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ASPERGILLUS ANTIGENS ASSOCIATED WITH ALLERGIC BRONCHOPULMONARY ASPERGILLOSIS

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1. ABSTRACT

Among the molds causing allergy, *Aspergillus fumigatus* (Af) constituted a major species present both indoors and outdoors. The antigens both secreted and bound to hyphae and conidia have been isolated for immunodiagnosis of allergic aspergillosis. The crude extracts have been used to demonstrate IgE antibody *in vitro* and for skin testing. The crude extracts contain many antigenic and non-antigenic components and toxins and have demonstrated inconsistent reactivities. In addition, similarity with other allergens also may complicate specific diagnosis. A number of methods including conventional purification and fractionation methods have been used to obtain relevant antigens. In recent years, monoclonal antibody dependent affinity purification and molecular biology methods have obtained considerable progress in the allergen purification and in developing specific and reliable immunoassays. However, international standards are still lacking and hence, comparison of results among laboratories are not possible yet.

2. INTRODUCTION

The prevalence of respiratory allergy to fungi is estimated in the range of 20-30% in atopic individuals and up to 6% in the general population (1-3). The major allergic manifestations induced by fungi are rhinitis, asthma, allergic bronchopulmonary aspergillosis (ABPA), and hypersensitivity pneumonitis (4,5). ABPA is caused by the sensitization of *Aspergillus* antigens in patients of atopic asthma. This can result from exposure to spores and mycelial fragments or through metabolites of *Aspergillus* species and some other fungi. The fungi implicated in inducing ABPA or similar syndromes are *Aspergillus fumigatus, A. niger, A. terreus, A. flavus, A. clavatus, A. nidulans, Candida albicans, Curvularia lunata, Epicoccum nigrum, Helminthosporium sp. and Penicillium sp.* (6-9). The laboratory diagnosis of ABPA and similar syndromes include the demonstration of total serum IgE, specific IgE and IgG, immediate skin reactivity, and serum precipitins to *Aspergillus* or other fungal antigens. Antigen induced *in vitro* stimulation of peripheral blood mononuclear cells (PBMCs), and enhanced eosinophil counts in blood of ABPA patients are considered other important parameters. A wide range of antigens from *Aspergillus* sp. with diverse biochemical structure and immunological properties, have been used in diagnosis of ABPA.

3. ANTIGEN PREPARATION

Culture filtrate, conidia and mycelial extracts from different strains of the same species of fungi or different batches of same strain show significant variation in their antigenic components. These variations are primarily due to growth conditions, nutrient media, processing of antigenic material, and differences in the strains selected for antigen preparation. The frequent mutation observed in fungi under different culture conditions also accounts for the variability of antigens within the same species (10). The presence of toxins and proteolytic enzymes also affect the potency and integrity of the fungal extracts (6,11,12).

The mycelia-spore extracts from Aspergilli and other fungi consist of mixture of proteins, glycoproteins, and polysaccharides (6,13-14). Synthetic media such as Czapek-Dox, glycine, asparagine or Association of Analytical Chemists (AOAC) broth (Difco) supplemented with different nutrients forms the suitable media for culture filtrate antigens. After the cultivation, the media components and other non-specific components can be selectively removed using 10 kDa cut off dialysis membrane (13,15-16). It is essential to remove them since they can interfere in immunoassays or *in vivo* tests (skin tests). The use of synthetic media and incubation at 37°C are reported to give more suitable antigens. The results of studies have suggested different incubation period ranging from 1-4 weeks to obtain *Aspergillus* antigens. It has been established that the most reliable cell associated antigens for immunoassays can be generated from 4 day-old shake cultures. Whereas secreted antigens are better obtained from 2-4 week old stationery culture of the fungi (13,17-20). Extracts are supposed to be standardized using a combination of *in vivo* and/or *in vitro* procedures recommended by FDA, WHO, and International Union of Immunological Societies (21). Although not standardized, the various commercially available crude extracts are used frequently for laboratory diagnosis of ABPA.

4. CHARACTERIZATION OF ANTIGENS

Aspergillus fumigatus is the dominant fungal species responsible for ABPA. Therefore, many studies have been directed to characterize the most relevant allergens (antigens). Both culture filtrate and mycelial antigens from Af have been analyzed by SDS-PAGE, isoelectricfocusing, and two-dimensional gel electrophoresis (22-25). Immunoelectrophoresis of culture filtrate Af antigens using sera from ABPA patients demonstrated approximately 35 precipitin arcs, of which 8-10 showed specific IgE reactivity. Crude extract of Af resolved in 200 protein components on two-dimensional gel electrophoresis (25-27). Kim and Chaparas identified 52 antigens, whereas Kauffman and co-workers could demonstrate 22 and 44 antigens, respectively in different extracts of Af using crossed immunoelectrophoresis (16,26,28). However, the water-soluble mycelial and spore extracts showed over 30 antigens on immunoblotting with patients' sera (13,29,30).

