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Immunological Characterization of a Recombinant 27-Kilodalton Antigenic Protein from *Paracoccidioides brasiliensis*

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We report the expression in *Escherichia coli* of a 27-kDa antigenic protein from *Paracoccidioides brasiliensis*. When analyzed by immunoblotting, this recombinant antigenic protein was recognized by antibodies present in the sera of 40 of the 44 paracoccidioidomycosis patients studied. No cross-reactions were observed with sera from patients with other mycoses (histoplasmosis, aspergillosis, cryptococcosis, sporotrichosis, and chromoblastomycosis) or with tuberculosis.

Paracoccidioides brasiliensis is the dimorphic fungus responsible for paracoccidioidomycosis, one of the most important systemic mycoses in Latin America, where the disease is geographically confined (3). Several serological assays have been used for the diagnosis of paracoccidioidomycosis. These assays employ a variety of antigenic preparations obtained by diverse methods and devised with the aim of improving the sensitivity, specificity, and reproducibility of the different diagnostic assays. Since most of the antigens used in the diagnostic tests come from crude preparations, standardization of the results is very difficult (10).

Recombinant proteins could provide a highly reproducible source of specific, chemically characterized antigens which would not only be easier to obtain than conventional filtrate antigens but would also be more reproducible. Once cloned, these proteins have the additional advantage of a prompter production (48 h versus 15 to 20 days). In search of a *P. brasiliensis*-specific antigen, Puccia et al. purified a 43-kDa glycoprotein (gp43) that showed high sensitivity and specificity (12, 13); Taba et al. also cloned the corresponding gene for this antigen (18), and the sequence is now available at GenBank (accession number U26160).

It has been documented that the production of reactive antigens from *P. brasiliensis* is a difficult task because of the variability that exists from lot to lot (10). Some critical factors such as the culture medium employed, the fungal isolate, and the time of growth may influence antigen production (12). It has been our experience that even if special care is given to the above details and the same steps are rigorously followed, many lots must be discarded because of their lack of reactivity. Serological tests are very useful in the diagnosis and follow-up studies of patients with paracoccidioidomycosis (5), and as such, highly reactive antigens are necessary. It seemed highly desirable to be able to obtain cloned proteins in suitable vectors, so that reproducibility could be achieved. Working in such a direction, we constructed a *P. brasiliensis* cDNA library from which a clone expressing an antigenic protein of 27 kDa was obtained, and it was characterized by immunoblotting in the presence of sera from patients with active paracoccidioidomycosis. This protein was recognized by 91% of such serum samples but ignored by sera from patients with other mycoses or tuberculosis.

Microorganisms and culture conditions. *P. brasiliensis* (ATCC 32069) from the collection of the Mycology Section of the Corporación para Investigaciones Biológicas was grown in the mycelial phase, in the modified liquid synthetic medium of McVeigh and Morton (16). Subcultures were done weekly; for the preparation of the antigen the cultures were kept for 10 to 12 days at room temperature (18 to 20°C) in a gyratory shaker.

mRNA isolation and library construction. Total RNA was obtained according to standard techniques (11). In brief, cells from a 10-day-old *P. brasiliensis* mycelial culture incubated as described above were harvested, frozen in liquid nitrogen, and broken with mortar and pestle. The mRNA was isolated by using an oligo(dT) cellulose column. A cDNA library was made by using a λ Zap II synthesis kit and packed in a Uni-Zap-XR vector. Plating, determination of titers, and amplification of the library were done with the *Escherichia coli* SURE strain with 90-mm-diameter NZY dishes (Stratagene, La Jolla, Calif.). The library yielded 90 to 95% recombinant plaques.

Screening of the cDNA library and selection of clones. Nitrocellulose membranes were applied on top of a culture of recombinant plaques and left overnight at 39°C. Membranes thus prepared were probed with a pool of sera, obtained from 16 paracoccidioidomycosis patients, known to be reactive in serological testing. The screenings were performed with the streptavidin-alkaline phosphatase system (Amersham, Aylesbury, United Kingdom). Additional analyses and characterization were carried out in the phagemid pBluescript II SK (Stratagene).

