

# Identification and Partial Characterization of Diagnostically Relevant Antigens of *Aspergillus fumigatus*

Banani Banerjee,<sup>1</sup> Anbu Chetty,<sup>2</sup> Arvind P Joshi<sup>1</sup> and Usha S Puranam.<sup>1</sup>

*Aspergillus fumigatus* induces a number of diseases in man. Allergic asthma, allergic bronchopulmonary aspergillosis (ABPA) and hypersensitivity pneumonitis in man are mainly associated with hypersensitive reactions to antigens of *A.fumigatus*.<sup>1-4</sup> Early diagnosis of aspergillosis is still an intriguing problem. This may be due to lack of consistent antigenic preparations and inadequate studies on immunological responses in the patients with respect to well defined antigens of *A.fumigatus*. The high levels of total IgE immunoglobulins and antigen specific IgE and IgG present in patients with ABPA have been used to differentiate these patients from those with aspergilloma, atopic asthma and from normal individuals.<sup>5,6</sup> A number of immunodiagnostic procedures such as agar gel diffusion, complement fixation, hemagglutination, radioallergosorbent test (RAST), immunofluorescence, radioimmunoassay, and enzyme linked immunosorbent assay (ELISA) have been reported for the detection of specific IgE and IgG antibodies in the sera of aspergillosis patients.<sup>6-12</sup> The commercial antigenic extracts have low protein content and the antigenic compo-

**SUMMARY** Metabolic antigens of *Aspergillus fumigatus*, soil strain 2605 and sputum isolate, were evaluated for their diagnostic applicability using hyperimmune sera and sera of adults and pediatric patients of allergic bronchopulmonary aspergillosis. An indirect ELISA was standardised by using 2-10 µg/ml of coating antigen for detection of specific IgG and IgE antibodies in the sera of patients. The ratios of absorbance for specific IgE and IgG antibodies by ELISA (normal to patients) were observed to be in the range of 1:2 to 1:5 and 1:3 to 1:8 respectively. These antigenic preparations were further analyzed to identify and characterize the individual components by immunoblotting. This analysis indicated the presence of allergenic and antigenic determinants in the antigens of molecular weights 70, 34, and 28Kd. The utility of the antigens of soil strain for diagnostic purpose is suggested.

nents vary from strain to strain and in various preparations from a single strain.<sup>13</sup> Therefore a well defined extract of the fungus is necessary for the immunodiagnosis of Aspergillus induced diseases.

Bronchopulmonary aspergillosis in childhood may be one of the chronic conditions leading to damage of the lung tissue. The diagnosis of ABPA is often not made as very low levels of serum antibody against *A.fumigatus* are observed in patients at early stages of the disease. Hence sensitive methods like enzyme linked immunosorbent assay (ELISA) and western blot may be required for detecting low levels of serum IgG and IgE antibodies against *A.fumigatus* for diagnosis of ABPA. In this paper the preparation of potent antigenic

extract from culture filtrate of *A.fumigatus* (soil and sputum strains) and the identification of the relevant IgE and IgG responsive antigens for early diagnosis of allergic bronchopulmonary aspergillosis under age group of 15 are explored.

## MATERIALS AND METHODS

*A.fumigatus* antigens from two different strains were used for the present studies: i) sputum isolate of an Aspergillosis patient was ob-

From the <sup>1</sup>CSIR Centre for Biochemicals, V.P.Chest Institute Building, Delhi University Campus, Delhi 1100 07, India. <sup>2</sup>Department of Pediatrics, All India Institute of Medical Sciences, New Delhi 1100 29, India.

Correspondence : Dr. Usha S Puranam.

tained from the Mycology Department of Vallabhai Patel Chest Institute; ii) soil strain 2605 (ITCC) was obtained from the Mycology and Plant Pathology Department of the Indian Agricultural Research Institute, New Delhi. These strains were grown as long term stationary cultures (4 to 5 weeks) on synthetic medium at 37° C. The culture filtrate (CF) was collected at different stages of growth, dialyzed against deionized water at 4° C and lyophilized.

Lyophilized crude CF extract was dissolved in water to a final concentration of 20-30 mg/ml and an equal volume of saturated ammonium sulphate was slowly added with stirring. The precipitate was separated by centrifugation and washed twice with 50% saturated ammonium sulphate and resuspended in distilled water. The protein content was determined by the dye binding method of Bradford<sup>16</sup> and various protein components were examined by PAGE and IEF techniques. Antigenicity of the extracts was evaluated by immunological methods using antisera raised in rabbits.

