Characterization of the Cellular Antigens of *Paracoccidioides brasiliensis* Yeast Form

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Antigenic components of the yeast extract of *Paracoccidioides brasiliensis* Linder 2511 cultured for 3, 8, 20, 30, and 60 days were examined by the Western blot (immunoblot) technique. The 3-day extract was chosen for characterization of the antigenic components because its stability did not vary with time and it contained all antigens identified by patient sera. Antibodies to cross-reacting antigens of *P. brasiliensis* extracts were detected in sera from patients with histoplasmosis, candidiasis, and aspergillosis. The 58-, 57-, 21-, and 16-kilodalton (kDa) antigens were specific for *P. brasiliensis*, while the 48- and 45-kDa antigens were specific for paracoccidioidomycosis. The Western blot technique is a useful tool for the diagnosis of disease and revealed heterogeneity in the responses of patient sera. The combination of the 58-, 57-, and 45-kDa proteins confirmed a diagnosis of paracoccidioidomycosis (87% of the cases).

Paracoccidioidomycosis (PCM) is a systemic granulomatous chronic disease caused by the dimorphic fungus *Paracoccidioides brasiliensis*. The disease is limited to Latin American countries, from Mexico (20° north latitude) to Argentina (35° south latitude). The more important endemic regions are Brazil, Colombia, and Venezuela (10, 18).

The definitive diagnosis of PCM is made by the identification of typical yeast cells in biopsies or other pathological material. Since in many cases such a demonstration of the fungus is difficult, diagnosis is usually made by serological methods. Numerous serological tests for PCM diagnosis have been described, but immunodiffusion and immunoelectrophoresis are the most frequently used. Antigens from both the mycelial and yeast phases of the fungus (culture filtrate or yeast cells) have been used for these immunological tests (2, 4, 6, 12, 13, 19, 20, 27).

Antigens of diagnostic value have been identified in the exocellular or culture filtrate. Restrepo and Moncada (20) described, through immunodiffusion, the first specific antigen (band 1), which appears to be similar to specific antigen E characterized by immunoelectrophoresis by Yarzabal et al. (29, 30). More recently, Puccia et al. (16) identified and characterized by immunoprecipitation a 43-kilodalton (kDa) glycoprotein produced in the yeast culture filtrate of *P. brasiliensis* B339. However, although only one antigen was described, it is known that *P. brasiliensis* contains a complex mixture of antigens. The cytoplasmic yeast antigens have been studied by only two groups (11, 19), without being characterized.

At present, the immunoblot technique provides a means of analyzing serological responses to complex antigenic mixtures. This method is a useful tool for the identification of immunogenic fungal components that elicit a specific antibody response (17). It has been successfully applied to the study of the immune responses to several fungi—*Candida*, *Aspergillus*, *Sporothrix*, and *Rhizopus* spp. (17, 22, 25)—but the immunoblot technique has not been used for the characterization of the antigens of *P. brasiliensis*.

The principal objective of this work was to identify and characterize the specific antigens of the yeast cells of *P. brasiliensis* Linder 2511. For this purpose, immunoblots of the cellular extracts of *P. brasiliensis* and other pathogenic fungi (*Candida albicans*, *Cladosporium carrioni*, *Sporothrix schenckii*, *Aspergillus niger*, *A. nidulans*, *A. fumigatus*, *A. flavus*, *Histoplasma capsulatum*, and *Blastomyces dermatitidis*) were tested with sera from patients with PCM and sera from patients with other mycoses. The identified specific antigens were characterized by their pIs.

**MATERIALS AND METHODS**

**Fungal strains.** *P. brasiliensis* Linder 2511, *C. albicans* 3009 serotype B, *C. carrioni* 3050, *H. capsulatum* 3049, and *S. schenckii* 3087 were obtained from the Center of Amazonian Research for Tropical Diseases, Puerto Ayacucho, Venezuela; *A. niger*, *A. nidulans*, *A. fumigatus*, and *A. flavus* were obtained from the Institut Pasteur, Lille, France; and *B. dermatitidis* ATCC 18188 was obtained from the American Type Culture Collection, Rockville, Md.