Expression of the recombinant 27-kDa protein. One of the positive clones obtained from the *P. brasiliensis* cDNA library was isolated, and by means of a helper phage, the pBluescript phagemid was excised from the λ Zap vector (17). Upon restriction analysis of the phagemid with *Eco*RI and *Xho*I, one insert of 1 kb was liberated. Characterization of the recombinant protein synthesized by *E. coli*, using total extracts from the recombinant bacteria, showed a new band around 27 kDa with or without IPTG (isopropyl-β-D-thiogalactopyranoside) induction. A band of similar size was found when Western blot (immunoblot) analysis (1) was performed on the total extract in the presence of a pool of sera from 16 paracoccidioidomycosis patients (Fig. 1).

SDS-PAGE and immunoblot assay. Gels (12% polyacrylamide) were run by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) according to standard tech-

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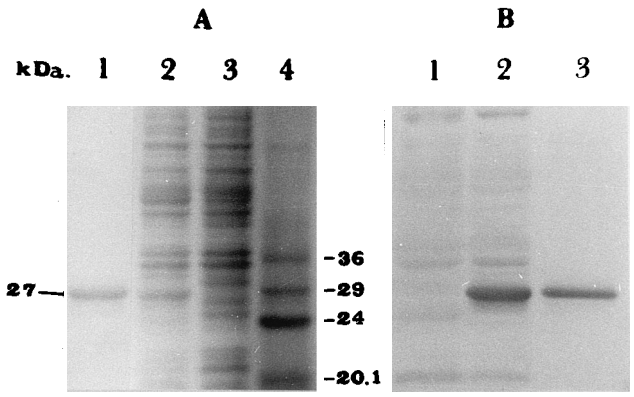

FIG. 1. SDS-PAGE and Western blot analyses. (A) SDS-polyacrylamide gel stained with Coomassie brilliant blue. Lane 1, pure protein obtained with preparative electrophoresis (Prep Cell apparatus). The molecular mass of the recombinant protein is 27 kDa. Lane 2, *E. coli* containing the phagemid expressing the recombinant 27-kDa protein. Lane 3, *E. coli* SURE containing the plasmid Bluescript II SK. Lane 4, molecular mass markers. (B) Western blot analysis with pool of sera from paracoccidioidomycosis patients. Lane 1, *E. coli* SURE containing the plasmid Bluescrip IISK; no reaction. Lane 2, specific reaction with the 27-kDa recombinant protein. Lane 3, reaction with the purified protein obtained by preparative electrophoresis.

niques (9). The proteins subjected to electrophoresis were either stained with Coomassie brilliant blue (Sigma) or transferred to nitrocellulose paper by using a transblot apparatus (Bio-Rad Laboratories, Richmond, Calif.). The quality of the transfer was evaluated by Ponceau S staining (1). The antigen concentrations were adjusted according to the protein content measurement (Bradford technique [2]) so as to load 0.2 μg per track. Nitrocellulose sheets were cut into strips of 0.4-mm width and blocked overnight with 5% skim milk. Each strip was assayed with sera of patients with paracoccidioidomycosis in 1:500, 1:1,000, and 1:2,000 dilutions (per ml). The specific antibody reaction was developed with the blotting detection kit for human antibodies (Amersham).

Serum samples. A total of 44 serum samples collected at the time of diagnosis from patients with mycologically confirmed (by direct KOH examination and isolation in culture) active paracoccidioidomycosis were used in this study. Three of these samples came from patients with the acute (juvenile) form of the disease, and the remaining samples were obtained from patients with the chronic (adult) form of the disease (14). Thirty-four additional serum samples, also obtained at the time of diagnosis from patients with other mycoses such as histoplasmosis (10 individuals), aspergillosis (5 individuals), cryptococcosis (5 individuals), sporotrichosis (9 individuals), and chromoblastomycosis (5 individuals), were evaluated. Eight serum samples from patients with proved tuberculosis were also studied. As a negative control, we used a pool of sera from 20 healthy blood donors.

