***Aspergillus fumigatus* antigens**

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Cytosolic fractions of mycelial extracts from *Aspergillus nidulans*, *A. flavus*, and three different isolates of *A. fumigatus*, grown to stationary phase in Czapek-Dox-AOAC medium, were tested by immunoblotting for the presence of antigens reactive to 80 serum samples from aspergilloma patients. Fifty control serum samples were used to determine the specificity of the reactions. In the *A. fumigatus* cytosolic fraction a group of four main antigenic bands (p90, p60, p40 and p37) was consistently recognized (in total or partial form) by 90% of the serum samples from the aspergilloma patients. This group of antigens was designated as the 'cytosolic fraction complex' (CFC). As confirmed by two-dimensional electrophoresis followed by immunoblotting with aspergilloma serum samples, each of the four antigenic bands is formed of several isoforms of acidic glycopeptides with slightly different pIs. All the isoforms are at least N-glycosylated, as demonstrated by endoglycosidase H removal of a considerable amount of sugar residues. The relationship of these antigens with certain other *A. fumigatus* antigens previously reported in the literature, and their potential use in the immunodiagnosis of aspergilloma, are discussed.

Keywords: *Aspergillus*, aspergilloma, immunodiagnostics, fungal antigens

INTRODUCTION

The standardization of antigenic mixtures (mainly from *Aspergillus fumigatus*) reactive to serum samples from patients with different forms of aspergillosis has been a primary goal for researchers involved in the immunodiagnosis of these diseases. Complex preparations have been successfully used to detect different classes (IgG and IgE) of specific antibodies by several methods including immunodiffusion, immunoelectrophoresis, RIA, ELISA and BALISA (Kurup & Kumar, 1991; Andriole, 1992). The antigens present in these mixtures were in their native form and the molecules directly responsible for the immune reactions were identified indirectly by subsequent analytical procedures. High-resolution techniques such as SDS-PAGE, analytical isoelectrofocusing and two-dimensional electrophoresis have also been applied to the analysis of these complex mixtures. However, to our knowledge, only the separation power of SDS-PAGE has been combined with the specificity of immune reactions in Western blot assays to define with better precision which particular molecules are responsible for the reaction with the specific antibodies from aspergillosis-affected indi-

viduals (Burnie *et al.*, 1989; Hearn *et al.*, 1990; Fratamico & Buckley, 1991; Latgé *et al.*, 1991; Hearn, 1992; Kobayashi *et al.*, 1993). Because of the need to establish an integrated approach to the identification of defined *Aspergillus* antigens, the current study was undertaken. In this work we have tried to combine the best of the different previous approaches: (i) standardization of antigenic mixtures with reference to the selected species (Kim & Chaparas, 1979; Burnie *et al.*, 1989; Hearn, 1992), isolates, growth conditions (Kim & Chaparas, 1978; van der Heide *et al.*, 1985a, b; Calera *et al.*, 1994), subcellular fractions and extraction methods; (ii) the use of SDS-PAGE, and two-dimensional electrophoresis methods, followed by immunoblotting to analyse in depth the composition of the antigenic mixtures; (iii) the use of a high number of serum samples, perfectly documented, from clinical situations or control patients. Using these approaches, we have been able to detect and unequivocally identify useful antigens in the previously analysed extracts.

METHODS

Organisms and growth conditions. Three *Aspergillus fumigatus* isolates were used: AFC is a strain from the American Type Culture Collection (ATCC 9197). AFR is a clinical isolate from

Abbreviation: CFC, cytosolic fraction complex.

the sputum of a patient with pulmonary aspergilloma; AFN was isolated from a lung biopsy of a patient with invasive pulmonary aspergillosis and AIDS, kindly provided by J. M. Torres-Rodriguez (IMIM, Barcelona, Spain). *Aspergillus nidulans* G1059wt (*adF17 pabaA1 yA2*) was obtained from A. J. Clutterbuck, Glasgow, UK. *Aspergillus flavus*, isolated from a lymphadenopathy in a patient with breast cancer and cutaneous invasive aspergillosis was a gift from A. del Palacio (12 de Octubre Hospital, Madrid, Spain).