The crude mycelial extracts of Af were fractionated by isoelectricfocusing (IEF) and the reactivity of three major fractions was compared using ABPA patients' sera. Several of these fractions reacted with ABPA sera and also showed reactivity with antibodies against other fungi. These fractions share some of the epitopes with other fungal antigens. Kurup and associates and Wilson and co-workers fractionated the Af antigens using affinity chromatography/isoelectricfocusing and reported strong IgE/IgG reactivity to these fractions (18,23,31). The fractions with molecular masses of 25-470 kDa Af antigens were evaluated and found to be useful for ABPA diagnosis (32-33). In order to characterize the antigens, fractions of Af antigens were obtained using gel filtration, concavalin A affinity chromatography, IEF, and trichloroacetic acid precipitation (26). It was observed that high MW (>20 kDa) components reacted strongly with ABPA sera in ELISA and frequently demonstrated precipitin reactions by agar gel double diffusion. The low MW fractions showed weak binding to polystyrene ELISA plate. However, Longbottom reported that ABPA sera show strong IgE binding to low MW peptides by ELISA. The results also indicate that these peptides were more specific, hence, suitable for a correct diagnosis of ABPA (34).

Antigens from other common *Aspergillus* species such as *A. flavus, A. niger*, and *A. oryzae* were also investigated. *A. flavus* antigens demonstrated IgE antibody in 44% of the asthmatic patients' sera by immunoblotting (35). Skin prick tests and IgE immunoblotting revealed the importance of *A. niger* allergens in asthmatic subjects (12). *A. oryzae* has been detected as an important occupational allergen associated with work related respiratory symptoms in bakery employees (36).

5. PURIFICATION OF ANTIGENS

5.1. Conventional purification

Various methods have been used to fractionate and purify the relevant antigens from Af for immunodiagnosis of ABPA. Most of the conventional methods involved harsh physical and chemical treatments, which has adverse effect on antigenicity. Earlier attempts to fractionate and purify Af antigens using gel filtration and Diethyl amino ethyl (DEAE) chromatography were not very successful. However, better results were obtained with preparative isoelectricfocusing (22-23,37-38). An antigen component was isolated from culture filtrate by IEF using a p^H gradient of 4-6.5 (37). This fraction bound to concavalin A and probably had a tetrameric configuration with approximate MWs of 20,40, and 80 kDa. ELISA using this fraction on solid phase showed high levels of IgG and IgE in ABPA patients' sera. An immunologically relevant antigen fraction was isolated from cytoplasmic preparation of *A. fumigatus* by hydrophobic interaction chromatography and Con A affinity chromatography. This antigen could be identified from culture filtrate also by crossed immunoelectrophoresis. ELISA using this fraction and sera from ABPA patients showed high IgG and IgE titers, whereas sera from cystic fibrosis and aspergilloma with concomitant ABPA showed elevated IgG but only moderate increase in IgE titers.

Several glycoprotein components were reported from Af, which showed reactivity with ABPA patients' sera (32,38-40). A major 24 kDa allergen of Af (Ag3) was isolated from precipitinogenic components which bound to Con A (41). Among the four antigens purified by Longbottom and co-workers, Ag3 and Ag5 are thermolabile Con A non-binding antigens of 18 kDa and 35 kDa, respectively (41-42). They showed reactivity with ABPA patients' sera, hence useful as diagnostic antigens. The non-Con A binding fractions of low MWs (<14 kDa) also showed reactivity with sera from ABPA patients. High MWs 200-450 kDa antigens isolated from 3rd week culture of Af were reported useful for detecting antibodies in sera

of ABPA and invasive aspergillosis patients (13). In another study, Con A binding fraction with molecular weight 35 and 65 kDa demonstrated specific IgE and IgG binding in sera of ABPA patients (39).

