The recombinant antigen ASPND1r from *Aspergillus nidulans* is specifically recognized by sera from patients with aspergilloma

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A 996 bp *Aspergillus nidulans* cDNA encoding the ASPND1 immunodominant antigen was cloned and expressed in *Escherichia coli* as a fusion protein with the enzyme glutathione S-transferase (GST) from *Schistosoma japonicum*. The GST-ASPND1 fusion protein was purified from isolated bacterial inclusion bodies by preparative SDS-PAGE. After cleavage with thrombin, the ASPND1 recombinant antigen (ASPND1r) and the GST protein were separated by SDS-PAGE and immunoblotted with a number of different human sera. The sera from 22 (88%) of 25 patients with an aspergilloma recognized the ASPND1r recombinant antigen on immunoblots. Forty-nine normal human sera and 14 sera from patients with other infections were unreactive. The ASPND1r expressed in *E. coli* could therefore be used, in combination with previously reported recombinant antigens, as a standardized antigen for serological and clinical diagnosis of *Aspergillus*-associated diseases.

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

INTRODUCTION

The aspergilloses are a complex array of diseases ranging from pulmonary aspergilloma to invasive aspergillosis, and including various allergic reactions (Kwon-Chung & Bennett, 1992). Different species of the genus *Aspergillus*, a ubiquitous mould common in soil and plant debris, are the aetiological agents of these infections, whose diagnosis depends on clinical and immunological findings. Specific circulating antibodies against *Aspergillus fumigatus* antigens have been recognized in most of these diseases (Kurup & Kumar, 1991). However, for many years the lack of standardized antigens has thrown doubt on the reliability of antibody assays (Kwon-Chung & Bennett, 1992). Owing to the intrinsic problems associated with the preparation of antigenic mixtures, many attempts have been made to standardize fungal extracts or to purify defined antigens. Among others, the CAT1 (the subunit of a catalase) (López-Medrano *et al.*, 1995b) and superoxide dismutase

(Holdom *et al.*, 1995) antigens have been purified to homogeneity from *A. fumigatus* water-soluble extracts. Currently, the best way to circumvent these problems relies on the use of purified homogeneous recombinant antigens and allergens of known biological activity. The *Asp f I* ribotoxin (Moser *et al.*, 1992), the *Asp f II* allergen (Banerjee *et al.*, 1996), and the heat-shock protein (HSP-1) (Kumar *et al.*, 1993) from *A. fumigatus* have been obtained as recombinant antigens. All these antigens have been shown to be consistently reactive with sera from patients with different forms of aspergillosis but not with sera from control or healthy individuals (Moser *et al.*, 1994; Banerjee *et al.*, 1996; López-Medrano *et al.*, 1996; Hamilton *et al.*, 1995).

Although *Aspergillus nidulans* is not the most frequently isolated species from aspergillosis patients, it does constitute an alternative model to enable various aspects of the pathogenesis and immunodiagnosis of *Aspergillus* infections to be studied. In support of this assumption, we have previously demonstrated that *A. nidulans* and *A. fumigatus* (the main aetiological agent) share a family of closely related antigens able to induce a strong and specific humoral response in different forms of *Aspergillus* infections (Calera *et al.*, 1997). Antigens belonging to this family, mainly p40 and p37 (López-

Abbreviations: GST, glutathione S-transferase; IOD, integrated optical density.

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Medrano *et al.*, 1996) and *Asp f II* (Banerjee *et al.*, 1996) from *A. fumigatus* and ASPND1 from *A. nidulans* (Calera *et al.*, 1994), have been shown to be specific markers for clinical infections caused by *Aspergillus*, regardless of the species originally involved. *Asp f II* antigen has been produced in recombinant form (Banerjee *et al.*, 1996), thus avoiding the problems of the batch-to-batch variability associated with its production from the original fungal host. We have recently cloned and characterized the *A. nidulans* gene coding ASPND1 and reported its similarity to *A. fumigatus Asp f II* allergen (Calera *et al.*, 1997). In this work, we describe the overexpression in *Escherichia coli* of an ASPND1 cDNA and evaluate the ability of this recombinant antigen (ASPND1r) to be specifically recognized by immune human sera.

