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Use of the 27-Kilodalton Recombinant Protein from *Paracoccidioides brasiliensis* in Serodiagnosis of Paracoccidioidomycosis

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Paracoccidioidomycosis (PCM) is one of the most important endemic mycoses in Latin America; it is usually diagnosed by observation and/or isolation of the etiologic agent, *Paracoccidioides brasiliensis*, as well as by a variety of immunological methods. Although the latter are effective, two circumstances, cross-reactions with other mycotic agents and antigen preparation that is marked by extreme variability among lots, hinder proper standardization of the procedures. To circumvent this lack of reproducibility, molecular biology tools were used to produce a recombinant 27-kDa-molecular-mass antigen from this fungus; a sizable quantity of this antigen was obtained through fermentation of *Escherichia coli* DH5α, which is capable of expressing the fungal protein. The latter was purified by the Prep-Cell System (Bio-Rad); the recovery rate of the pure protein was approximately 6%. A battery of 160 human serum samples, consisting of 64 specimens taken at the time of diagnosis from patients with PCM representing the various clinical forms plus 15 serum specimens each from patients with histoplasmosis and aspergillosis, 10 each from patients with cryptococcosis and tuberculosis, 6 from patients with coccidioidomycosis, and 40 from healthy subjects, were all tested by an indirect enzyme-linked immunosorbent assay with the purified 27-kDa recombinant protein. The latter was used at a concentration of 1.0 μg/well; there were three serum dilutions (1:1,000, 1:2,000, and 1:4,000). The experiment was repeated at least twice. The average sensitivity for both experiments was 73.4%; in comparison with the healthy subjects, the specificity for PCM patients was 87.5% while for patients with other mycoses, it was 58.7%. Important cross-reactions with sera from patients with aspergillosis and histoplasmosis were detected. The positive predictive value of the test was 90.4%. These results indicate that it is possible to employ recombinant antigenic proteins for the immunologic diagnosis of PCM and, by so doing, achieve high coverage rates. Furthermore, antigen reproducibility can now be ensured, thus facilitating inter- and intralaboratory standardization.

Paracoccidioidomycosis (PCM), a disease caused by the thermally dimorphic fungus *Paracoccidioides brasiliensis*, is prevalent in most countries of Latin America, where it afflicts mainly adult males engaged in agriculture (5, 18, 20, 22, 27). The disorder causes an important number of deaths (9, 14, 18, 22, 26, 27). The disease varies in severity, and both the host and the pathogen contribute to this end, as reflected in the various clinical forms (5, 14, 15, 18, 20, 27). Prompt and accurate diagnosis is of the outmost importance since it allows initiation of specific therapy and thus avoidance of continuous organ system damage (5, 15, 26, 27). Laboratory diagnosis relies on the visualization of the causative agent in direct KOH preparations or biopsy specimens, as well as on its isolation in culture (5, 18, 20). Serologic testing also plays an important role in diagnosis and is widely used for this purpose (5, 25) and also for follow-up studies (5, 20, 26).

In spite of their usefulness, serologic tests have certain important limitations such as cross-reactivity with tests for other mycotic disorders, mainly histoplasmosis, and the difficulties encountered in the proper standardization of the various tests and reagents (5, 10, 25). One of the most difficult problems to address is the lack of reproducibility of *P. brasiliensis* antigens (12, 13). Similar antigenic preparations exhibit strain variation, with antigens differing according to fungal form, incubation time, and culture medium employed; even under controlled conditions and following identical protocols, there is variability in the final products (12, 13). Several important attempts have been made to circumvent this situation by employing purified and well-characterized immunologically reactive antigens derived from this pathogen, such as the 43-kDa (gp43) and the 58-kDa glycoprotein antigens, described by Camargo et al. and others (6, 29, 34) and by Figueroa et al. (12), respectively. In the last decade, the former antigen has been used successfully in various types of serologic tests and its reproducibility is higher than that of other preparations (6, 25).