Purification of a 18 kDa Con A non-binding protein from different strains of Af has been attempted using various techniques such as gel filtration, ion exchange chromatography, gel permeation chromatography, sephacryl S-200 column chromatography, and phenyl sepharose chromatography (10, 43-45). A diagnostically relevant protein of MW 18 kDa was purified to homogeneity from culture filtrate of Af. This allergen was fractionated from a clinical (strain no. 285) as well as the soil strains (ATCC, strain no. 2605) using a combination of phenyl sepharose chromatography and high performance liquid chromatography (10). In another study, a 20 kDa (Con A non-binding) antigen was purified to near homogeneity by size exclusion chromatography using a sephacryl S-200 column and IEF in p^H range of 2.5-6.5 gradient. IgE binding activity of this fraction has been confirmed with ABPA patient's sera by radio-allergosorbent test (RAST) (46).

5.2. Antigen purification using monoclonal antibodies

Murine monoclonal antibodies raised by hybridoma technology were used in affinity chromatography to obtain pure and diagnostically relevant antigens of Af (47-49). An IgM class of monoclonal antibody reacted with 12 of 16 antigens fractionated from nine isolates of Af and with antigens from two isolates of *A. flavus*. However, it did not react with *A. niger, A. terreus*, and *Candida albicans* antigens (50). The antigens present in the cell wall of Af hyphae and conidia reacting to the monoclonal antibodies were reported (51). One of these antigen was recognized by these monoclonal antibodies as an antigen with oligogalactosyl side chain residues of Af galactomannan (GM), while the second monoclonal identified the mannopyranosyl residues common to GM of Af and *C. albicans* mannan. Several monoclonal antibodies belonging to IgM and IgG class have been generated. An immunodominant 58 kDa antigen of Af hyphae and conidial wall has been recognized using these antibodies (48). Three of the monoclonal antibodies belonging to IgG_1 subclass showed cross reactivity to 55 kDa antigen of *C. albicans*. Con A sepharose beads coated with Af were used for immunization of mice to obtain monoclonal antibody specific to glycoprotein antigens by affinity chromatography (52). This novel method of immunization can overcome the difficulties involved in large-scale purification of antigens.

Two Con A binding components from Af antigens reacted with a IgM class of monoclonal antibody and demonstrated IgG and IgE binding activity on immunoblotting (50). A Con A non-binding antigen (22 kDa) purified using monoclonal antibody affinity chromatography showed more reactivity with sera from ABPA patients than with aspergilloma and controls (53,54). Monoclonal antibodies against Af glycoproteins showed cross reactivity with other fungi. Six monoclonal antibodies produced against Af were analyzed for cross reactivity against other fungal allergens (53). Two of the antibodies in this group reacted with antigens of other *Aspergillus* species such as *A. niger, A. flavus, A. glaucus, A. clavatus, A. fischeri*, and *A. terreus* and also with *C. albicans, Penicillium notatum*, and *Trichoderma viridis*.

Monoclonal antibodies have also been used in combination with biochemical purification techniques to study the immunologic reactivity of Af antigens (44). It was observed that 18 and 45 kDa antigens were the important markers for clinico-immunologic studies of *Aspergillus* induced diseases. The 18 kDa major allergen (*Asp f 1*) of Af was reported to release into the medium in early phase of fungal growth, therefore, the culture filtrate of aerated cultures may be used for its purification. The 18 kDa protein (*Asp f 1*) has also been identified as a major antigen in the urine of patients with invasive aspergillosis (55). Recently *Asp f 1* and *Alt a 1* levels were measured in commercially available unstandardized extracts, reference extracts and spores of Af and *Alternaria alternata* by a monoclonal antibody based ELISA (56). A monoclonal antibody was produced against *Asp f 1* (18 kDa with ribonuclease activity) and this antigen was purified using monoclonal Ab immunoaffinity column (43). The purified *Asp f 1* induced specific IgE response in 85% of ABPA patients. Another diagnostically relevant allergen is 37 kDa Con A non-binding protein purified by fractionation and monoclonal antibody based immunoaffinity chromatography (57). However, such antigens are yet not commercially available for serodiagnosis of ABPA.

6. CHEMICAL CONSTITUENTS OF ANTIGEN

The various purified and fractionated antigens from Af are listed in Table 1.