General chemicals used in this study were of reagent grade and were obtained from local companies. Special chemicals such as substrates and immunochemicals were purchased from Sigma Co., and nitrocellulose membranes were from Schleicher & Schuell Co.

#### Rabbit Antisera

Hyperimmune sera to third week culture filtrate (IIIWCF) antigens were raised in New Zealand white rabbits. A 10 mg extract in 2 ml of saline was emulsified with Freund's complete adjuvant and injected subcutaneously in multiple sites. This procedure was repeated for four consecutive weeks, with Freund's incomplete adjuvant followed by weekly booster doses until a satisfactory antibody titre was obtained as determined by CIE tests.

#### Human Sera

Sera of patients suffering from ABPA were obtained from the pediatric chest clinic. All India Institute of Medical Sciences, New Delhi. The clinical criteria applied were: 1) episodic asthma with fleeting pulmonary infiltrate; 2) immediate and delayed skin reactivity to *A. fumigatus* (Af) antigens; 3) precipitin antibodies against Af; 4) eosinophilia; 5) elevated total IgE; 6) central bronchiectasis. Sera from adult patients suffering from ABPA were also analyzed. These sera were obtained from RBTB Hospital, Patel Chest Institute, New Delhi and the Chest clinic of the Guntur Medical College, Andhra Pradesh. ABPA patients fulfilled the clinical and immunological criteria.<sup>17</sup> The normal controls included in this study were from blood banks and other laboratory workers and children under age group 15 not having any respiratory problems. Their sera were found to be negative in precipitin reactions with *A. fumigatus* antigens.

#### Immunochemical characterization of the protein extracts

Antigenicity of the preparation was evaluated by crossed immunoelectrophoresis (CIE) and Ouchterloney's immunodiffusion.<sup>18,19</sup>

#### Enzyme Linked Immunosorbent Assay

The wells of microtitre plates (Nunc co.) were coated with 200 µl of culture filtrate antigens (2-10 µg/ml) diluted in 0.05 molar carbonate buffer pH 9.6 and incubated overnight at 4° C. Plates were washed 3 times with PBS containing 0.05% Tween 20 (assay buffer) and blocked with 3% BSA in assay buffer for 1 hour, then incubated with sera from pediatric patients (1:25) and (1:50) and adults (1:100) diluted in the assay buffer for 3 hours at 37° C. The microtitre plate was washed as before. 200 µl antihuman IgG peroxidase (1:1000) or protein A peroxidase diluted 1:500 in assay buffer was then added to the plate and incubated for

30 minutes at 37° C. After washing thrice, 200 µl of freshly prepared substrate containing O-phenylenediamine (40 mg in 50 ml of phosphate citrate buffer pH 4.5 and 40 µl of hydrogen peroxide) was added and incubated for 30 minutes in the dark. The reaction was stopped by adding 50 µl of 6N HCl and absorbance was measured at 490 nm against the wells of the last row of the plate which are the buffer blanks by ELISA reader. Peroxidase conjugated antibody and protein A peroxidase systems were found to be satisfactory with respect to sensitivity. Incubation periods for protein A peroxidase could also be reduced to 20 minutes.

#### Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis was performed in 12% polyacrylamide separating gel in 0.756M Tris pH 8.8, 0.2% SDS with a 3% stacking gel in 0.25M Tris pH 6.8 and 0.2% SDS. Samples were boiled in 0.25M Tris pH 6.8 and 5% 2-mercaptoethanol for 2-3 min. Gels were stained with Coomassie brilliant blue R-250 dye.

#### Enzyme linked immuno electrotrans-blot (EITB)

Immunotransblot was performed as described by Towbin *et al.* with some modifications.<sup>20</sup> Proteins were first resolved on SDS-PAGE and then transferred to nitrocellulose membranes (0.45 µM) using transfer buffer containing 25 mM Tris and 125 mM glycine (pH 8.3) and 20% methanol. The transblot cell (E-C transblot cell) was filled with transfer buffer and the gel sandwiched between NC membrane and Whatman filter paper in a cassette. The transverse electrophoresis was carried out at 500 mA for 5 hr at 4° C. The NC blot was then placed in blocking solution containing 3% BSA in 0.01M Tris HCl (pH 7.4), 0.15 M NaCl (TBS) for 3 hr at room temperature. The protein NC blot was washed thrice with TBS containing 0.3% Tween

20 with an interval of 10 minutes each. The membrane was then incubated with the patient's sera at 1:50 dilution for detecting IgG antibodies and 1:10 dilution for IgE antibodies overnight at 4° C. The strips were washed thrice with washing buffer for 10 minutes each and incubated either with protein A peroxidase (1:100) or antihuman IgG (1:50) for the detection of specific IgG antibodies in patient and with anti human IgE peroxidase (1:125) for detecting IgE antibodies for one hour at room temperature. Diaminobenzidine with hydrogen peroxide was used as the chromogenic substrate for peroxidase.

