In a recent study recombinant Asp f 1, Asp f 2, Asp f 3, Asp f 4 and Asp f 6 were evaluated for their IgE binding with sera from ABPA patients, *A. fumigatus* skin prick test positive asthmatics and normal controls (95). The results demonstrate that Asp f 2, Asp f 4 and Asp f 6 can be used for serodiagnosis of ABPA, while IgE binding of Asp f 1 and Asp f 3 are not specific. In a recent study a panel of purified recombinant and natural allergens from most common sources such as tree, grass, weed pollen, mites, animals, fungi and insects were microarrayed on glass chips in order to develop a miniaturized allergy diagnosis test (96). This test allows the detection and monitoring of allergic patients' IgE reactivity profiles to specific allergens using single measurements and minute amounts of clinical samples. However, studies have shown discrepancies between serum IgE binding and biological activity of the recombinant allergens in sensitized patients and hence, measurement of specific serum IgE antibody levels may not be sufficient to determine the clinical reactivity of allergic patients against a particular allergen (97).

11. EPITOPES OF *ASPERGILLUS* ALLERGENS

Protein structure in general can be described at different levels based on their primary structures (amino acid sequence), protein fold, domain structure and surface structure. The surface structure of the allergen is the most relevant for antibody binding, particularly the part of the surface that interacts with the antibody. The regions on an allergen molecule that interact with cells of the immune system or antibodies are defined as epitopes. The allergen interacts with the immunoglobulin by either its three dimensional structure (B-cell epitope) or after ingestion and processing by antigen presenting cell (APC) as peptide fragments linked to major histocompatibility (MHC) molecules to T-cell (T-cell epitope). The allergen specific immune responses start with the interaction of peptide MHC complexes on APC with T-cell receptors (TCR) on T cells. The T-helper (Th) cells become activated to proliferate and secrete cytokines when co-stimulatory signals are provided by APCs. In contrast, T-cell non-responsiveness can be produced if co-stimulatory signals of APC are blocked or by the administration of higher doses of antigenic peptides (98-100). Recent studies have revealed that T-cell epitopes of an allergen can also function as strong inducer of T-cell non-responsiveness in humans (101,102). The Asp f 1 specific T cell lines established from ABPA have been characterized as $CD4^+$ Th2 like cells in their cytokine synthesis pattern (103). 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 (104). However, Asp f 1 peptides differentially induce $CD4^+$ Th2 and $CD4^+$ Th1 cells have recently been reported in a mouse model (105). 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* (106).

Unlike T-cell epitopes, B-cell epitopes may be primarily conformational or sequential (107). The knowledge of IgE binding epitopes of the major Af allergens may be of great importance for increasing the sensitivity of diagnostic test by designing model allergens representing such epitopes. On the other hand, identification of IgE binding peptides that are unable to cross-link IgE might be useful for suppressing the release of mediators on subsequent contact with complete allergen molecules.

12. STRUCTURE/FUNCTION ANALYSIS OF ALLERGENS

Three dimensional structural analysis of allergens are usually investigated by X-ray crystallography or by nuclear magnetic resonance (NMR) using overexpressed allergens as metabolically labeled proteins. Structural analysis of

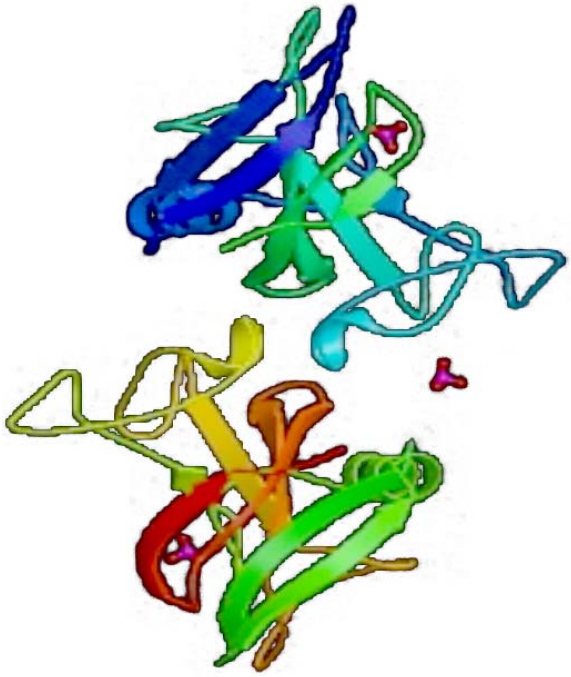

Figure 1. Structural core of restrictocin, with a three –turn alpha helix packed against a five-stranded antiparallel beta sheet. The large positively charged concave loops near the active site construct a platform with a concave surface for substrate binding.

crystallographic data from a number of allergens have shown that important environmental allergens contain multiple epitopes which cluster at exposed surface areas of the molecule and allow simultaneous binding of more than two IgE antibodies. The same is true for small Birch pollen allergen profilin or its fragments, which simultaneously can bind several IgE antibodies with different epitope specificities (107).