METHODS

Organisms and growth conditions. *Aspergillus nidulans* G1059wt (*adF17*, *pabaA1*, *yA2*) was obtained from A. J. Clutterbuck (University of Glasgow, Scotland) and grown as previously described (Calera *et al.*, 1997). The following *E. coli* strains were used: strain Y1090 was used for propagation of bacteriophage λ gt11; all plasmid subcloning and overexpression experiments were performed in strain DH5 α ; strain MV1190 was used to obtain single-strand DNA for sequencing; and strain Y1089 was used to obtain lysogen. All bacterial strains were grown at 37 °C in Luria broth medium (Sambrook *et al.*, 1989).

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 (8 × 6 × 0.1 cm) using the discontinuous buffer system of Laemmli (1970). Molecular mass protein standards were normal or prestained Bio-Rad low-molecular-mass markers. Proteins in gels were detected by a sensitive silver stain (Morrisey, 1981) or by staining for 30 min with 0.5% Coomassie brilliant blue R-250 in acetic acid/2-propanol/water (1:3:6) and destaining in acetic acid/methanol/water (10:5:85).

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

Human serum samples. A total of 89 human serum samples was provided by the microbiology laboratories of different Spanish hospitals; 25 of the 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 precipitin arcs against *A. fumigatus* mycelial or culture filtrate antigens. For controls, 64 serum samples were used: 49 were from healthy individuals (blood donor bank), four from patients with fungal sepsis due to *Candida albicans*, three from patients with bacteraemia (two cases due to *Pseudomonas* spp. and one to *Lysteria monocytogenes*), four from pulmonary tuberculosis cases, two from leukaemia cases, one from a patient with positive C-reactive protein, one from a patient with positive rheumatoid factor and two with otomycosis due to *As-*

pergillus. No antibodies against *Aspergillus* antigens were detected in these sera using the immunodiffusion tests described above.

Isolation of total RNA. Total fungal RNA was isolated from *A. nidulans* mycelia grown in CDA liquid culture for 24 h at 37 °C. Hyphae were harvested and ground to a fine powder in liquid nitrogen in a tightly closed Waring blender. Total RNA was isolated from the mycelia by the hot phenol purification method (Timberlake & Hamer, 1986).

Construction and screening of the cDNA expression library. Poly(A)⁺ RNA was isolated from total RNA obtained from *A. nidulans* mycelia using the mRNA Separator Kit (Clontech). Double-stranded cDNA was synthesized from 5 μ g poly(A)⁺ RNA using the cDNA Synthesis System Plus (code RPN 1256 Y/Z, Amersham). After addition of *EcoRI* adapters according to the cDNA Rapid Adaptor Ligation Module (code RPN 1712, Amersham), the cDNA was ligated with λ gt11 arms, digested with *EcoRI*, dephosphorylated (cDNA Rapid Cloning Module λ gt11, code RPN 1714, Amersham) and packaged *in vitro* according to the manufacturer's instructions (λ -DNA *in vitro* Packaging Module, code RPN 1717, Amersham), yielding a library with a packaging efficiency of 4.6×10^6 p.f.u. (μ g cDNA)⁻¹. Immunological screening of the expression library was performed with the immunopurified monospecific antibody using standard protocols (Sambrook *et al.*, 1989).

Positive plaques were identified and recovered from agar plugs. The positive phages were purified following the glycerol step gradient method as described by Sambrook *et al.* (1989). DNA inserts were excised with *EcoRI* and subcloned into pBluescript SK(+) and KS(+) by standard procedures (Sambrook *et al.*, 1989).

Preparation of bacteriophage λ gt11 lysogens. Lysates containing fusion proteins encoded by phage λ gt11 lysogens were prepared as described by Huynh *et al.* (1985).

DNA preparation and analysis. Normal DNA manipulations were carried out as indicated by Sambrook *et al.* (1989). Plasmid DNA was extracted from *E. coli* DH5 α according to the instructions of the Wizard DNA Purification System (Promega). Restriction enzymes and T4 DNA ligase were obtained from Boehringer and used under the recommended conditions. Restriction fragments of genomic DNA carrying the *aspid1* gene were subcloned into pBluescript SK(+) and KS(+) vectors (Stratagene). Single-stranded DNAs from overlapping deletions generated according to the double-stranded nested deletion kit (Pharmacia) were sequenced by the dideoxy chain-termination method (Sanger *et al.*, 1977) using the Pharmacia T7 polymerase sequencing kit and following the supplier's instructions, with ³⁵S-labelled dATP (Amersham) as labelled dNTP.