Advancements in molecular biology have allowed production of reproducible and characterized antigenic proteins through cloning and sequencing. In the case of *P. brasiliensis*, the gp43 antigen was the first product to be thus studied in 1989 by Taba et al. (33); however, the clone was lost, and only recently (in 1996) was it prepared anew by Cisalpino et al. (7). Shortly thereafter, our group cloned and sequenced a 27-kDa antigenic protein from the fungus (23); in preliminary studies, this product was recognized by antibodies present in sera from patients with PCM and appeared to be free of significant cross-reactivity (28). The present report extends the findings described above and shows that batch production of the recombinant 27-kDa antigenic product (p27) is feasible. Additionally, the availability of this antigen allowed us to standardize an en-

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TABLE 1. Fermentation conditions

Parameter	Value or type
Medium	Luria broth
Shaking	100–350 rpm
Temp	37°C
pH	7.0
Foam control[a]	0.01% SAG 710
Dissolved oxygen[b]	90%
Air vol	15 liters/min
Pressure of input water	16 lb/in^2
Pressure of steam	30 lb/in^2
Net fermentation vol	5 liters
Time	6 h
Regulator base	3 N ammonia hydroxide
Regulator acid	3 N hydrochloric acid

[a] SAG 710 antifoam (Wiptcol Corp.).
[b] The dissolved oxygen is reported as a percentage of environmental oxygen.

zyme-linked immunosorbent assay (ELISA) for the diagnosis of PCM.

MATERIALS AND METHODS

Recombinant protein. The original 27-kDa recombinant protein was cloned in the pBluescript II phagemid (Stratagene, La Jolla, Calif.) (1) and expressed in *Escherichia coli* Sure (23) by using a cDNA library constructed in the λZap II vector (Stratagene) (2, 32).

However, further trials with this strain of *E. coli* revealed that there was no expression of the transcript, and a different host cell, *E. coli* DH5α, was employed. The new host successfully carried and expressed the recombinant protein. Plasmid DNA extraction and host cell transformation were done in accordance with the methods of Sambrook et al. (31).

Batch production and characterization of the recombinant protein. The recombinant bacterium was scale-grown in a Bioflo 4000 bioreactor (New Brunswick Scientific Co., New Brunswick, N.J.), under the conditions described in Table 1. To the culture medium employed, Luria broth base (Miller's Luria broth base; Oxoid Limited, Basingstoke, Hampshire, United Kingdom) (21), we added 50 to 1,000 μg of ampicillin (aminobenzylpenicillin; Sigma, St. Louis, Mo.) per ml to control the purity of the transformed bacterium and, at the higher concentrations, to retard the loss of expression (19). Finally, an antifoaming product, SAG 710 (Wiptcol Corporation, OSI Specialties, Sisterville, W. Va.), was employed (38).

The inoculum consisted of a 24-h culture of the recombinant bacteria incubated at 37°C under continuous shaking (orbital incubator model INR-200; Sanyo-Gallenkamp plc, Leicester, Leicestershire, United Kingdom) that had an optical density (OD) at 595 nm of 0.6. The optimal proportion of the inoculum was 3 to 10% of the total fermentation volume (24). During fermentation, the bioreactor controls all the parameters that influence the process (30).

At the end of the process, cell mass was determined by dry weight and the yield of protein was calculated by the Bradford technique (Bradford kit 500-0001; Bio-Rad, Hercules, Calif.) (37). The expression of the recombinant protein was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (with a 12% polyacrylamide gel) run under denaturing conditions (37). The antigenic activity of the protein thus obtained was assessed by transferring the gels to nitrocellulose membranes (Hybond-C; Amersham Life Sciences, Buckinghamshire, United Kingdom) by Western blot analysis (4, 35) if the protein was recognized by the antibodies present in a pool of sera from patients with PCM.

Characterization and purification of the batch-produced recombinant protein. Purification was done by preparative electrophoresis using the Prep-Cell system (model 491; Bio-Rad) (3). The separating gel at 12% was prepared under denaturing conditions with a total of 35 to 90 mg of bacteria. The purified protein was precipitated with 2.25 volumes of chilled acetone per 150-μl sample volume and left at −20°C for 6 h; the sediment was then centrifuged at 9,280 relative centrifugal force at 5°C for 5 min. The supernatant was discarded, and the sediment was collected and resuspended in the sampling buffer and electrophoresed in an SDS–12% PAGE gel (37). The gels were stained with Coomassie blue. Once the purified fractions corresponding to the recombinant protein were identified, they were dialyzed against phosphate-buffered saline (PBS) diluted 1:5 and concentrated by per-evaporation to 1/10 their original volume and their protein content was measured by the Bradford technique (Bio-Rad Bradford kit) (37).

Serum specimens employed. As shown in Table 2, 160 serum specimens were examined, 64 from patients with the various clinical forms of PCM, 15 each from histoplasmosis and aspergillosis patients, 10 each from cryptococcosis and tuberculosis patients, 6 from coccidioidomycosis patients, and 40 from healthy subjects.