**Culture conditions.** *P. brasiliensis*, *C. albicans*, and *B. dermatitidis* yeast forms were routinely subcultured every 3 days onto PGY (21) agar slants at 36°C. Yeast cells (10⁶) were precultured in 500-mI Erlenmeyer flasks containing 100 ml of TG medium (7.5% tryptose [Difco Laboratories, Detroit, Mich.], 5% glucose) at 36°C for 2 days on a gyratory shaker (New Brunswick Scientific Co., Inc., Edison, N.J.) at 120 rpm. Cells (10⁶) of these precultures were transferred to 100 ml of fresh TG medium and incubated as described above for 3, 8, 20, 30, or 60 days at 36°C. *A. niger*, *A. nidulans*, *A. fumigatus*, *A. flavus*, *H. capsulatum*, *C. carrioni*, and *S. schenckii* were subcultured in Sabouraud dextrose broth (Biomerieux, Lyon, France) agar slants each month at 21°C. The precultures were first grown in liquid Sabouraud medium for 5 days at 25°C on a gyratory shaker at 150 rpm. They were then cultivated in Sabouraud medium for 3 days at 36°C (*Aspergillus* spp.), 10 days at 25°C (*Cladosporium* sp.) and 5 days at 25°C (*Histoplasma* and *Sporothrix* spp.).

**Antigen preparation.** The fungal cells were killed with 0.1% sodium azide (Merck-Schuchardt, Darmstadt, Federal Republic of Germany) for 30 min. After centrifugation at 16,000 × g for the yeast form or after filtration for the mycelium, the cells were washed three times with 0.15 M NaCl. The pellet was suspended in 0.15 M NaCl containing 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma Chemical Co., St. Louis, Mo.) and 1 mM Na-p-tosyl-L-lysine-chloromethyl ketone (TLCK; Sigma) (20% [wt/vol]). Cells
were frozen and broken by mechanical disruption (X-Press; Biox, Paris, France). After being examined for complete disruption, cells were stored at −20°C and used within 3 months.

SDS-PAGE. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), 80 μg of protein from the broken cells (cytoplasm, membrane, and cell walls) was boiled for 5 min in sample buffer (62.5 mM Tris hydrochloride [pH 6.8] 2.3% SDS). The samples were subjected to electrophoresis on a 4 to 15% polyacrylamide gradient gel in the presence of 0.1% SDS as described by Laemmli (9). Standard markers were a mixture of high- and low-molecular-mass proteins (330 to 14.4 kDa; Pharmacia, Uppsala, Sweden).

Two-dimensional electrophoresis. The isoelectric focusing procedure was carried out as follows. Urea (15 mg; Bio-Rad Laboratories, Richmond, Calif.) was added to 10 μl of broken cells containing 150 μg of protein. The mixture was solubilized in 100 μl of lysis buffer (9.8 M urea, 2% Triton X-100, 2% Ampholines [pHs 5 to 7 and 3 to 10]). Isoelectric-focusing gels were prepared in glass tubes (2.5 mm by 12 cm). Samples were focused at 200 V for 15 min, 300 V for 30 min, 400 V overnight, and 800 V for 1 h. The running buffer was that described by O’Farrell (15). The second dimension was performed in the same SDS-PAGE gradient as that described above.

Immunoblotting. Proteins from SDS-PAGE were electrotransferred onto nitrocellulose membranes (0.45-μm pores; Schleicher & Schuell, Inc., Keene, N.H.) in a transblotting chamber (Bio-Rad) with 25 mM Tris–192 mM glycine (pH 8.3)–20% methanol (vol/vol) (26). The markers transferred were stained with India ink (8). For immunoblot assays, the nitrocellulose membranes were saturated with 5% skimmed milk–0.3% Tween 20 in 10 mM phosphate buffer (pH 7.2)–0.15 M NaCl (PBS-M-T 0.3%). The membranes were cut into 5-mm strips and incubated for 2 h at 21°C with sera diluted 1/500 in PBS-M-T 0.1%. The strips were washed four times for 10 min each time in PBS-M-T 0.1% and incubated for 1 h at 21°C in peroxidase-conjugated human anti-immunoglobulin A (IgA), -IgM, or -IgG or anti-IgG (Biosys, Compiègne, France) diluted 1/500 in washing buffer. After incubation, the peroxidase activity was developed with 5 mg of 3,3′-diaminobenzidine tetrahydrochloride (Sigma) in 10 ml of phosphate-buffered saline and 40 μl of H₂O₂ (30 volumes).

Serum samples. The PCP serum used was a pool of 10 serum specimens. Individual serum specimens from 30 other PCP patients were selected on the basis of a positive clinical history and positive immunodiffusion tests. Serum specimens from PCM patients, histoplasmosis patients (9 serum specimens), and health individuals (15 serum specimens) were provided by the Institute of Dermatology and by the mycology laboratory of Hospital Vargas, Caracas, Venezuela. Seven serum specimens from patients with aspergillosis and seven from patients with candidiasis were obtained from the mycology laboratory of Institut Pasteur, Paris, France. All serum specimens were divided into aliquots and stored at −20°C.