Construction of expression vector pGSTag-cDNAaspid1. The cDNA encoding mature ASPND1 was purified using the double Gene Clean procedure (Bio101), digested with *NcoI* and ligated into plasmid pGSTag (Ron & Dressler, 1992) by standard protocols. Vector pGSTag-cDNAaspid1 was used to transform competent *E. coli* DH5 α and transformants were selected on LB plates with ampicillin (100 μ g ml⁻¹) according to standard protocols (Sambrook *et al.*, 1989). Correct transformants were isolated after analysis of plasmid DNA from randomly selected transformants and digested with *BamHI* (within the pGSTag polylinker) and *PvuII* (within the cDNA 5' end).

Production and purification of glutathione S-transferase (GST)-ASPND1r. A 20 ml sample of an overnight culture of the

correct transformant *E. coli* DH5 α grown at 37 °C in LB containing 0.1 mg ampicillin ml⁻¹ was used as preinoculum for 180 ml of fresh LB containing 0.1 mg ampicillin ml⁻¹. After 2 h growth at 37 °C in a shaking incubator, IPTG was added at a final concentration of 0.1 mM to induce GST-ASPND1r synthesis. Cells were pelleted and lysed after 4 h IPTG induction, according to the method described by Marston (1987). The fusion protein was found to accumulate as inclusion bodies, which were purified by centrifugation at 4000 g for 15 min at 4 °C. Purified and washed inclusion bodies were resuspended in SDS gel loading buffer and GST-ASPND1r was purified to homogeneity by preparative electrophoresis.

Preparative electrophoresis. Recombinant ASPND1 was purified by preparative electrophoresis in a Prep Cell apparatus (model 491, Bio-Rad) according to the manufacturer's instructions. An isotropic 10% (w/v) polyacrylamide cylindrical gel (110 mm long, 37 mm diameter) was prepared using the discontinuous buffer of Laemmli (1970). The sample was run at 10 W constant power for 14 h at room temperature. To prevent overheating, buffer was recirculated through the cooling core of the apparatus. Elution of the protein was monitored by UV absorbance (Waters 490 programmable multiwavelength detector, Millipore) and 1.5 ml fractions were collected (Frac-100 collector, Pharmacia). All fractions were subjected to SDS-PAGE and those containing the purified protein were mixed together and stored at -70 °C.

Thrombin digestion of the fusion protein (GST-ASPND1r). The GST-ASPND1r protein purified by preparative electrophoresis was in soluble form at a concentration of 0.1 mg ml⁻¹ in 25 mM Tris buffer, pH 8.3, containing 192 mM glycine and 0.1% SDS. This solution was concentrated and dialysed with a Centricon-30 microconcentrator unit (Amicon) according to the manufacturer's instructions. For digestion, 1 mg fusion protein was routinely digested with 0.03 bovine thrombin units (Boehringer Mannheim) in 20 mM Tris/HCl pH 8.4, 150 mM NaCl, 2.5 mM CaCl₂ and 1 mM DTT for 15 h at 22 °C.

Densitometric quantification of the blots. Densitometry was done with a Bioimage system using the Whole Band Analyzer software (Millipore). Integrated optical density (IOD) arbitrary units were used to express the results.

RESULTS

Molecular cloning and sequencing of cDNA for ASPND1 immunodominant antigen from *A. nidulans*

Screening of 2.2×10^4 recombinant λ gt11 plaques of the cDNA library with affinity-purified antibodies revealed six strongly hybridizing clones designated F1-F6. Lysogens were prepared as described in Methods to ascertain if all the clones encoded the ASPND1 antigen and to determine which ones contained longer encoding cDNAs. To determine whether expression of the fusion protein was under control of the *lacZ* promoter, lysates of the recombinant lysogens were examined by immunoblotting after heat induction at 45 °C followed by growth at 37 °C for 60 min in the absence or presence of 1 mM IPTG (Fig. 1). A lysogen expressing only the β -galactosidase was used as negative control. The six selected clones expressed the correct epitope, as demonstrated by their reactivity with the purified monospecific

