

Selected recombinant *Aspergillus fumigatus* allergens bind specifically to IgE in ABPA

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Summary

Background Allergic bronchopulmonary aspergillosis (ABPA) is a hypersensitivity lung disease resulting from exposure to *Aspergillus fumigatus* allergens. Patients with ABPA show elevated *Aspergillus*-specific serum IgE, a major criterion used in the diagnosis of the disease. Crude culture filtrate and mycelial antigens have been used widely to demonstrate IgE antibody to *Aspergillus* in the sera of patients. While these antigens have been useful in the diagnosis of ABPA, occasionally they present inconsistency in their reactivity and lack of specificity. Although in recent years, a number of purified *A. fumigatus* allergens have been produced by molecular cloning, no attempt was made to evaluate them systematically. **Objective** To evaluate the recombinant proteins from *A. fumigatus* for their IgE antibody binding, we studied sera from ABPA patients and controls by antigen specific enzyme linked immunosorbent assay (ELISA).

Methods Recombinant *Aspergillus* allergens Asp f 1, f 2, f 3, f 4, and f 6 were studied for their specific binding to IgE in the sera of ABPA patients, *A. fumigatus* skin prick test positive asthmatics, and normal controls from the USA and Switzerland. The sera were blinded and studied by ELISA in two different laboratories.

Results All the recombinant allergens showed IgE antibody binding with sera from patients with ABPA, whereas only fewer asthmatics and normal sera showed significant binding. The three selected recombinant allergens together reacted with all the ABPA patients studied.

Conclusions The results demonstrate that Asp f 2, f 4, and f 6 can be used in the serodiagnosis of ABPA, while IgE antibody binding to Asp f 1 and f 3 was not specific.

Keywords: allergic asthma, allergic bronchopulmonary aspergillosis, ELISA, recombinant *Aspergillus* allergens, specific IgE

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Introduction

Aspergillus fumigatus (*A. fumigatus*) a ubiquitous fungus, is implicated in a number of clinical forms of diseases in humans including allergic bronchopulmonary aspergillosis (ABPA) [1]. ABPA is a hypersensitivity lung disease

occurring in patients with asthma or cystic fibrosis. The diagnostic criteria include: (1) asthma; (2) fleeting pulmonary infiltrates; (3) immediate skin test reactivity to *Aspergillus* antigen; (4) elevated total serum IgE; (5) *Aspergillus*-specific IgE and IgG; (6) peripheral blood eosinophilia; (7) precipitating antibody to *A. fumigatus* and; (8) central bronchiectasis [2,3]. All these criteria may not always be present in all patients and some patients have no bronchiectasis but may present criteria 1–7 [4]. Although sharply

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increased amounts of specific serum IgE to *A. fumigatus* is considered a significant and reliable criterion for the diagnosis, the lack of standardized, dependable and specific antigens is a limiting factor in the demonstration of *Aspergillus*-specific IgE in the sera of ABPA patients. Until recently, the antigens used in the *in vitro* and *in vivo* diagnosis were mostly uncharacterized extracts from the mycelium of the fungus or the secreted antigenic materials present in the culture media [5]. The currently used antigens are fraught with inherent variability resulting possibly in unreliable results from laboratory to laboratory [6]. Attempts made in the past decade using a number of fractionation methods resulted in isolating partially purified antigens for improved reliable assays. However, most of these immunochemical methods were time consuming and failed to improve the results substantially [7]. Of late, molecular biology methods have been used to clone the genes encoding relevant allergens of *A. fumigatus* [8–14]. Considerable success resulted from this approach [15,16], although concerted efforts have not yet been made to evaluate these antigens for their IgE binding through interlaboratory studies. The present communication is the result of the evaluation of five different recombinant allergens from *A. fumigatus* namely Asp f 1, Asp f 2, Asp f 3, Asp f 4, and Asp f 6 by ELISA using sera from patients with ABPA and control individuals from the United States and Switzerland. The results demonstrate significant IgE levels in the sera of ABPA patients against Asp f 2 and Asp f 4, whereas with the addition of Asp f 6 all ABPA patients showed significant levels of specific antibodies.

Materials and methods

Patient and control sera

The sera used to test IgE antibody against various *A. fumigatus* antigens were from the USA and Switzerland. A total of 24 ABPA patients were studied along with 16 asthmatics with immediate skin test reactivity to *A. fumigatus* antigens and 10 normal healthy controls. All patients' sera were evaluated for IgE antibody to various allergens by both laboratories. ABPA was ruled out in all *Aspergillus* skin test positive asthmatics because they did not have sufficient criteria for ABPA. The majority of ABPA patients in the present study fulfilled at least seven of the eight diagnostic criteria proposed by Patterson *et al.* and Rosenberg *et al.* [2,3]. The normal control subjects presented no history of allergy or asthma. The institutional human studies committees at the different institutions approved the study.