Absorption experiment. Sera were absorbed with a mixture of antigens from A. niger, A. nidulans, A. fumigatus, A. flavus, H. capsulatum, C. carrionii, S. schenckii, C. albicans, and B. dermatitidis. Twenty microliters of serum and 200 μl of the antigenic mixture (5 mg/ml) were incubated for 30 min at 22°C. After centrifugation (10 min at 10,000 × g), the supernatants were subjected to second and third incubations and fresh antigenic mixtures. These absorbed sera were stored at −20°C.

RESULTS

The crude cellular extracts obtained at 3, 8, 20, 30, and 60 days of fungal growth were subjected to SDS-PAGE, and the separated components were stained for protein with Coomassie blue (Fig. 1). The patterns at 3 and 8 days were similar, except for the 330- to 94-kDa region, in which several bands were absent at 8 days. This difference can be explained in part by a loss of cell viability (50%), with subsequent cell lysis and protein degradation. The smear observed in the 20-day extract (28% viability) indicated a partial degradation of the cellular extract, while in the 30- and 60-day extracts, complete degradation was observed.

Pooled serum specimens from 10 PCM patients and from 10 healthy people were tested for total IgA, IgM, and IgG reactivity to P. brasiliensis proteins blotted onto nitrocellulose (Fig. 2). The response of the PCM pool was greatest for the 3- and 8-day extracts (Fig. 2, lanes 3a and 8a). This pool recognized 38 antigens. However, 19 of these antigens were also recognized by the pooled sera from healthy controls (Fig. 2, lanes 3b and 8b). Fourteen of these 19 antigens were not recognized in the extracts up to the 20-day extract by the PCM pool. However, the 3-day (a time which corresponds to the end of the exponential phase) extract was selected because it contained all the antigens recognized by PCM sera and was less influenced by fungal proteolysis. The 3-day cultures were 80% viable. The individual IgG responses of 30 patients to the 3-day extract revealed the heterogeneity of the antigen-binding patterns of the different sera (Fig. 3A). Fifteen major antigens reacted with PCM sera and corresponded to apparent molecular masses of 220, 114, 94, 84, 82, 76, 58, 57, 48, 45, 37.5, 27.5, 21, 18.5, and 16 kDa (Fig. 3B). Patient sera were grouped according to clinical form (14), but no correlation could be observed between the number or intensity of the reactive antibodies and the clinical forms (unifocal or multifocal).

Three types of experiments were executed to determine if these antigens were specific for P. brasiliensis: (i) immunoblotting of sera from patients with mycoses; (ii) absorption of PCM sera with other fungal extracts; and (iii) immunoblotting of other fungal extracts with PCM sera. Blots of the 3-day extract of P. brasiliensis were tested with nine serum specimens from patients with histoplasmosis, seven from
patients with aspergillosis, and seven from patients with candidiasis. Only one serum specimen from a patient with candidiasis recognized a paracoccidioidal protein of 220 kDa (data not shown). The sera of some patients with aspergillosis bound weakly to the 220-, 94-, 82-, and 27.5-kDa paracoccidioidal antigens (data not shown). Histoplasmal sera recognized paracoccidioidal antigens of 220 (three cases), 114 (one case), 94 (two cases), 76 (two cases), and 37.5 (one case) kDa (Fig. 4). Sera from healthy controls did not bind many of these antigens (220, 94, 82, 76, 37.5, and 27.5 kDa) (data not shown). PCM sera were absorbed with a mixture of

FIG. 2. Immunoblot analysis of the combined IgA, IgM, and IgG responses to cellular extracts from 3-, 8-, 20-, 30-, and 60-day-old cultures of P. brasiliensis. Lanes: a, PCM pool; b, pool from healthy controls. MW, Molecular weight standards (in thousands).

FIG. 3. Immunoblot analysis of the IgG responses to cellular extracts from a 3-day-old culture of P. brasiliensis. (A) Immunoblots of sera from patients with different chronic clinical forms. (−), Healthy controls. (B) Antigenic pattern of all the antigens recognized by the PCM sera. MW, Molecular mass.

FIG. 4. IgG responses of patients with other mycoses. Hst, Patients with histoplasmosis. Pa, Controls of two individual PCM serum specimens. MW, Molecular mass.