Fig. 1. Western blot analysis of cell extracts from six *E. coli* Y1089 lysogens (F1-F6) containing an inducible vector for the β -Gal-ASPND1r fusion protein. A control without insert is shown (Control lanes). Cultures were not induced (-) or induced (+) with 1 mM IPTG. Affinity-purified anti-ASPND1 antibody was used at a dilution of 1:1000. Experimental details are described in Methods.

antibody. Phage DNA from clones F3 and F5 (producing larger fusion proteins) and from clone F4 (producing the smallest fusion protein) was isolated. Digestion of these DNAs with *Eco*RI yielded three unique fragments of about 1.0, 0.95 and 0.5 kbp respectively. The three fragments were subcloned into pBluescript SK(+) and KS(+) in the *Eco*RI site and sequenced. The exact size of each fragment was 996 bp for clone F3, 878 bp for clone F5, and 429 bp for clone F4. All three clones came from the same mRNA (as determined by comparison of their nucleotide sequences) and contained the 3' poly(A) tail. Differences in size were due to the presence of different 5' ends, probably caused by arrest of the reverse transcriptase at different positions in the mRNA template. The nucleotide sequence of the largest clone (F3) has been deposited in the EMBL database (accession no. Z80229). It displays an open reading frame of 789 bp preceded by 2 bp of 5' sequence and followed by 205 bp of 3' untranslated sequence, terminating with a putative polyadenylation signal and a poly(A) tail. The absence of a consensus translational start signal (ATG) indicated that the F3 cDNA was not a complete copy of the ASPND1 mRNA. Nucleotide sequence analysis of this clone confirmed that it encoded ASPND1 antigen since the first 14 residues of the predicted amino acid sequence of the translation product coincided exactly with the corresponding residues of the previously determined N-terminal amino acid sequence of purified ASPND1 (Calera *et al.*, 1997).

Comparison of the cDNA and genomic sequences (Calera *et al.*, 1997) showed that the missing region in clone F3 would correspond to a signal peptide of 16 amino acids preceding the mature product.

Expression of ASPND1 antigen in *E. coli*

Plasmid vectors have been constructed that express antigens as fusion proteins with the enzyme GST from *Schistosoma japonicum*, thereby enabling affinity-purification under non-denaturing conditions (Johnson *et al.*, 1989). The fusion protein GST-ASPND1r was prepared by introducing the *Nco*I-digested insert from

Fig. 2. (a) Schematic representation of the sequence of the 5' and 3' ends and the corresponding linkers (boxed) in both chains of the cDNA of λ gt11 clone F3. The Met and Ala residues (shown in bold) are encoded within the linker sequence. Restriction sites are also shown. (b) Detailed scheme of the ASPND1r expression plasmid containing the ASPND1 cDNA (bold) under transcriptional regulation of the *lacZ* promoter. F3 cDNA corresponding to ASPND1 was cloned in the *Nco*I site of vector pGSTag. The target site for thrombin cleavage is shown by the arrow. A 34 amino acid stretch (shown in lower-case letters) remains linked to the ASPND1 sequence after thrombin cleavage.

clone F3 into the *Nco*I site of vector pGSTag (Fig. 2). Induction with IPTG at 37 °C for various periods of time followed by SDS-PAGE of the total protein revealed a major IPTG-induced band at 63.7 kDa (Fig. 3). This band is 5.2 kDa larger than the estimated size deduced from the sequence data. This fact was also noticed with the original ASPND1 antigen and is due to the abnormal electrophoretic mobility of ASPND1 in SDS-PAGE (Calera *et al.*, 1997). The soluble supernatant from the lysate of induced cells showed very low levels of reactivity with the specific antibody. The fusion protein accumulates as inclusion bodies, which are easily visible by light microscopy, thus preventing the possibility of affinity purification even after its solubilization with Sarkosyl (Frankel *et al.*, 1991; Frangioni & Neel, 1993). Inclusion bodies were then purified as described in Methods (Fig. 3, lane IB) and the fusion protein was