### Isoelectric Focusing (IEF)

The isoelectric points of the component proteins of potent extracts of *A. fumigatus* (soil and sputum strains) were determined by analytical isoelectric focussing. The samples were run on a 7% polyacrylamide gel with a pH gradient 3-10 at 10° C for 5 hr at 250 volts.

### RESULTS

The protein concentrations in III WCF of *A. fumigatus* (soil and sputum strains) was observed to be in the range of 10-12 mg percent by Bradford method. Antigenicity of the CF proteins was evaluated at different stages of growth against the rabbit hyperimmune sera by transblot technique (Fig. 1). In II WCF major proteins in the MW range of 66-50Kd were antigenic in nature. Besides these antigens proteins in the MW range of 30-20Kd and 18-21Kd were also found to have antigenicity in III WCF. Analytical isoelectric focusing of the potent antigenic preparations revealed the presence of proteins in the isoelectric point range from 4 to 7.

The protein concentration was adjusted to 2-10 µg/ml for coating the wells of microtitre plates for ELISA. ABPA patients (pediatric) during acute attacks and those im-

proving steadily were evaluated for their IgE and IgG antibody titres. The ratio of IgG specific antibodies in normals to patients under acute attack ranged from 1:5 to 1:8. In some cases the ratio of normal to patient IgE was as high as 1:10. The ratios of absorbance of normal to adult patients are shown in Table 1.

The IgG reactivity of ABPA pediatric and adult patients' sera against culture filtrate proteins of both the soil and sputum strains of *A. fumigatus* are shown in Fig. 2. The antigens of MW 70Kd from both the strains of *A. fumigatus* showed strong reactivity with the patients' sera. Antigens of the sputum strain in MW ranges 55-40Kd and 20-12Kd exhibited immunological reaction with IgG antibodies. Some of the normal sera showed a low grade of nonspecific reactivity with the CF antigens of sputum strain. The IgG reactivity of the pooled sera of adult ABPA patients with CF antigens of sputum and soil strains are shown in Fig. 2 (lanes 1&2). The sputum strain showed high reactivity in the ranges of 90-70Kd, 60-35Kd and 20-12Kd. Antigens of MW 45-35Kd of both the strains showed reactivity with IgG in the sera of adult patients. However, these antigens showed relatively less reactivity with sera of pediatric patients (Fig. 2, lanes 3&4). The specific IgE and IgG reactivities of the pooled pediatric patients'

sera with (an absorbance of 0.5 and 1.5 by ELISA respectively) against culture filtrate antigens of the sputum strain of *A. fumigatus* are shown in Fig. 3. Strong IgG reactivities were observed in the MW range 30-14Kd. IgG reactivity was also present to antigens of MW 70, 60, 45 and 34Kd. IgE reactive antigens were observed to have MW of 70, 34 and 28Kd. These observations indicate that antigens with molecular weights 70,