TABLE 2. Human serum samples tested

Source of sample[a]	No. of samples
Patients	
PCM	64
Chronic multifocal form	45
Chronic unifocal form	12
Subacute form	7
Histoplasmosis	15
Aspergillosis	15
Cryptococcosis	10
Coccidioidomycosis	6
Tuberculosis	10
Healthy subjects	40
Total	160

[a] With the exception of the samples from tuberculosis patients, all samples were taken at the time of diagnosis.

ELISA testing. The purified recombinant antigen was initially titrated to determine its reactivity by using microtitration plates (Nunc Immuno Module Star Well Maxisorp; Nunc-Intermed, Naperville, Ill.). Dilutions of the 27-kDa recombinant antigen varied and ranged between 1 and 0.125 μg per well. Serum dilution ranged from 1:1,000 to 1:4,000; the second antibody was used at concentrations of 1:8,000 to 1:64,000. A carbonate buffer described by Voller et al. was used throughout (36).

The indirect ELISA procedure described by Engvall and Perlmann was used (11). Plates were sensitized with the purified protein diluted in carbonate buffer, pH 9.6 (36), in concentrations ranging from 0.5 to 4 μg per well. Incubation was carried out for 30 min at 25 to 26°C and left at 4°C overnight. The plates were allowed to stabilize at 22 to 26°C for 30 min. They were then saturated with 5% bovine serum albumin dissolved in 1× PBS plus 0.05% Tween and incubated at the same temperature for 2 h (11, 36).

The plates were washed three times with the same PBS formulation, and the excess fluid was eliminated. Then, 100 μl of each one of the three serum dilutions (1:1,000, 1:2,000 and 1:4,000) was applied to the respective well. Incubation was for 1 h at 22 to 26°C. Washing and drying as described above were repeated, and then 100 μl of the peroxidase-labelled second antibody at the indicated dilution (peroxidase-conjugated Affinipure goat anti-human antibody; Jackson Immunoresearch, West Grove, Pa.) was added to each well. The plates were incubated at the same temperature for 1 h.

After another cycle of washing and drying, the reaction was visualized with 100 μl of OPD (*o*-phenylenediamine; Sigma) 10 mg in 4 μl of hydrogen peroxide (Sigma), and dissolved in phosphate citrate, pH 5.0. Incubation was for 15 min at 22 to 26°C. The reaction was then stopped by using 100 μl of H_2SO_4 (4 N) per well. The plates were read at 450 nm in an ELISA reader (Bio-Rad microplate reader model 550). All tests were run in duplicate.

Statistical procedures. The significance of positive values was determined by the Student *t* test (Microsoft Excel). Sensitivity and specificity were determined by the statistical method of Gallen and Gambino (16).

RESULTS

Recombinant protein. The expression of the 27-kDa recombinant protein by the transformed *E. coli* DH5α was poor during fermentation in cultures that had low concentrations of ampicillin; it was then necessary to increase its concentration to 1,000 μg/ml. A larger bacterial mass was thus obtained, and the expression of the 27-kDa protein became significant. Furthermore, it was shown that this protein was antigenically active as it was recognized by anti-*P. brasiliensis* antibodies present in a pool of 16 PCM patient serum specimens (data not shown).

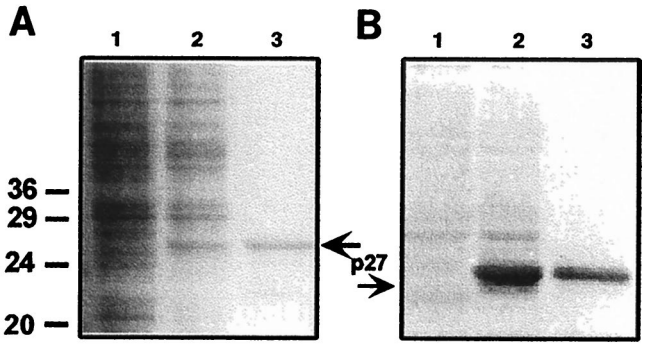

FIG. 1. SDS-PAGE gel (A) and immunoblot (B) of the 27-kDa recombinant protein of *P. brasiliensis*. (A) SDS-12% PAGE gel with Coomassie blue stain; (B) immunoblot developed with a mixture of sera from patients with PCM. Lanes: 1, *E. coli* nonrecombinant; 2, *E. coli* recombinant; 3, purified protein. This figure was obtained through scanning with the NIH Image 1.61/ppc computer program (Power Macintosh) (ftp://zippy.nimh.nih.gov).