isolated by preparative SDS-PAGE (Fig. 3, lane P). The yield of purified GST-ASPND1r was around 90–120 mg l⁻¹. Since the GST protein is a very strong antigenic molecule (it is used for the immunodiagnosis of some forms of parasitosis such as schistosomiasis; Lin *et al.*, 1993), we decided to separate the ASPND1 recombinant protein (ASPND1r) from the GST moiety to prevent the appearance of false-positive reactions. Taking advantage of the existence in the pGSTag vector of a target sequence for cleavage with thrombin, we digested the fusion protein with this protease to obtain an ASPND1 recombinant antigen (Fig. 4). ASPND1r contained 34 extra N-terminal amino acids corresponding to the coding region determined by the DNA fragment containing the different cloning sites of the pGSTag vector between the site of thrombin cleavage and the *Nco*I restriction site (Fig. 2).

Fig. 3. SDS-PAGE (16%, w/v, polyacrylamide gel) of cell lysates of *E. coli* transformants (corresponding to 50 μ l culture) containing the GST-ASPND1r expression vector not induced (lane C) or induced with 1 or 0.1 mM IPTG for the number of hours indicated (lanes 1 h to 8 h). Lane IB, SDS-PAGE of purified inclusion bodies; lane P, purified GST-ASPND1r fusion protein. The gel was Coomassie stained. Experimental details are described in Methods.

Fig. 4. SDS-PAGE (16%, w/v, polyacrylamide gel) of the purified GST-ASPND1r fusion protein (3 μ g per lane) before (lane -T) and after (Lane +T) cleavage with 1 cleavage unit of thrombin (approx. 0.03 units of Boehringer enzyme) per mg fusion protein. The gel was silver-stained.

Specific recognition of ASPND1r by immune human sera

Purified GST-ASPND1r fusion protein was digested with thrombin and the cleaved polypeptides (GST and ASPND1r) were separated by SDS-PAGE (Fig. 4) and transferred to Immobilon. Several parallel blots were cut into numerous strips, each of which was incubated with a different human serum sample at a dilution of 1:200. Fig. 5 shows the results obtained after developing with peroxidase reagents. Of the 25 serum samples from patients with confirmed aspergilloma, 22 (88%) were reactive with ASPND1r, with the appearance of a single band in the immunoblot. Reaction intensities varied among patients. None of the 49 serum samples from healthy individuals showed reactivity (data not shown). Of 15 serum samples from patients with different clinical

situations, only one, from a patient with positive rheumatoid factor, was clearly reactive to ASPND1r. The remaining 14 control sera from miscellaneous infections showed negligible or no reactivity (data not shown). Densitometric quantification of the blots is shown in Fig. 6. An IOD of 0.04 (Fig. 6, dashed line) corresponds to the mean (0.01) plus four standard deviations (4×0.007) calculated for the 64 control serum samples from 49 blood bank donors and 15 patients with diseases unrelated to *Aspergillus*. Of the 25 serum samples from patients with aspergilloma, 22 gave IOD values equal to or greater than 0.04 (Fig. 6, left panel), whereas only one of 64 negative controls gave a value greater than 0.04 (Fig. 6, right panel). In view of these data, the assay was considered positive if the detected ASPND1r band had an IOD equal to or greater than 0.04 and negative if the IOD was lower than 0.04.

Positive and negative Western blots were compared in 2×2 tables versus the 25 patients with proven *Aspergillus* infections and the 64 controls (χ^2 with Yates correction: 85.65, $p < 0.001$). Calculation of the odds ratio value (OR = 462) and OR confidence intervals [127–1676 ($z = 1.96$, $P = 0.05$) and 88–2422 ($z = 2.54$, $p = 0.01$)] allowed us to determine the existence of an association between aspergilloma and Western blot positivity. The immunoblot assay was highly specific (98.4%) and efficient (95.5%) although not highly sensitive (88%), indicating that ASPND1r could be an antigen of potential interest in the serodiagnosis of aspergilloma, although it should be used as part of a complex antigenic mixture to increase the sensitivity of the assay.

The isolated GST protein reacted to different extents with 11 of the 25 (44%) immune serum samples and seven of the 15 (46.7%) serum samples with clinical characteristics unrelated to *Aspergillus*. No reactivity was observed with serum samples from healthy individuals (data not shown).