6.1. Glycoprotein antigens

Several glycoprotein antigens from Af have been isolated using Con A affinity chromatography (25,58-59). The high MWs glycoprotein antigens from hyphae or culture filtrate preparations were purified using conventional methods and used to detect specific IgE in ABPA patients' sera. An acidic glycoprotein (45 kDa) was isolated from cell sap protein by Calvanico and co-workers (58). It contained four polypeptides and showed reactivity with sera of ABPA patients. Longbottom and co-workers identified four glycoprotein antigens, viz. Ag3, Ag4, Ag7, and Ag13 using gel filtration, IEF, and affinity chromatography (27,41-42,60). Ag7 (150-200 kDa) and Ag13 (70 kDa) bound to Con A and reacted with antibodies in ABPA and aspergilloma patients. Kurup and co-workers resolved Af extract into 18 fractions, of which 12-14 bands showed Con A binding indicating their glycoprotein nature (18). Using two-dimensional electrophoresis, they isolated a fraction containing 20, 40, and 80 kDa size components, which showed high antibody binding in sera of ABPA patients (37). The same group reported two glycoproteins of 35 and 65 kDa, which showed IgE/IgG binding in ABPA patients' sera (22). Schonheyder and Andersen (32) isolated an antigen component of Af with a molecular mass of 25-50 kDa using hydrophobic interaction chromatography. Out of 14 fractions detected on PAGE, three showed Con A binding property.

Table 1. Summary of purified/fractionated *Aspergillus* antigens

Antigen	Method of purification	Molecular weight	IgE/IgG reactivity	References
Culture filtrate	CIE	-	8 to 10 antigens showed IgE binding, 35 precipitin arcs	22-25
CF, Mycelial-spore extracts	CIE	-	52,22,44 precipitin arcs	16,26,28
Water soluble Mycelial-spore extract	PAGE, Immunoblotting	-	30 antigens showed IgG binding	13,29-30
Mycelial extract	Preparative IEF, affinity chromatography	25-470kDa	3 antigens, Strong IgE/ IgG binding	31-33
Culture filtrate antigen	Gel filtration, Con A chromatogrphy, IEF,TCA precipitation	5-20 kDa low molecular weight	Showed weak precipitin reaction, strong IgE binding	26,34
Con A non binding	Affinity chromatography, SDS-PAGE	12-40 kDa	Showed specific IgE/IgG binding	88
Mycelial antigen	Chromatography	250 kDa high mol. Weight	Showed binding with sera from Aspergilloma patients	33,79
Water soluble components	Immunoblot	90 kDa, immunodominant antigen (Catalase)	Reacted with sera from Aspergilloma patients	74
Membrane vesicle antigen	Immunoblot	73,43 kDa (Protease)	Reacted with sera from ABPA Patients	59
Galactomanan antigen	Chromatography	50kDa	Showed IgE binding and precipitin reaction	64
Cell sap protein(Cytoplasmic antigen)	Chromatography, IEF	45 kDa (4 polypeptides)	Showed IgE/IgG binding with ABPA Sera	58
Intracellular/ Cytoplasmic Con A binding antigen	Gel filtration, IEF, affinity chromatography	150-200kDa (Ag7), Ag13 70kDa	Reacted with sera from ABPA and Aspergilloma patients	27,41-42,60
Water soluble component	Gel filtration, SDS-PAGE	55kDa	Showed IgE binding with ABPA sera	87
Culture filtrate	Gel filtration, SDS-PAGE	45 kDa, Glycoprotein	Showed IgE binding with ABPA sera	61
Con A non binding allergen	Affinity chromatography, Monoclonal Ab column	37 kDa	Strong IgE binding with ABPA sera	57
Culture filtrate	IEF, Affinity chromatography, CIE	IEF 6.5, 3 proteins 20, 40, 80 kDa	Strong IgE/IgG reactivity with ABPA sera, IgG reactivity with Aspergilloma patients sera	37
Culture filtrate	Immunoblot	50-66 kDa 20-30 kDa	IgG reactivity with rabbit sera	86
Culture filtrate (Cytotoxic protein)	Ammonium sulphate precipitation, Con A sepharose/phenyl sepharose chromatography, HPLC	18 kDa	Reacted with monoclonal antibodies raised against *Asp f1*	89

Using gel filtration and SDS-PAGE, a major allergenic protein (group 55) has been purified from water extract of Af. This protein has N-glycosidically linked oligosaccharides and showed specific IgE binding on immunoblot with ABPA patients' sera (10). In another study, a glycoprotein allergen of 45 kDa (group 45) was purified and characterized (61). However, on deglycosylation, this protein lost the IgE reactivity, but the IgG binding has been retained. A glycoprotein of 66 kDa (group 66) was isolated from Af by ammonium sulfate precipitation followed by immunoaffinity column (specific antibody coupled to cyanogen bromide activated sepharose) chromatography (62). This antigen showed IgE reactivity when tested by crossed-radio-immunoelectrophoresis and ELISA using sera of patients with cystic fibrosis as well as ABPA. Recently, a glycoprotein (93 kDa) was purified from water extract of Af (strain NCPF 2109) using single step affinity column where mannose specific lectin was coupled to agarose (63). The protein showed specific IgE binding with sera from ABPA patients.