Aspergillus fumigatus allergens

Asp f 1 a ribotoxin [8], Asp f 3 a protein homologous to peroxisomal protein of *Candida boidinii* [13], and Asp f 6 a

protein having homology to superoxide dismutase [17], were cloned from a cDNA library of *A. fumigatus* displayed on phage surface as described elsewhere [14,18]. In addition, two other proteins expressed by *A. fumigatus* genes, with unknown biological function and without sequence similarity to any of the described proteins, also were studied. These two are Asp f 2 [12,19] and Asp f 4 [15,16]. The cDNAs encoding the proteins were subcloned in suitable vectors, the nucleotide sequences and correct constructs were used to express [His]₆-tagged fusion proteins [12,14]. Fusion proteins were purified in a single step by Ni²⁺ chelate affinity chromatography [20]. Asp f 2 was expressed in *Pichia pastoris* while the other allergens were expressed in *Escherichia coli* [21]. The purity and molecular sizes were verified by SDS-PAGE analysis and reactivity to various sera was ascertained by Western blot analysis using sera from ABPA patients and normal controls (Fig. 1).

Detection of IgE antibody in sera

ELISA was used to demonstrate the IgE binding of the various allergens by the two laboratories. The ELISA used in the US laboratory has been described previously [12,22]. Briefly, the microtitre plates were coated with 5 µg/mL recombinant allergens dissolved in phosphate buffered saline (PBS) pH 7.2, for 1 h at room temperature followed by incubation at 4 °C overnight. After washing, the wells were blocked with PBS-Tween 20 containing 0.5% bovine serum albumin. This was followed by washing as before and the addition of appropriately diluted sera. The plates were again incubated for 3 h at room temperature followed by washing and the addition of a second antibody (biotinylated mouse antihuman IgE; Pharmingen, San Diego, CA). The

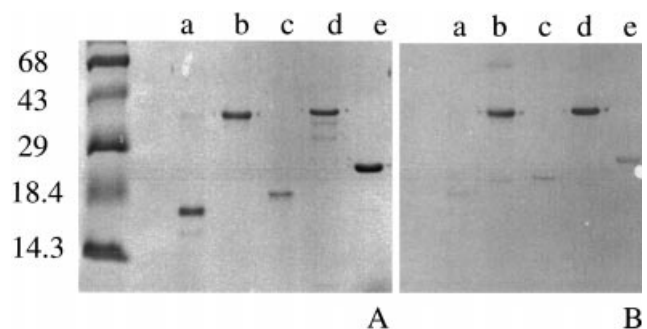


Fig. 1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (A) and Western blot (B) using *A. fumigatus* recombinant allergens Asp f 1, f 2, f 3, f 4, and f 6. A, SDS-PAGE showing molecular weight markers (kd) at the left and (a) Asp f 1 (b) Asp f 2 (c) Asp f 3 (d) Asp f 4 (e) Asp f 6. B, Western blots showing the reactivity with a positive ABPA serum pool; Asp f 1 (a) showing very weak signal whereas Asp f 2 (b) Asp f 3 (c) Asp f 4 (d) and Asp f 6 (e) showing moderate to strong signals.

plates were incubated for 1 h followed by washing as before. Streptavidin-peroxidase was added and incubated as before and the wells were again washed. The enzyme reactivity was developed using enzyme substrate and the Absorbance (A_{490}) of the developed colour was measured after stopping the reaction with 2 M H_2SO_4 . The concentrations of the reagents and conditions of the assay were determined by checkerboard titrations using known positive and negative sera. Different buffers and molarity of the coating buffers were evaluated before selecting PBS, which gave comparable binding to the solid phase by all five antigens tested. The method followed in the Swiss laboratory was by a standard direct solid phase ELISA. Briefly the method is as follows: antigens were coated on the wells of microtitre plates overnight at 4°C at a concentration of 10 µg/mL in PBS (pH 8.0). The plates were washed with PBS (pH 7.4) containing 0.05% Tween-20. The wells were blocked with 2% (w/v) skimmed milk in PBS containing 0.5% Tween-20 for 1 h at 37°C. After washing, the sera from the patients and controls were titrated and studied for specific IgE antibody to the allergens using a mouse monoclonal antibody (mAb TN-142 antihuman IgE) and the reactivity was visualized with alkaline-phosphatase labelled goat antimouse IgG (Pierce, Rockford, IL, USA) [23]. The ELISA plates were developed with 1.5 mg/mL 4-nitrophenyl phosphate disodium salt hexahydrate (NPP, Merck Darmstadt, Germany) in diethanolamine buffer pH 9.8 for 45 min and the reaction stopped by adding 50 µL 2 M NaOH. Absorbance of the developed colour was measured at 405 nm and results expressed as ELISA units per ml (EU/mL). The ELISA unit was obtained by comparing the absorbance of an in-house reference standard arbitrarily defined as 100 EU/mL for each allergen tested [13,23].