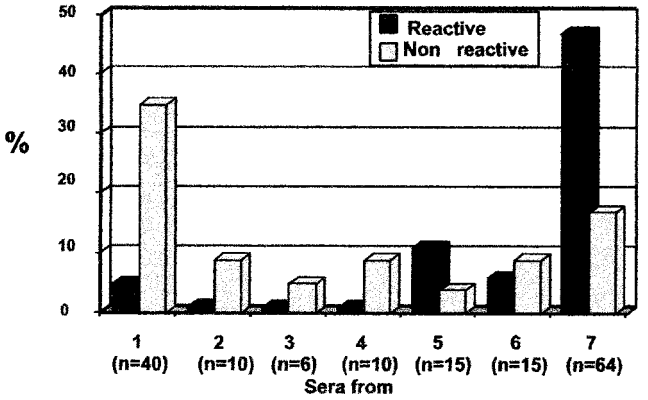

FIG. 3. Comparison of reactivity and nonreactivity of serum samples. The numbers along the bottom of the figure represent the following sources of serum samples: 1, healthy subjects; 2, tuberculosis patients; 3, coccidioidomycosis patients; 4, cryptococcosis patients; 5, aspergillosis patients; 6, histoplasmosis patients; 7, PCM patients.

Batch production of the recombinant protein. The conditions under which the transformed *E. coli* cells were grown are given in Table 1. Optimal mass production was obtained after 6 h of fermentation when the OD was at its highest and then tapered off until the end of the fermentation at 8 h. The antifoaming agent did not influence the expression of the protein (data not shown).

At this time, the dry weight of the bacterial mass varied between 4 and 5 g/liter of culture medium. Total bacterial mass was 20 to 25 g/5 liters.

Characterization and purification of the recombinant protein. Once batch production was accomplished, purification ensued as described previously (Prep-Cell). From approximately 45 mg of the bacterial mass, 3.0 mg of purified protein (6.6%) was obtained. The final product was tested by Western blotting in the presence of the positive control (pooled PCM patient sera) and found to be reactive (Fig. 1).

Diagnostic tests. As shown in Fig. 2, the indirect ELISA procedure revealed that the sera from patients with PCM as well the positive control (pool of PCM patient sera) demonstrated higher reactivity in comparison with those of the specimens from patients with other mycoses or tuberculosis and from healthy individuals, regardless of the serum dilution.

According to statistical procedures, the cutoff point of sensitivity and specificity was estimated to be 1.4 times the median point of those of the control sera.

The results of the ELISA with the p27 recombinant protein (Fig. 3) revealed that 47 (73.4%) of the 64 PCM patient serum samples were reactive while 35 (87.5%) of the 40 samples from healthy individuals were nonreactive. Important cross-reactions were noticed in the case of serum samples from aspergillosis and histoplasmosis patients, with 11 of 15 (73.3%) and 6 of 15 (40%) samples giving positive results, respectively.

The ELISA had specificities of 87.5% for PCM patients when compared with healthy subjects ($P < 0.001$), 58.7% for patients with other mycoses ($P < 0.005$), and 90% for patients with tuberculosis ($P < 0.001$).

Table 3 shows the sensitivities, specificities, and predictive values obtained for patients with the various clinical forms of PCM. Sensitivities were 66.7% for the chronic unifocal form, 71.4% for the acute form, and 75.6% for the multifocal form. In comparison with the heterologous sera, specificity was higher for sera from patients with the acute form (87.5%) than for those from patients with the chronic unifocal (83.3%) and chronic multifocal (84.4%) forms. Positive predictive values were similar, being 80% for patients with the unifocal form and 83.3 and 82.9% for patients with the acute and multifocal forms, respectively. When these parameters were used without regard for the clinical form, the sensitivity was 73.4%, the specificity was 87.5%, and the positive predictive value was 90.4%.

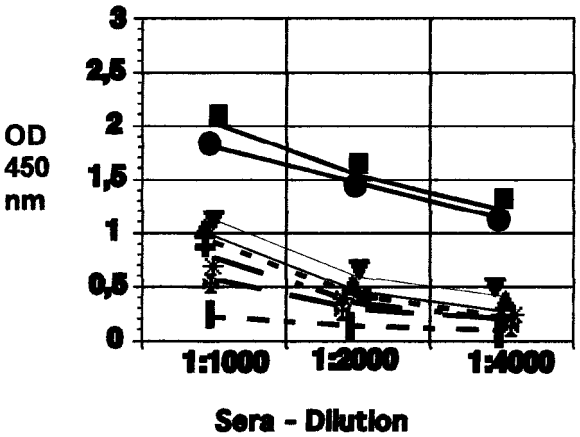

FIG. 2. Indirect ELISA with *P. brasiliensis* 27-kDa recombinant protein. The sera evaluated were positive control p27 (pool of 15 sera from PCM patients) (●), PCM patient sera (■) ($n = 64$), histoplasmosis patient sera (▲), ($n = 15$), aspergillosis patient sera (▼) ($n = 15$), cryptococcosis patient sera (▮) ($n = 10$), coccidioidomycosis patient sera (✚) ($n = 6$), tuberculosis patients (*) ($n = 10$), and healthy subjects (✠) ($n = 40$).