Preparative electrophoresis. Preparative electrophoresis was performed in a Prep Cell apparatus (model 491; Bio-Rad) according to the manufacturer's directions. A 12% polyacrylamide solution was poured into the cylindrical ring of the electrophoresis cell, up to 5 cm, and then the solution was covered with water and allowed to polymerize overnight. A stacking solution was poured to make a 1-cm-high gel. Once the stacking gel had polymerized, it was loaded with the protein mixture of pelleted cells from a 20-ml *E. coli* culture in mid-log phase resuspended in the sample buffer. Electrophoresis was performed at 10 to 12 W for 12 h. Five-milliliter fractions were collected and analyzed by SDS-PAGE.

Purification of the 27-kDa protein. The 27-kDa polypeptide was isolated from the fractions that had been separated by preparative electrophoresis according to their molecular weight. The elution profile showed a single band in fractions 22 to 30 when SDS–12% PAGE analysis was performed. In immunoblot analysis, the sera from paracoccidioidomycosis patients also reacted as a single band (Fig. 1). The selected fractions were precipitated with acetone at −20°C, and the pellets were resuspended in phosphate-buffered saline.

Statistical methods. Statistical analysis of sensitivity, specificity, and predictive values was determined by the method of Galen and Gambino (8).

Reactivity of sera from paracoccidioidomycosis patients against the recombinant protein. Preliminary trials with various antigen and antibody concentrations were performed. The optimal reactivity was achieved at a 1:1,000 dilution of the serum and at 0.20 μg of the cloned crude antigen per well (data not shown).

In this study, the protein expressed in *E. coli* was as sensitive as the previously employed crude antigen, an antigen which reported sensitivities of 83 to 93% (5). Immunoblot analysis of the recombinant 27-kDa protein showed specific reactivity against sera from 40 of 44 (91%) paracoccidioidomycosis patients tested. The four negative serum samples were checked again by immunodiffusion assays by using our standard crude yeast-derived antigen (15), and they again yielded a positive band. We also used immunoblotting and immunodiffusion, both of which can exhibit different sensitivities in each assay. It appears, therefore, necessary to compare various techniques in order to evaluate these negative results (10).

The 27-kDa cloned antigen was specific for paracoccidioidomycosis when antigen at the concentrations indicated previously was used. Sera from patients with the other mycoses did not cross-react, nor did the tuberculosis patients' sera (Table 1). According to Galen and Gambino's method (8), sensitivity was 91.6%, specificity was 100%, and predictive value was 100%.

A purified antigenic preparation of the 43-kDa glycoprotein (12) has been used by Camargo et al. (4), who reported a sensitivity of 97.1% when the antigen was evaluated by immunodiffusion. Using the purified 43-kDa antigen, Taborda and Camargo evaluated the reactivity of paracoccidioidomycosis patients' sera by dot immunobinding and found that all 50 serum samples tested were reactive (19). In a different study which used immunoblotting with a yeast-derived antigen, the sensitivity was reported to be 87% when antigens of 57, 48, and 45 kDa were employed. When only the 57-kDa antigen was used, such sensitivity was lower, 70% (6). Ferreira-da-Cruz et al., working with immunoblot analysis, found a 45-kDa antigen to be specific (2.5% cross-reaction) and sensitive (90.6%) in patients with paracoccidioidomycosis (7).

TABLE 1. Comparison of the reactivities of sera from patients with different mycoses and tuberculosis with a *P. brasiliensis* cloned antigenic protein[a]

Mycosis or tuberculosis	No. of serum samples	No. (%) of reactive specimens
Paracoccidioidomycosis	44	40 (91)
Histoplasmosis	10	0 (0)
Sporotrichosis	9	0 (0)
Aspergillosis	5	0 (0)
Cryptococcosis	5	0 (0)
Chromoblastomycosis	5	0 (0)
Tuberculosis	8	0 (0)

[a] Immunoblotting was used for the analysis.

Although it may appear that cloned antigens are more expensive, the fact that all lots will bear the same reactive protein and, thus, that the protein could be reproduced from lot to lot clearly indicates that molecular biology has its place in facilitating the procurement of suitable antigens for serological testing.

We are currently working on the characterization of the clones obtained in this study by further screenings of our library, as well as on the production of other libraries, with the aim of cloning a range of *P. brasiliensis* antigenic proteins. Such clones would permit the development of an improved antigenic preparation consisting of more than one cloned protein, in order to produce a reagent suitable for all diagnostic purposes.

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