6.2. Carbohydrate antigens

Polysaccharide fractions isolated from cell wall or cytoplasm of *Aspergillus* species were reported to demonstrate cross reactivity with other fungal antigens (64-69). This limits their scope as diagnostic allergens for ABPA or aspergilloma. Galactomannan antigen was isolated from mycelial extract using sequential chromatography on anion exchange and Con A affinity columns. Cell wall galactomannan of Af has structural similarities to galactomannan of *A. flavus, A. niger*. Azuma and co-workers have purified a galactomannan of approximate molecular mass of 50 kDa. It showed skin reactivity and precipitin reactions with patients' sera (64). The detection limit of galactomannan in serum of patients with invasive aspergillosis has been suggested as 10-40 ng/ml. Recently, fungal extracellular polysaccharides (1-->3)-glucan have been reported as good marker to estimate overall levels of fungal concentration in floor dust of patient's residences (70).

6.3. Enzymes as allergens

A large number of enzyme allergens viz. proteinase, elastase, ribonuclease, chymotrypsin, catalase, and superoxide dismutase have been identified from Af (10,33,43,71-77). Out of 18 components detected from *Aspergillus*, 13 reacted with sera of ABPA patients (78). Schonheyder and co-workers recognized a 250 kDa enzyme, possibly a tetramer of catalase from Af (33,79). The catalase antigen showed IgE binding with sera of patients with cystic fibrosis as well as ABPA (30,80). Recently, a 90 kDa catalase antigen was reported from Af, which detected specific antibodies in more than 90% sera of aspergilloma patients (74).

IgE reactivity to glucoamylase, cellulase, and hemicellulase from *A. niger* was established by skin tests and immunoblotting. Recently, β-xylosidase (*Asp n* 14) from *A. niger* used as baking additives was reported to induce sensitization in 4% of symptomatic bakers (81).

An antigenic component of Af with chymotrypsin activity was isolated by Tran-van Ky and co-workers (78). It reacted with sera of aspergilloma patients, but not with ABPA. Hamilton and coworkers has isolated superoxide dismutase from Af by IEF, gel filtration, and fast protein liquid chromatography (FPLC) (71). This enzyme (19 kDa) reacted strongly with sera of confirmed cases of ABPA on immunoblot. Proteases have been reported actively involved in pathogenesis of aspergillosis. Two proteases of 25 and 50 kDa sizes respectively, were partially purified from Af, which showed reactivity with aspergillosis patients' sera (79). The component of 73 and 43 kDa isolated from conidial walls of Af were recognized as acid proteases (25). These two enzyme allergens demonstrated IgE reactivity in ELISA with ABPA patients' sera. Alkaline serine proteinases with allergenic properties have been reported from Af (*Asp f 13*), *A. flavus* (*Asp fl 13*), and *A. oryzae* (*Asp o 13*). Similar serine proteinases with sequence homology to *Aspergillus* proteinase have been isolated from *Penicillium citrinum, P. notatum,* and *P. brevicampactum* (82). The 34 kDa alkaline serine proteinases were identified as major allergens from *A. flavus* and *A. oryzae* (83-84). From *A. niger*, a vacuolar serine proteinase (*Asp n 18*) has been identified as major allergens by immunoblotting using sera of asthmatic patients (12,81). Protease allergens are reported to be involved in fungal penetration of the host barriers by enzymatic hydrolysis of major structural proteins in lung tissue. But their role in cellular and humoral immune responses in ABPA patients is yet to be studied in detail. However, several proteases have been identified from Af using conventional purification methods or recombinant DNA technologies (72,75-77,85).

7. CONCLUDING REMARKS

The extracts from the whole mycelia and spore mass (surface growth) or metabolic products secreted into the medium during growth of Af can be used for detecting antibodies in the sera of patients. The use of synthetic media and incubation at 37°C has been reported to give more reliable antigens. The most authentic cell associated antigens for immunoassays have been obtained from four-day-old aerated cultures. The secreted antigens are better available from 2-4 week old stationary cultures. The progress made in recent years may enable to replace crude mixture of allergens with suitable recombinant proteins from cDNA libraries of *A. fumigatus*. The availability of purified relevant antigens may influence the dependability and reproducibility of the diagnosis. Furthermore, such allergens may contribute to better immunotherapy measures and also in understanding the pathogenesis of the disease.

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