The organisms were maintained on solid YED medium [1% (w/v) D-glucose, 1% (w/v) Difco yeast extract and 2% (w/v) agar]. To obtain high yields of conidia, the fungi were grown on solid *Aspergillus* complete minimal medium or on AMM [0.1% glucose, 0.6% NaNO₃, 0.052% MgSO₄, 0.052% KCl, 0.15% KH₂PO₄, traces of FeSO₄ and ZnSO₄, and 1.5% (w/v) agar (pH 6.5)]. Plates were incubated at 28 °C for at least 4 d.

For liquid growth cultures, Bacto Czapek-Dox broth/Bacto Synthetic broth AOAC (1:1) (CDA) medium was used (obtained from Difco). As shown in our previous studies on the influence of the culture medium on the presence of antigens specifically reactive to *Aspergillus* extracts (Calera *et al.*, 1994), Czapek medium seems to strongly induce the biosynthesis of CFC antigens. For *A. nidulans*, CDA medium, both solid and liquid, was supplemented with 10 mg *p*-aminobenzoic acid and 200 mg adenine per litre.

All the *Aspergillus* species were grown by inoculation of 10⁵ conidia ml⁻¹ in 1-litre Erlenmeyer flasks containing 300 ml of the corresponding liquid medium followed by incubation at 37 °C in an Adolph Kühner orbital shaker at 280 r.p.m. Mycelia were harvested from liquid medium cultures by filtering through Whatman GF/C paper and washed thoroughly with double-distilled H₂O. The wet cake was immediately frozen and kept at -70 °C until used.

Preparation of cytosolic extracts. Frozen mycelia were thawed and mixed with lysing buffer [100 mM Tris/HCl pH 7.5 containing 1 mM EDTA, 5 mM dithiothreitol, 1 mM freshly added phenylmethylsulfonyl fluoride (PMSF, Sigma) 5 µg Aprotinin ml⁻¹, and 5 µg Pepstatin A ml⁻¹ (both from Boehringer Mannheim)] to give a dense suspension. Samples were then disrupted in a French press (SLM Aminco) using the 20000 p.s.i. (138 MPa) cell, previously refrigerated at -20 °C, at a pressure of 16000 p.s.i. (110 MPa). Complete breakage was monitored by microscopy. Broken mycelia were centrifuged at 150000 *g* for 1 h to pellet cell-walls and total membranes; the resulting supernatant was considered as the cytosol. For SDS-PAGE, cytosolic fractions were extracted by boiling in 2% (w/v, final concentration) SDS, clarified (20000 *g* for 30 min) and stored as described above. Protein was quantified by a modification of the Lowry method (Peterson, 1977). Extracts containing less than 1 mg protein ml⁻¹ were concentrated by precipitation with 7 vols cold acetone at -70 °C for at least 3 h. Precipitated protein was pelleted by centrifugation for 20 min at 12000 *g* at 4 °C, dried in a vacuum evaporator (Savant instruments), carefully resuspended in 2% SDS to the desired concentration and clarified by centrifugation at 20000 *g* for 30 min.

Treatments with endoglycosidase H. After acetone precipitation, a dry sample of cytosol was solubilized in buffer [50 mM sodium citrate pH 5.5 supplemented with β-mercaptoethanol (0.1 M, final concentration) and a 1.2-fold excess of SDS relative to total protein concentration]. The sample was heated at 100 °C for 3 min. After cooling the sample, 1 mU endoglycosidase H (endo-β-N-acetylglucosaminidase H from *Streptomyces plicatus*, Boehringer Mannheim) was added per µg protein, and 0.5 mM

PMSF (final concentration) was added to prevent proteolytic degradation. The mixture was then incubated at 37 °C for 24 h.

SDS-PAGE. Electrophoreses were carried out on a Protean II or Mini-Protean apparatus (Bio-Rad) on isotropic 14% or 16% (w/v) acrylamide slab gels (16 × 18 × 0.1 cm or 8 × 6 × 0.1 cm) using the discontinuous buffer system of Laemmli (1970). Molecular mass protein standards were Bio-Rad Low Molecular Weight standards. Proteins in gels were detected by a sensitive silver stain (Morrissey, 1981) or by staining for 30 min with 0.5% Coomassie Brilliant Blue R-250 in acetic acid/2-propanol/water (1:3:6, by vol.) and destaining in acetic acid/methanol/water (10:5:85, by vol.).