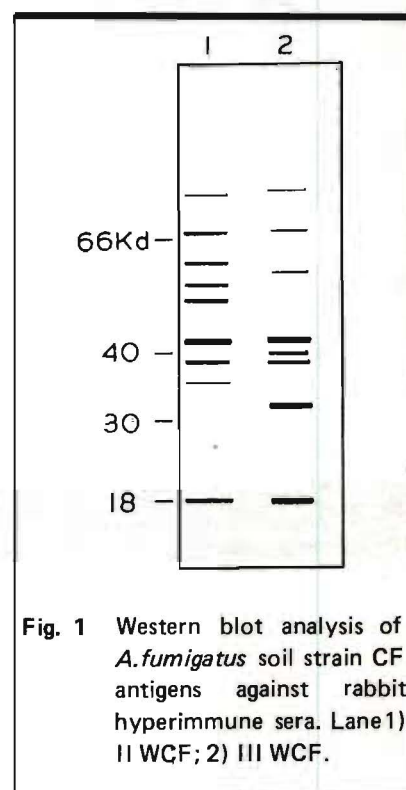
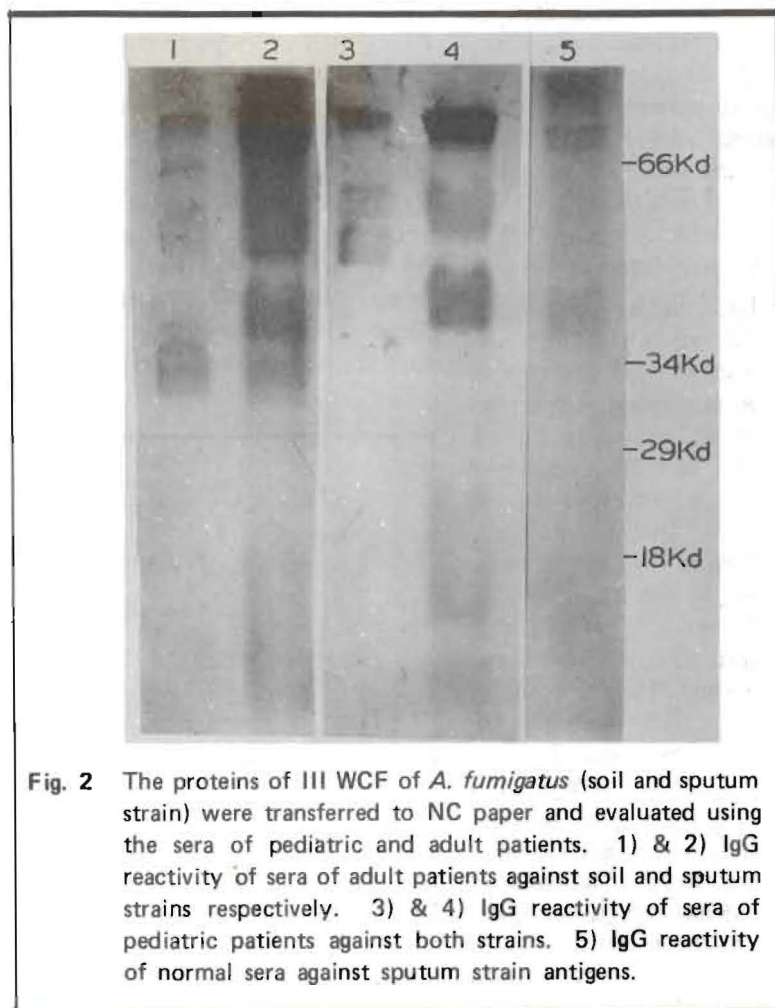


Fig. 1 Western blot analysis of *A. fumigatus* soil strain CF antigens against rabbit hyperimmune sera. Lane 1) II WCF; 2) III WCF.

Table 1. ELISA values for specific IgE and IgG antibodies of patients of Aspergillosis.

Study groups	n	Mean OD at 490 nm		Ratio of OD of normal to study groups	
		IgE	IgG	IgE	IgG
1. ABPA Pediatric (acute attack)	5	0.255	1.530	1 : 5	1 : 8
2. ABPA Pediatric (steady improvement)	10	0.093	0.560	1 : 2	1 : 3
3. ABPA Adult	8	0.240	1.488	1 : 4	1 : 7
4. Normal	10	0.048	0.188	--	--



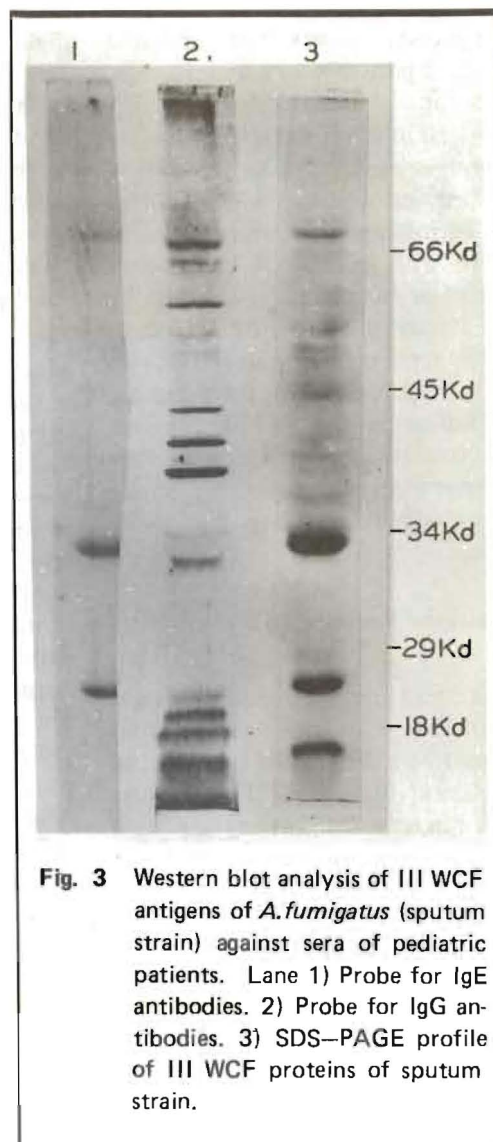
**Fig. 2** The proteins of III WCF of *A. fumigatus* (soil and sputum strain) were transferred to NC paper and evaluated using the sera of pediatric and adult patients. 1) & 2) IgG reactivity of sera of adult patients against soil and sputum strains respectively. 3) & 4) IgG reactivity of sera of pediatric patients against both strains. 5) IgG reactivity of normal sera against sputum strain antigens.