TABLE 3. p27 recombinant protein: sensitivity, specificity, and predictive value of the ELISA

PCM clinical form	Sensitivity (%)	Specificity (%)	Positive predictive value (%)
Acute	71.4	87.5	83.3
Chronic unifocal	66.7	83.3	80.0
Chronic multifocal	75.6	84.4	82.9
All forms	73.4	87.5	90.4

DISCUSSION

The p27 recombinant protein, cloned and sequenced previously by our group (23, 28) and expressed in the *E. coli* DH5α vector, was produced in sizable quantities by fermentation techniques. After purification by preparative electrophoresis, yields were in the range of 6%. The purified protein was found to be reactive when used as an antigen in the immunologic diagnosis of PCM patients.

The p27 product is the second *P. brasiliensis* recombinant protein that has been cloned and sequenced; the first one was gp43, an immunodominant antigen cloned in 1989 (33) and characterized further by Cisalpino et al. (7). The gp43 fraction has been extensively and successfully employed as an antigen in the serological diagnosis of PCM, but for this particular purpose, it has been extracted directly from *P. brasiliensis* cultures and not by recombinant processes (6, 25).

Standardization of antigens for the immunologic diagnosis of PCM has been difficult to accomplish partly because of the variations in antigen lots. As demonstrated by Franco et al. (13), differences in activity, specificity, and potency of the final products vary even with rigorous adherence to the same protocol. Such variation hinders inter- and intralaboratory comparisons (10, 13, 25). This problem could be solved by using recombinant antigens since, once cloned, the same antigenic moiety will be expressed by the transformed vector (31). Furthermore, large quantities can be produced, thus permitting standardization of diagnostic methods (8, 33).

As shown here, the ELISA required minimal amounts (1 μg/well) of the recombinant antigen and was able to detect anti-*P. brasiliensis* antibodies in the sera of 73.4% of the PCM patients tested at the time of diagnosis. This figure is lower than the one reported for the traditional tests that employ complex antigens, such as complement fixation and agar gel immunodiffusion, which detect antibodies in 90 to 95% of the cases (5, 10, 25). The p27 recombinant protein may well represent a single dominant antigenic epitope and, as such, may not be recognized for all patients. In the future, it might be necessary to use a cocktail of recombinant proteins to achieve higher sensitivity values.

In spite of the fact that previous testing by Western blotting (28) revealed no cross-reactions with sera from patients with mycoses other than PCM, the ELISA procedure turned out to be more sensitive, as it revealed important cross-reactivity with the sera from patients with aspergillosis (73.3%) and histoplasmosis (40%). This is a point that should be addressed, since the small number of specimens tested in each case could have influenced statistical interpretations. Nonetheless, all the serological methods presently in use have shown cross-reactions with sera from patients with these mycotic disorders, regardless of the antigen utilized (10, 12, 25). More recently, aspergillosis patient sera have been shown to react in a monoclonal antibody-based technique employed for the detection of *P. brasiliensis* circulating antigens (17).

In comparison with the specificity for patients with other mycoses, the ELISA was less specific (58.7%) for PCM patients than expected, even if test results were still statistically significant ($P < 0.005$). The assay was much more specific for PCM patients when compared with healthy subjects (87.5%, $P < 0.001$) and patients with tuberculosis (90%, $P < 0.001$). For all persons tested, specificity was higher (73.4%) as was the positive predictive value (90.4%).

When the PCM patient sera were grouped according to clinical forms (14), variations were observed although the lower sensitivity values exceeded 66%. The lower positive predictive values (80%) were obtained in patients with the unifocal form since, in this form, cellular immunity is still functional and as a consequence the patients are capable of restricting fungal development (14).

It may well be that this particular 27-kDa antigenic epitope is not recognized by all PCM patients. These results further strengthen the need for antigens that contain multiple reactive epitopes.

The potential contributions of molecular biology to the production of suitable antigens for immunologic testing of this and other deep-seated mycoses is now beginning to be understood, and their future appears to be promising.

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