220, 84, 37.5, and 18.5 kDa disappeared and the 82- and 76-kDa antigens showed decreased binding. The proteins of 114, 94, 58, 57, 48, 45, 27.5, 21, and 16 kDa persisted after absorption (Fig. 5B, lanes 1 to 3). No reactions were observed with the absorbed control sera (Fig. 5A, lanes 5 to 7). Finally, cellular extracts of *A. niger, A. nidulans, A. fumigatus, A. flavus, C. c.у. woricci*, *S. schenckii, C. albicans,* and *B. dermatitidis* separated by SDS-PAGE were incubated with a PCM pool (Fig. 6). Except for *S. schenckii,* common antigens were identified in all fungal species (2 for *C. albicans,* 10 for *B. dermatitidis,* 7 for *C. c.у. woricci,* 4 for *A. niger,* 1 for *A. nidulans,* 5 for *A. fumigatus,* and 1 for *A. flavus,* e.g.), the 114-kDa protein was present in *B. dermatitidis,* the 84-, 82-, and 76-kDa proteins were present in *A. fumigatus* and *B. dermatitidis,* and the 48- and 45-kDa proteins were present in *A. fumigatus, C. albicans,* and *B. dermatitidis.* However, the 58-, 57-, 21-, and 16-kDa antigens were not found in these species.

A frequency histogram showing the frequency with which individual PCM sera recognized the different antigens is presented in Fig. 7. The 18.5- and 16-kDa antigens were not included because they were recognized by only 5% of the patient sera. Cross-reactivity between paracoccidioidal antigens and antibodies from patients with other mycoses is also presented in Fig. 7. The antigens of 220 (45%), 114 (38%), 94 (25%), 82 (50%), 76 (70%), 37.5 (30%), and 27.5 (20%) kDa reacted with antibodies in the sera of patients with paracoccidioidal, candidiasis, and/or histoplasmosis. The absorbed PCM sera bound to 114-, 94-, 58-, 57-, 48-, 45-, 27.5-, and 21-kDa proteins. The 82- and 76-kDa antigens were weakly reactive. By this method, the 58 (17%), 57 (70%), 48 (50%), 45 (20%), and 21 (8%) kDa antigens were not recognized by the sera of patients with other mycoses and were therefore useful for PCM diagnosis.

The isoelectric points of the major specific PCM antigens were determined. A two-dimensional electrophoresis blot incubated with a serum specimen from a patient with recognized the 58 (Fig. 8A, arrow)- and 57 (Fig. 8A, arrowhead)-kDa antigens showed that their pIs were 5.2. Another serum specimen which recognized the 48- and 45-kDa antigens showed that the 48 (Fig. 8B, arrows)-kDa antigen was composed of two molecular species with pIs of 6.1 and 6.2, while the 45 (Fig. 8B, arrowheads)-kDa antigen was composed of three molecular species with pIs of 6.1, 6.2, and 6.4. The control serum did not recognize these antigens (data not shown).

**DISCUSSION**

The kinetics of the production in vitro of the cellular antigens of *P. brasiliensis* showed that these antigens were
stable for 20 days and that complete degradation was observed after 30 days (Fig. 1). Western blotting (immunoblotting) permitted the identification of 16 stable antigens present even after cell lysis (8- and 20-day-old cultures). The stability over time of the specific antigens was shown in an exocellular yeast preparation of Gp43 (6, 24). This glycoprotein is present until 25 days after it appears to be relatively resistant to secreted proteases, since it is not degraded after overnight incubation of the culture supernatants at 37°C (24). However, Restrepo et al. (19) reported that the cytoplasmic yeast extracts of a 3-day-old culture were of a perishable nature and that repeated additions of proteolytic inhibitors (PMSF and TLCK) were necessary. No degradation of my cellular extracts (3 days old) was observed after 3 months of storage at −20°C in the presence of PMSF and TLCK. It is possible that the addition of PMSF and TLCK allowed greater proteolytic inhibition of endogenous proteases, since PMSF is an inhibitor of serine proteases (chymotrypsin) while TLCK inhibits trypsin and other endoproteases (papain).