Sodium dodecylsulphate polyacrylamide gel electrophoresis and Western blot

All five recombinant *A. fumigatus* antigens were studied for their electrophoresis profile in a 12% polyacrylamide gel containing SDS as described before [13,19,22]. Electrophoresis of 10 µg of each antigen was carried out and the gels were stained with Coomassie blue and the proteins compared with molecular weight standards. The proteins resolved in the gel were electrophoretically transferred to nitrocellulose membranes using a transblot system (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The membranes were blocked and reacted with patient and control serum pools [13,19,22]. The antibody binding was demonstrated using secondary antibody-enzyme conjugate and the colour developed and the reactivity of different sera with the different allergens compared [22].

Statistical analysis

The mean ELISA Absorbance values and mean ELISA units were calculated and a cut off value above mean + 2 SD of normal subjects to each allergen was taken as significant. The different groups were compared for differences using 't' test. A *P*-value of less than 0.05 was considered significant when patient groups were compared. The ELISA results obtained by the two laboratories were compared by linear regression analysis using Statworks program (Cricket Software Inc., Philadelphia, PA, USA).

Results

Recombinant Aspergillus fumigatus allergens

The recombinant proteins expressed in different vectors resolved in SDS-PAGE are shown in Fig. 1a. The molecular weights deduced comparing standards are in the range of 17–40 kDa and are in good agreement with the values calculated from the nucleotide sequences [8,12,13,15,17,21]. The Western blot studies using selected individual sera showed diverse reactivity. A selected ABPA pool demonstrated strong binding to IgE from sera of ABPA by Asp f 2,

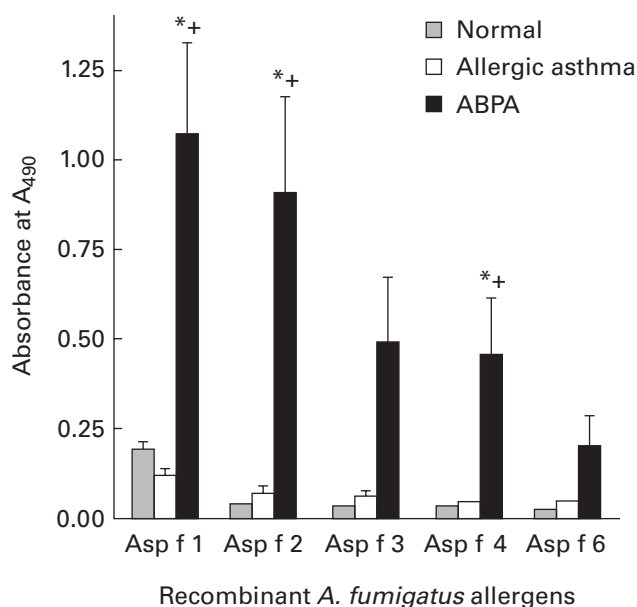


Fig. 2. The Absorbance (A_{490}) values of ELISA carried out at the US laboratory using ABPA, skin test positive asthmatics without ABPA and normal controls against recombinant *A. fumigatus* allergens, Asp f 1, f 2, f 3, f 4, and f 6. All the values of ABPA patients were significantly elevated compared to normal controls and asthmatics with positive immediate skin test reactivity. Statistical significance *P* < 0.05, between ABPA and normals (*) and between ABPA and *A. fumigatus* skin test positive asthmatics (+).

Table 1. Sera from ABPA, skin test positive asthmatics and normal controls showing binding of IgE to recombinant *A. fumigatus* allergens

A. fumigatus allergens	Percent positive in Switzerland			Percent positive in US		
	ABPA	Asthmatics	Normal	ABPA	Asthmatics	Normals
Asp f 1	83*	81	10	68	0	0
Asp f 2	87	6	0	87	36	0
Asp f 3	83	37	10	81	27	10
Asp f 4	92	0	0	62	18	0
Asp f 6	58	0	0	68	27	0

*Percent of subjects demonstrating significant IgE binding. Mean + 2 SD of normals was taken as positive.

f 4, and f 6 whereas weak binding only was detected with Asp f 1 and Asp f 3 (Fig. 1b). None of the pooled sera tested from *Aspergillus* skin test positive asthmatic and normal controls showed any reactivity with these antigens.