Two-dimensional gel electrophoresis. Two-dimensional gel electrophoreses were performed as described by Bravo (1984) and O'Farrell (1975). Briefly, the first-dimension separations for resolving acidic proteins were done on 150 × 2.3 mm 4% (w/v) polyacrylamide gels containing 6% (w/v) ampholytes (2% pH 3.5–5; 2% pH 4–6; 2% pH 5–7) at 5.28 mA for 4–5 h. The second-dimension separations were carried out in a 16% (w/v) SDS-polyacrylamide gel (20 × 14.5 cm) and electrophoresed at room temperature overnight. Carbamylate 2D standards (carbamylated creatine phosphokinase and carbonic anhydrase, Pharmacia) were used as internal markers.

Human serum samples. A total of 130 human serum samples was provided by microbiology laboratories of different Spanish hospitals. Eighty of these serum samples were from aspergilloma cases. Aspergilloma was demonstrated by chest X-ray changes showing a discrete lesion with a halo and positive cultures for *A. fumigatus* obtained repeatedly either from sputum cultures or by bronchoscopy. The serological response against *Aspergillus* antigens was demonstrated by immunodiffusion tests with commercial antigens, showing at least three precipitating arcs against *A. fumigatus* mycelial or culture filtrate antigens. Fifty serum samples were used as controls: 28 were from healthy individuals (blood donor bank), 17 from asthmatic and cystic fibrosis patients and five from pulmonary tuberculosis cases. No antibodies against *Aspergillus* antigens were detected in these sera, using the same immunodiffusion tests as described above.

Electrophoretic blotting procedures and immunological detection of proteins. Proteins from extracts were first subjected to electrophoresis (SDS-PAGE or two-dimensional) as described and then transferred to nitrocellulose sheets (0.45 µm; Schleicher & Schuell) in a Trans-Blot cell (Bio-Rad) as previously described (Calera *et al.*, 1994).

Glycoprotein detection. Protocol B of the DIG glycan detection kit (Boehringer Mannheim) was used according to the manufacturer's instructions.

Photography. Stained gels were photographed with a 32 Asa black-and-white Panatomic-X (Kodak) film using an orange (Coomassie stain) or blue (silver stain) filter. Nitrocellulose-developed blots were photographed with an orange filter or with no filter.

RESULTS

Identification of *A. fumigatus* polypeptides highly reactive in immunoblots with sera from aspergilloma patients

Cytosolic fractions from *A. nidulans*, *A. flavus* and three isolates of *A. fumigatus* grown to stationary phase in Czapek-Dox-AOAC (CDA) medium were separated by SDS-PAGE and analysed by silver staining and by immunoblotting using aspergilloma serum samples. The

Fig. 1. SDS-PAGE (16%, w/v) of the cytosolic fractions from three *Aspergillus* species grown with shaking at 280 r.p.m. in Czapek-AOAC medium for 7 d at 37 °C (initial inoculum 10^5 conidia ml^{-1}). The separated proteins were either silver stained (4 μg protein per lane) or transferred to nitrocellulose paper (20 μg protein per lane) and probed with the following human sera at 1/250 dilution: H-40, a representative aspergilloma serum sample; Pool, a pool of 10 different aspergilloma sera; and Control serum, a representative serum sample of healthy blood-bank donors. Lanes: ANid., *A. nidulans*; AFLav., *A. flavus*; AFC, AFR and AFN, three different isolates of *A. fumigatus* (see Methods).

Fig. 2. SDS-PAGE (16%, w/v) and Western blots of native and endoglycosidase H treated cytosol of *A. fumigatus* isolate AFR. Lanes: C, native cytosol; EHT, cytosol after digestion with endoglycosidase H (1 mU per mg protein); EH, control endoglycosidase H. The gel was stained with silver nitrate (left panel) and the blots were either stained for glycoproteins (DIG-glycan detection kit, Boehringer-Mannheim; right panel) or probed with a human aspergilloma serum sample (1/250, H-29; middle panel).

silver-stained polypeptide pattern (Fig. 1) was quite similar for the cytosolic fractions from *A. fumigatus* (AFC, AFR, AFN), but characteristic for each fraction. *A. nidulans* (AN) and *A. flavus* (AFL) gave completely different patterns. Fig. 1 shows that the major silver-stained polypeptides in cytosolic fractions from *A. fumigatus* were also the most reactive antigens to specific IgG antibody from a representative serum sample (H-40) or a pool at 10 different aspergilloma sera (1/250 dilution). *A. flavus* and *A. nidulans* did not show any consistent reaction.