Testing with anti-IgA, -IgM, and -IgG revealed that 38 antigens were recognized by pooled PCM sera and 17 were recognized by sera from healthy controls (Fig. 2). Analysis of the IgA response only, however, demonstrated that the anti-IgA was bound directly to proteins in the range of 67 to 14.4 kDa (data not shown) in a nonspecific manner. This result could explain the high number of bands revealed by the negative pooled sera (Fig. 2). In the IgG response analysis, only 12 antigens were recognized by the pooled PCM sera (Fig. 5) and 15 antigens were recognized when individual responses of 30 PCM serum specimens were examined (Fig. 3). The negative controls recognized only four antigens, and the anti-human IgG did not bind to the yeast antigens. Several authors (1, 6, 13) have shown that IgG is the immunoglobulin class prevalent in the response of PCM sera. Biagini et al. (1) found a positive correlation between the level of anti-PCM IgG and the positivity of the tub precipitin test, suggesting that the antibody was of the IgG class. Fiorillo and Martinez (7) showed by counterimmunoelectrophoresis that IgG was the PCM-specific immunoglobulin.

I found no correlation between the immunoglobulin response and clinical manifestations, in contrast to what others have noted in disseminated candidiasis (25) and extracutaneous sporotrichosis (22), in which specific antigens are markers of clinical forms. This difference may reflect my relatively small sample size.

Cross-reactivity has been a recognized problem in serological diagnostic tests of fungal disease for many years, and P. brasiliensis is no exception (28). I found (Fig. 6) common antigens with all the fungi studied, except for S. schenckii. The most similar protein pattern and the highest number of recognized antigens were found with B. dermatitidis. Five of the 10 antigens of B. dermatitidis identified by a PCM pool had the same molecular masses as P. brasiliensis antigens (114, 84, 82, 48, and 45 kDa). These two fungi are morphologically similar and were previously classified in the same genus (23). I think that common molecular mass bands between the two fungi represent common antigens, not just common epitopes. However, since blastomycosis and PCM are rarely found in the same endemic area, these common antigens should not be a problem for the diagnosis of PCM by serology.

The serological diagnosis of PCM is usually made with P. brasiliensis antigens tested against PCM sera and sera of other mycoses. Specific antigens are defined by the lack of a cross-reaction between P. brasiliensis and heterologous antibodies. In my study, specific antigens were defined on the basis of the lack of a cross-reaction with heterologous antibodies and/or antigens and absorption experiments (Fig. 5). The 57-, 57-, 57-, and 16-kDa antigens were not identified in the other fungal species tested (A. niger, A. nidulans, A. fumigatus, A. flavus, C. carrionii, S. schenckii, C. albicans, and B. dermatitidis), nor were they recognized with aspergillosis, candidiasis, or histoplasmosis sera. These antigens were considered specific for P. brasiliensis. The 48- and 45-kDa antigens of C. albicans, B. dermatitidis, and A. fumigatus were recognized by PCM sera but not in P. brasiliensis extracts by sera from patients with other mycoses. Therefore, the 48- and 45-kDa antigens may be considered specific for PCM.

The 57-kDa antigen was recognized by 70% of the sera and was often associated with some of the other specific antigens (only sera from six patients recognized this antigen alone). The combination of the 57-, 48-, and 45-kDa proteins was frequent, and these antigens were present in 87% of patients with PCM. The remaining 13% of patients tested were positive for other specific antigens.

Only two groups have studied the cytoplasmic antigen of the yeast form. McGowan and Buckley (11) purified on concanavalin A-Sepharose a cytoplasmic glycoprotein which, in an immunodiffusion assay, appeared to be similar to the exocellular antigen (band 1) described by Restrepo and Moncada (20). However, the molecular mass of this purified glycoprotein was not determined. In a preliminary study, Burgos et al. (3) showed by immunoprecipitation that a 66-kDa cytoplasmic protein was responsible for the formation of band 1. Puccia et al. (16), working with the exocellular yeast antigen, identified and characterized a 43-kDa glycoprotein similar to band 1. My specific cellular antigens of P. brasiliensis were characterized by molecular masses and pls. The 58- and 57-kDa antigens were acidic proteins (pl 5.2) with only one molecular species, while the 48 (pls 6.1, 6.2, and 6.4)- and 45 (pls. 6.1 and 6.2)-kDa antigens had several molecular species. These molecular species did not disappear after absorption (data not shown). The two molecular species (pls. 6.4 and 6.2) of the 45-kDa antigen seemed to be similar to the molecular species of the 43-kDa glycoprotein of Puccia et al. (16). It would be interesting to test my 45-kDa protein with the anti-43-kDa hyperimmune serum of Puccia and co-workers (16, 24).

I have demonstrated that immunoblot analysis of the response of PCM sera is a useful tool for the identification of immunogenic cellular components that elicit specific antibody responses.

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LITERATURE CITED