IgE antibody by ELISA

The results of the ELISA carried out at the two laboratories using five recombinant allergens of *A. fumigatus* are shown in Figs 2 and 3. All ABPA sera showed strong IgE binding

to the recombinant allergens, while skin test positive asthmatic patients and normal controls showed only low levels of specific IgE ($P < 0.05$). However, at the Swiss laboratory allergic asthmatics with *Aspergillus* skin prick test positivity showed more elevated IgE against Asp f 1 and Asp f 3 compared to the other allergens (Figs 2 and 3; Tables 1 and 2). The results indicate considerable overlap among the allergens in their differential binding to individual serum. The present results also demonstrate that immune response in ABPA patients is heterogeneous. Majority of the ABPA patients demonstrated significant IgE against Asp f 2 (87%) when studied by both laboratories while for Asp f 4 62 and 92% showed significant reactivity with ABPA patients at the USA lab and Swiss lab, respectively. However, by inclusion of Asp f 6 in the panel along with Asp f 2 and f 4 all clinically diagnosed ABPA patients showed significantly elevated levels of specific IgE. The ABPA patient

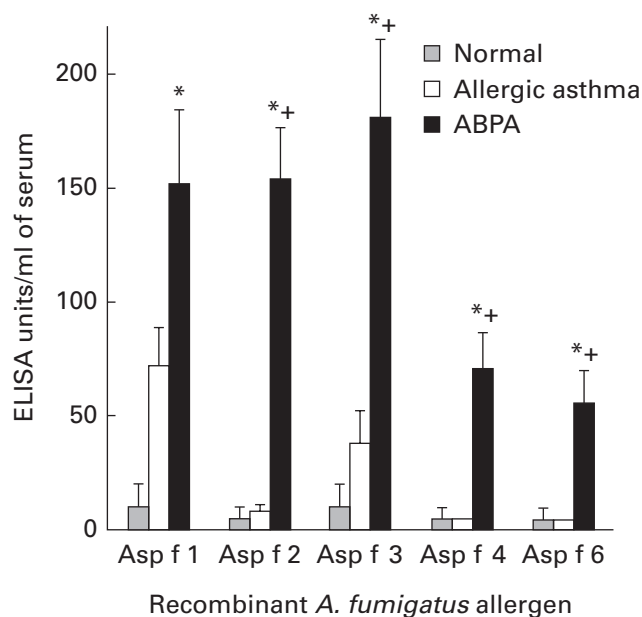


Fig. 3. The same as in Fig. 3 except that the ELISA was carried out in the Switzerland laboratory and expressed as ELISA units after comparing with the immunocap values of the standard serum. Statistical significance $P < 0.05$, between ABPA and normals (*) and between ABPA and *A. fumigatus* skin test positive asthmatics (+).

Table 2. IgE binding of the sera of patients with different recombinant *A. fumigatus* allergens and their combinations

Recombinant allergens	Subjects studied			
	ABPA		Asthmatics	
	Swiss lab	US lab	Swiss lab	US lab
Asp f 2	87*	87	6	36
Asp f 4	92	62	0	18
Asp f 6	58	68	0	27
Asp f 2 + f 4	96	94	6	36
Asp f 2 + f 6	96	94	6	36
Asp f 4 + f 6	100	94	0	27
Asp f 2 + 4 + 6	100	100	6	45

*Percent of subjects showing IgE binding. Mean + 2 SD of normals was taken as positive.

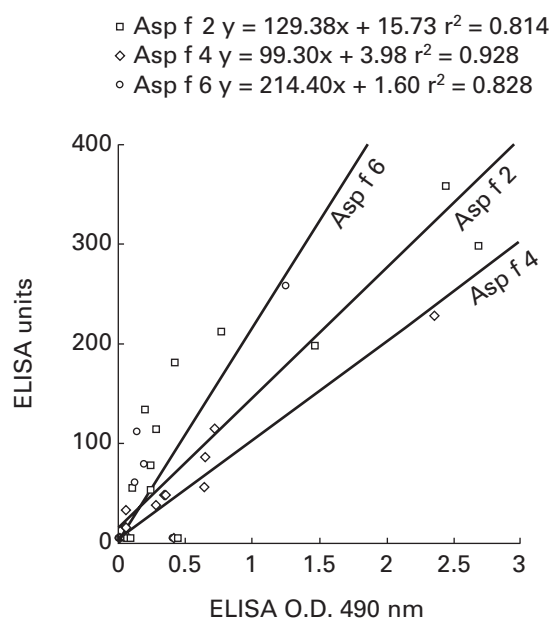


Fig. 4. Linear regression analysis of ELISA results obtained at the two laboratories using Asp f 2, Asp f 4, and Asp f 6 and sera from ABPA patients, skin test positive asthmatics, and controls. Results using other allergens also showed comparable results.