34 and 28Kd exhibit common reactivity for IgE and IgG antibodies during an acute attack.

### DISCUSSION

Mycelial, conidial and culture filtrate antigens of *A. fumigatus* have been reported by a number of groups. Some strains such as Ag-507, Ag-515 and Ag-534 seem to be of use for the preparation of antigens that can be employed in immunodiagnosis of ABPA.<sup>21</sup> The antigens from a soil strain (2605) and a sputum isolate used in this study for immunodiagnosis were extracted from culture filtrate of *A. fumigatus*. A maximum number of antigenic proteins was observed in III WCF by the transblot technique. IgG responsive antigens were identified by use of individual

or pooled sera of pediatric and adult ABPA patients. At 70Kd both the strains showed strong reactivity with pediatric sera. Besides 70Kd antigens, proteins in the range of 45-35 Kd of both strains showed IgG reactivity with the sera from adult patients. However the sputum strain had more antigenic proteins compared to the soil strain. Experiments with antigens of the soil strain using ID, ELISA and transblot techniques clearly indicated that these antigens can be successfully used for early diagnosis of ABPA patients. However, a detailed understanding by way of characterization and detailed comparisons with the antigens of the sputum isolate



**Fig. 3** Western blot analysis of III WCF antigens of *A. fumigatus* (sputum strain) against sera of pediatric patients. Lane 1) Probe for IgE antibodies. 2) Probe for IgG antibodies. 3) SDS-PAGE profile of III WCF proteins of sputum strain.

would further help in the utility of these antigens more specifically.

The presence of significant concentrations of IgG antibodies to the high molecular weight antigens was noticed in a few patients who were improving steadily. During the acute attack the sera of these patients showed an OD of 1.5 by ELISA and a wide range of antigenic reactions to the low molecular as well as high molecular weight proteins. Very high IgG reactivity was observed with the antigens in the molecular weight range of 30-14 Kd for the above mentioned patients. The presence of multiple bands showing IgG reactivity

with the sera of patients with ABPA has also been reported by Longbottom *et al.*<sup>22</sup> IgE reactivity with the antigens of the sputum strain was evaluated using sera of pediatric patients with an OD of 0.5 by ELISA. Antigens of MW 70, 34 and 28Kd showed IgE reactivity. These antigens also shared IgG reactivity. The 34Kd antigen reported in this paper showed characteristic properties similar to those of Ag 7 of Longbottom *et al.*<sup>23</sup> SDS PAGE of crude culture filtrate extracts showed proteins in the MW range 100-12Kd with prominent bands at 70, 50, 34, 28, 14Kd. The three proteins mentioned in this paper having both allergenic and antigenic determinants appear to be similar to the three antigenic fractions of a partially purified glycoprotein reported by Kurup *et al.*<sup>24</sup> The sera of a normal individual with an OD of 0.35 by ELISA showed IgG reactivity with the high and medium molecular weight antigens. This may be due to the non specific binding character of some of these antigens, as reported by Bardana *et al.*<sup>25</sup> or the occurrence of subclinical levels of these antibodies in this individual. However, the ratios of OD of normals to patients by ELISA was always taken into account in the western blotting experiments. The high MW antigen 260-160Kd from mycelial extract reported by Schonheyder *et al.*<sup>26</sup> was not present in our culture filtrate preparation. This high molecular weight antigen of mycelium does not appear to be excreted during metabolic processing. Western blot analysis with the antigens of *A. fumigatus* reported in this paper clearly indicate that there are three major antigens which can be of diagnostic importance. These antigens have MW 70, 34 and 28Kd respectively and they react with IgE and IgG from the patients' sera. Though the sources of antigens reported by Kurup *et al.*<sup>24</sup>, Longbottom *et al.*<sup>23</sup> and by us are different, the antigens of diagnostic importance appear to be similar as observed by molecular weight criteria.

There are many antigens which showed only IgG reactivity with patients' sera. Attempts are being made to separate the two activities (IgE and IgG) and characterize the antigenic determinants for possible use in diagnosis.

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