DISCUSSION

We have obtained an immunodominant *A. nidulans* antigen in recombinant form and have analysed its ability to react with immune human sera. The ASPND1r antigen was consistently recognized by serum samples from aspergilloma patients and proved to be a marker specific for *Aspergillus* infections in the sense that sera from healthy blood donors and patients with infections other than aspergilloses did not recognize it significantly. When compared to the original complex antigen mixture, the assay proved to be more sensitive (88% with ASPND1r vs 75% with the complex mixture; López-Medrano *et al.*, 1996; Calera *et al.*, 1997). This higher sensitivity is probably due to the fact that in SDS-PAGE ASPND1r gives a single narrow band, with a higher protein concentration, instead of the broad zone observed in the case of total extracts. The specificity of the assay was the same when either ASPND1r or the total extract was used. This result was predictable considering that only the reactivity of the ASPND1 zone

Fig. 5. Western-blot detection of ASPND1r (1.3 µg per lane) by 22 out of 25 serum samples from aspergilloma patients. All serum samples were tested at a dilution of 1:200. The lane numbers correspond to the numbers assigned to individual patients. GST, glutathione S-transferase protein.

Fig. 6. IOD values obtained with 25 sera from aspergilloma patients (left panel) and with 64 control sera (right panel) from blood bank donors and from individuals with diseases or infections unrelated to *Aspergillus*. Each point represents the mean of three separate experiments. The standard deviation of each point was <20% of the corresponding mean.

with different serum samples was taken into account for positivity when total extracts were used.

Aspergilloma patients are known to demonstrate numerous *Aspergillus*-specific precipitins and therefore provide a 'filter' for identifying clinically relevant antigens. Based on this assumption, we had previously identified certain immunodominant antigens from *A. fumigatus* after monitoring their consistent reactivity with a high proportion of adequately documented sera from aspergilloma patients (López-Medrano *et al.*, 1995a). One of these antigens – p90 or CAT1 – proved to be a relevant antigen as a specific marker for the immunodiagnosis of pulmonary aspergilloma (López-Medrano *et al.*, 1996) and was shown to possess catalase activity (López-Medrano *et al.*, 1995b). Using basically the same collection of aspergilloma sera, we also established the clinical relevance of the *A. nidulans* antigen designated ASPND1 (Calera *et al.*, 1994, 1997) – now expressed in recombinant form as ASPND1r – and demonstrated its similarity to the *A. fumigatus* Asp f II allergen (Calera *et al.*, 1997). The fact that the same array of serum samples scored positive for CAT1, ASPND1 and ASPND1r antigens reinforces their value as potential markers for *Aspergillus* infections. An

adequate number of sera from invasive aspergillosis and allergic bronchopulmonary aspergillosis (ABPA) patients should be tested in the future to evaluate the usefulness of ASPND1r as a marker for these types of disease.

Healthy individuals often show a certain degree of recognition to *Aspergillus* antigens (Hearn, 1988). Accordingly, the lack of reactivity to ASPND1r of the 49 serum samples from blood bank donors is very significant. The serum samples from three out of 25 (12%) patients with confirmed *Aspergillus* infections were not reactive to ASPND1r. This may be due to variations in the immunological responses of different individuals and would represent a low percentage of false-negative reactions. These would be negligible if this recombinant antigen were used for clinical purposes in combination with other previously reported antigens.

The frequently observed non-specific reactivity of GST protein with different types of serum samples reinforces the need to excise the GST–ASPND1r fusion protein before the immunoblot step to avoid false-positive reactions.

Several perfectly characterized antigens have been shown to be relevant for the immunodiagnosis of *Aspergillus* infections and their recombinant counterparts have been obtained: HSP-1 (Kumar *et al.*, 1993) Asp f II (Banerjee *et al.*, 1996), rAsp f I/a (Moser *et al.*, 1992, 1994) from *A. fumigatus* and ASPND1r (this work) from *A. nidulans*. Two more reliable *A. fumigatus* antigens, CAT1 (López-Medrano *et al.*, 1995b) and the superoxide dismutase antigen (Hamilton *et al.*, 1995), have been purified to homogeneity and will probably be produced as recombinant proteins in the near future. All these antigens should be evaluated together, or in appropriate combinations, for the manufacture of defined reagents for a consistent diagnosis of the different forms of aspergillosis.

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