Fungal ribotoxins display a three dimensional protein fold similar to those of the larger group of microbial noncytotoxic RNAses represented by T1 and U2 (Figure 1). This similarity involves the three catalytic residues and also the Arg 121 residue, whose counterpart in RNAse T1 , Arg 77, is located in the vicinity of the substrate phosphate moiety. The potential functional role of these catalytic residues are not known. In a mutational study substitution of Arg 121 of alpha sarcin with Gln or Lys resulted in no modification of the conformation of the protein but abolished the ribosome-inactivating activity of alpha sarcin. This study concludes that Arg 121 is a crucial residue for the characteristic cytotoxicity of alpha –sarcin and presumably of the other fungal ribotoxins. (109).

The enzymatically active recombinant ribotoxin cleaved the 28S rRNA within the universally conserved region resulted in cytotoxicity to EBV immortalized or PHA stimulated PBMC. However, expression of a mutant restrictocin, H136L, has no effect on cell growth of EBV immortalized or PHA stimulated PBMC suggesting that H136 may be the active site of restrictocin (110). The restrictocin family of proteins contains four cysteines at position 5, 75, 131, and 147. These cysteine residues are involved in the formation of two disulfide bonds, one between Cys 5 and 147 and another between cysteine 75 and 131. Experimental evidences have shown that one of the two-disulfide bonds is absolutely essential and sufficient to maintain the enzymatically active conformation of restrictocin (111). The allergenic conformation of Asp f 1 belonging to the restrictocin family of protein is not known. In a recent study, involvement of cysteine residues in IgE antibody binding of a major Af allergen, Asp f 2 have been studied using various deletion and point mutants of the allergen (112). A significant reduction in IgE antibody binding of mutants with substitution or deletion of the C-terminal cysteine residues indicated the involvement of cysteine disulfide bonds in conformational constraints for antibody interaction.

Three-dimensional structure of Af MnSOD has been determined at 2-A resolution by X-ray diffraction analysis (113). The structure was derived by molecular replacement using the structure of the human MnSOD as the model. The final refined model included four chains of 199-200 amino acids, four manganese ions and 745 water molecules with a crystallographic R factor of 19.4 % and a free R- factor of 23.3%. Like MnSODs of other eukaryotic organisms, Af MnSOD forms a homotetramer with the manganese ions coordinated with three histidines, one aspartic acid and one water molecule. The fungal and the human MnSOD share high similarity on the level of both primary and tertiary structure (113).

13. ALLERGEN SPECIFIC 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 an approach for safer immunotherapy (35,107). Allergen specific therapy may be aimed to the prophylaxis of atopy, the induction of tolerance or to the modification of ongoing immune responses (101,114-118). The mechanism by which specific immunotherapy (SIT) achieves clinical improvement may be due to a rise in allergen blocking through IgG4 antibodies, generation of IgE modulating CD8+ T cells and a reduction in the number of mast cells and eosinophils and release of mediators. Therefore, for successful and safe SIT, allergen variants could be created of which recognition sites for T cells remain intact, whereas binding sites for IgE antibodies are removed. Intact T cell epitopes are required to enable the induction of specific T cell tolerance or anergy against the antigen or allergen. More recent approaches such as modification of allergens by allergen engineering through site directed mutagenesis and gene immunotherapy using cDNA encoding relevant allergens (naked DNA therapy). Deoxyoligonucleotide (nonmethylated CpG) may also find application in immune modulation and immune intervention of allergic aspergillosis (119).

14. FUTURE DIRECTIONS

Despite the increasing knowledge of the molecular biologic properties of *Aspergillus* allergens, our understanding of the immunopathogenesis and pathophysiology of allergic aspergillosis is very much limited. Immunotherapeutic approaches using specific allergens needs further evaluation. 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 course of specific application of these allergens should depend on the clinical evaluation of synthetic peptides, recombinant allergens, degraded allergenic extracts or engineered allergens that can selectively induce tolerance or immunomodulation.

15. ACKNOWLEDGMENT

This work is supported by the Department of Veterans Affairs, Medical Research and by the American Academy of Allergy, Asthma and Immunology.

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Key words: ABPA; Recombinant allergens; Epitopes; Structure of allergens; Specific Immunotherapy, Review

Send correspondence to: Banani Banerjee, PhD, V A Medical Center, Research Service 151-I, 5000 West National Avenue, Milwaukee, WI 53295, Tel: 414-384-2000 Ext. 41510, Fax:414-382-5374, E-mail: banerjee@mcw.edu