When nitrocellulose blots identical to the one just described were reacted with 130 different serum samples, the responses to 80 serum samples from aspergilloma patients were similar to that shown in Fig. 1. In all cases, the cytosolic fraction of the three *A. fumigatus* isolates was the most consistently reactive. We designated the group of the four main reactive bands, with apparent molecular masses of 90, 60, 40 and 37 kDa, as CFC (cytosolic fraction complex). From this point these proteins will be named p90, p60, p40 and p37 *A. fumigatus* polypeptide antigens. Of the 80 aspergilloma sera, 53 % recognized all

Fig. 3. SDS-PAGE (1D, 20 µg protein per lane) and two-dimensional electrophoresis (2D, 500 µg protein per gel) of the cytosolic fractions of three different *A. fumigatus* isolates. The 1D and 2D gels in panels A, B and C (isolates AFN, AFC and AFR, respectively) were silver-stained. Panel D is an immunoblot of a 2D gel, identical to the one shown in panel C (isolate AFR), developed with a pool of four representative human aspergilloma sera (1/250); arrows indicate corresponding zones between the gel and the blot. CA, carbamylated carbonic anhydrase markers used as internal standard.

four bands of the CFC and 37% recognized at least p90 (alone or with p60), giving a combined total of 90% recognition; 10% of the aspergilloma sera did not give any reaction. By comparison, 70% of the 50 controls (35 sera) were non-reactive and 30% (15 sera) slightly recognized p60 (Fig. 1, control serum). These data suggest that p90 could be a useful aspergilloma marker, and that p40 and p37 could indicate a stronger immunological response to the infection.

All the CFC polypeptide components are glycosylated

To test if the CFC components were glycopeptides, we followed a protocol for specific staining of glycoproteins, using a commercial kit (Boehringer-Mannheim). As

shown in Fig. 2 (Glycoprotein stain, lane C), only p60 stained strongly; some extremely faint reactions were present around the rest of the CFC components (not visible in the figure). To confirm this point, we digested cytosolic extracts from the three isolates with an excess of endoglycosidase H. The result of the digestion is shown in Fig. 2 (only for isolate AFR) after SDS-PAGE separation and silver staining or immunoblotting with a representative aspergilloma serum (H-29). The electrophoretic mobility of all the components of the CFC was faster after endoglycosidase H treatment (lanes EHT) as compared with the original cytosol (lanes C). This result demonstrates that p90, p60, p40 and p37 are all glycoproteins with at least *N*-linked sugars. The low sensitivity of the commercial kit used was probably responsible for

the lack of staining of p90, p40 and p37. The fact that p60 was still stained after Endo H treatment indicates either that not all the N-linked sugars were removed or that O-linked sugars existed in the molecule.

Two-dimensional mapping of CFC glycopeptides

We analysed the cytosolic extracts of the three *A. fumigatus* isolates by two-dimensional electrophoresis. First-dimension (isoelectricfocusing) separation was done in the presence of urea on a gradient of acid pH (based on a mixture of 3.5–5, 4–6 and 5–7 ampholites). The second dimension was achieved by SDS-PAGE on 16% (w/v) polyacrylamide gels. Carbamylated carbonic anhydrase (Pharmacia) was used as an internal standard. Gels were either silver-stained or transferred to nitrocellulose and incubated with aspergilloma serum samples. Fig. 3 shows the virtual equivalence of the silver-stained maps of the cytosols from the three different isolates (AFN, AFC, AFR). By comparison with the one-dimensional SDS-PAGE included as a reference to the left of each two-dimensional gel it is evident that all the CFC components are not single polypeptides; rather, each of them consists of several isoforms (as confirmed by chemical or enzymic peptide mapping studies to be described elsewhere) of slightly different pI. p60 showed at least 11 different forms and p40 and p37 at least eight. The pI of these three groups of glycoproteins was acidic and very similar. p90 behaved as a family of at least five glycoproteins with slightly less acidic pIs. All the isoforms of the three isolates were reactive with serum samples from the different aspergilloma patients (the immunoblot with H-29 is shown in panel D of Fig. 3 as an example).