who had no demonstrable allergen-specific serum IgE to Asp f 1, Asp f 2, Asp f 3, and Asp f 4 showed significant level of specific IgE to Asp f 6. Asp f 2 reacted with only a few allergic asthmatic patients (6%) when tested by the Swiss laboratory, whereas as much as 36% showed reactivity when tested at the USA laboratory. Both laboratories noted similar reactivity with Asp f 3 when ABPA and asthmatic sera were tested. The results obtained from two laboratories using the same patients and controls against these three antigens, Asp f 2, f 4, and f 6 are shown in Fig. 4. The results demonstrate comparable correlation between the two laboratories. The reactivity of the selected antigens in the IgE assay is shown in Table 2. The combination of antigens selected, identified all the 24 ABPA patients by both laboratories, whereas the reactivity of asthmatics varied with antigens and tests and ranged from 6 at the Swiss lab to 45% at the USA lab.

Discussion

The results demonstrate that the recombinant allergens rAsp f 2, rAsp f 4 and rAsp f 6 from *A. fumigatus* used in the present study specifically bind IgE antibody in the sera of ABPA patients. However, the reactivity of these recombinant proteins varied considerably with different patients. Of the 24 ABPA patients studied, rAsp f 4 and rAsp f 2 demonstrated significant IgE in 22 and 21 patients, respectively. Asp f 6 was more specific for ABPA although, the

IgE binding was weaker than with the other allergens and the sensitization among ABPA ranged from 58–68%. The results indicate that the two allergens, Asp f 1 and Asp f 3, cannot be used to discriminate between asthmatic patients with *A. fumigatus* sensitization and ABPA by their IgE binding [15,16]. Furthermore, the results obtained with rAsp f 1 also showed partial overlap between normal controls and sensitized asthmatics (Fig. 2). The results from the two laboratories indicate difference in the reactivity of Asp f 1 and Asp f 3. These two allergens showed some reactivity with the sera of skin test positive asthmatics and normal controls at the Swiss laboratory, while they failed to demonstrate the same level of reactivity when tested at the US laboratory. Whether this disparity is due to the differences in the procedures used in the two laboratories or due to other factors is not known. Since rAsp f 1 showed enhanced reactivity with IgE of asthmatic and normal subjects, it may be considered as an indicator of sensitization rather than as a diagnostic allergen for ABPA. In contrast, Asp f 2, Asp f 4 and rAsp f 6 has superior specificity in distinguishing ABPA compared to rAsp f 1 and rAsp f 3, corroborating earlier reports [12,15,16,18,21]. When used together for the diagnosis, 100% sensitivity and specificity was noted with the assays carried out at both laboratories for ABPA diagnosis, while for asthmatics the specificity was 100% at both laboratories although the sensitivity ranged from 6 to 45%. The positive and negative predictive values for ABPA was 100%, while for skin test positive asthmatics these values were 64 and 45 for the US laboratory and 6 and 7 for the Swiss laboratory, respectively.

The previous reports involving rAsp f 2 [12,18,21] rAsp f 4 and rAsp f 6 [15,16] suffered from the limitation that they were tested with limited groups of patients diagnosed in individual clinics. This is also the first study on the serological demonstration of IgE in the serum of ABPA patients using recombinant allergens by two independent laboratories in USA and Europe. The results obtained confirm the specificity of the allergens rAsp f 2, rAsp f 4 and rAsp f 6 in detecting IgE in sera from patients with a clinical diagnosis of ABPA. It is suggested that these recombinant allergens can be developed as standards in the diagnosis of ABPA, although additional studies involving more sera from ABPA patients and *A. fumigatus*-sensitized individuals diagnosed in different clinics need to be carried out before a final decision on the diagnostic value of the allergens can be made. For standardization purposes, pure recombinant allergens are superior to native allergens as they are consistent and can be prepared in large quantities and used in fully automated diagnostic devices [24,25].

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