DISCUSSION

Our previous study on the influence of media and time and temperature of growth on the detection of *A. nidulans* antigens (Calera *et al.*, 1994) showed that the use of strictly controlled culture conditions in Czapek-AOAC medium, as recommended by Kurup & Kumar (1991), provides antigenic preparations that are reactive with serum samples from aspergilloma patients. Here we applied an identical approach to compare the reactivity of antigenic extracts of some species of *Aspergillus* commonly involved in pathological situations. The results obtained indicate that *A. fumigatus* provided the strongest reactivity with aspergilloma serum samples. Four polypeptides (p90, p60, p40 and p37) were present in the cytosolic fractions of three different *A. fumigatus* isolates grown under strictly controlled conditions. These polypeptides reacted strongly and repetitively with serum samples from patients with aspergilloma. The level of recognition was similar to that described in the literature when other detector antigens were used (Kim *et al.*, 1979; Calvanico *et al.*, 1981; Fratomico & Buckley, 1991). p90 was the most specific antigen, detected by a 90% of the aspergilloma serum samples and none of the controls. Only p60 seems to be recognized by 30% of the control serum samples (including healthy subjects or patients affected by diseases unrelated to *Aspergillus*). Either the high degree of

glycosylation of this antigen or the ubiquity of *Aspergillus* species, or both, could be responsible for this reactivity.

Two-dimensional analysis in combination with Western blotting with well-characterized human serum samples discloses the great complexity of the antigenic mixtures used and provides a powerful means to more accurately define the characteristics of the antigens described here. Some of these characteristics, mainly their acidic pI and glycoprotein nature, are in agreement with those of previously described antigens (Calvanico *et al.*, 1981; Fratomico & Buckley, 1991). In this respect, a direct comparison could be made between our silver-stained two-dimensional patterns and those described for CS and CS2 by Piechura *et al.* (1983, 1987) and Calvanico *et al.* (1981). However, we have directly shown the consistent reactivity of all the isoforms present in the cytosolic preparations with IgGs in the serum samples of aspergilloma patients. Additionally, our silver-stained SDS-PAGE profiles are reminiscent of those obtained by Piechura *et al.* (1987) when they analysed 96 h old *A. fumigatus* mycelia or by Fratomico & Buckley (1991), who analysed younger mycelial extracts of the same species. In this latter case, the immunoblots also coincide in the detection of a 58 kDa (very close to 60 kDa) antigen. These two antigens are probably the same, the slight difference in size being due to the conditions of preparation. In our hands, around 30% of the control serum samples also recognized this antigen. For this reason, its value for the diagnosis of aspergilloma or invasive aspergillosis is at least doubtful.

The glycosylation of *Aspergillus* antigens has been reported on several occasions (for a review see Kurup & Kumar, 1991). We have shown that at least part of the carbohydrate moiety of CFC antigens is N-linked. A similar result has been reported for an 88 kDa antigen purified from *A. fumigatus* (Kobayashi *et al.*, 1993). Owing to differences in subcellular location, pI and reactivity with candidiasis serum samples (unpublished results) no relationship seems to exist between this antigen and the one described by us as p90.

The reason for the pI differences between the isoforms of the described antigens is not clear. They could be due to phosphorylation [the 58 kDa antigen reported by Fratomico & Buckley (1991) contained phosphate groups], sulfatation or differential glycosylation. Further work needs to be done to ascertain this point.

The CFC components still reacted with the aspergilloma sera after treatment with endoglycosidase H. Total removal of the sugar from bands p90, p40 and p37 was demonstrated by gas-phase chromatography (unpublished results). These observations indicate that the immune recognition of these molecules seems to be due, at least in part, to their protein moiety. Similar results have been reported for other *A. fumigatus* antigens (Hearn *et al.*, 1990; Kobayashi *et al.*, 1993).

Our data indicate that CFC is an important antigenic complex of *A. fumigatus*, able to generate a strong antibody response in patients with aspergilloma. Owing

to the high level of recognition of some of these antigens (especially p90) by human serum samples, they could be useful for the diagnosis of some forms of aspergillosis, mainly pulmonary aspergilloma.

ACKNOWLEDGEMENTS

We wish to thank Carlos Belinchón for photographic services, Drs M. Rivera and J. M. Torres-Rodríguez for providing the human serum samples, and Dr Ramón Santamaría for critical reading of the manuscript. We are grateful to Nicholas Skinner for revising the English manuscript. This work was supported by grant BIO92-0042 from the CICYT of the Ministerio de Educación y Ciencia (Spain). M. C. Ovejero and R. López Medrano are recipients of predoctoral fellowships from the Ministerio de Educación y Ciencia and the Fondo de Inversiones Sanitarias de la Seguridad Social respectively.

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Received 13 March 1995; revised 18 May 1995; accepted 5 June 1995.