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Preparation and Use of Cytoplasmic Antigens for the Serodiagnosis of Paracoccidioidomycosis

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A cytoplasmic antigen of *Paracoccidioides brasiliensis* (strain MTC) prepared from the yeast phase grown in the chemically defined medium of McVeigh and Morton is described. This antigen can be easily prepared, does not vary from lot to lot, and can be lyophilized without loss of activity or potency. In the immunodiffusion test, the cytoplasmic antigen demonstrated a sensitivity of 97% and a specificity of 100% when tested against 218 sera from 139 cases of paracoccidioidomycosis. When 177 sera from patients with fungal diseases other than paracoccidioidomycosis were tested by immunodiffusion, there were no false-positive reactions. In an enzyme-linked immunosorbent assay, the antigen was equally effective in identifying cases, giving a sensitivity of 100% and a specificity of 99% when a 1:640 titer was considered the threshold for clinical significance. Antigenic components in the cytoplasmic extract of *P. brasiliensis* were examined after fractionation by concanavalin A-Sepharose 4B column chromatography. The fraction of the cytoplasmic antigen that binds to the concanavalin A column is material identical to the specific b_1 (antigen 1) precipitin band described by A. Restrepo and L. H. Moncada (Appl. Microbiol. 28:138–144, 1974).

Paracoccidioidomycosis, or South American blastomycosis, is a chronic granulomatous disease of the skin, mucous membranes, lymph nodes, and internal organs caused by the dimorphic fungus *Paracoccidioides brasiliensis*. Because the clinical manifestations of the disease are so protean, diagnosis on purely clinical grounds is difficult. Consequently, diagnosis is usually established serologically. The presence of specific precipitating antibodies demonstrated by immunodiffusion (ID) testing is considered diagnostic for the disease. Antigens from both the mycelial and yeast phases of the fungus have been used in serological tests (1, 5, 6, 8). The antigen described by Restrepo and Moncada (5) and Restrepo-Moreno and Schneidau (6) is derived from the yeast form of the organism and is carbohydrate in composition. Unfortunately, this antigen varies considerably in activity and quality from lot to lot due to difficulties with production. The medium used to grow the organism must be dialyzed before use, and the organism must be grown for 4 to 6 weeks. By the use of the culture filtrate antigen of Restrepo, three precipitin bands (b_1, b_2, b_3) have been identified when tested against sera from patients with paracoccidioidomycosis. The bands are identified according to their migration in ID plates (5). Yarzabal et al. (8) described an antigen that is also used for detection of precipitating antibodies to *P. brasiliensis*. This antigen is derived from the mold phase of the organism and is a protein with alkaline phosphatase activity. A purified fraction of this preparation, called antigen E, is reported to be specific for cases of paracoccidioidomycosis.

A recent report by Blumer et al. (1) describes a rapid method for production of a specific ID test antigen derived from the mycelial phase of *P. brasiliensis*. Their method results in the production of an antigen that is identical to antigen 1 (b_1) (described by Restrepo and Moncada [5]) and shares antigenic components with the specific E antigen described by Yarzabal et al. (8). The antigen produced by the Blumer method appears to be specific for detecting cases of paracoccidioidomycosis when used in ID testing; however, its use may be limited to that one method of testing. When this antigen was tested by complement fixation against human sera from heterologous fungal diseases, numerous false-positive and nonspecific reactions were seen (1). The most common of the cross-reactive sera were from cases of histoplasmosis, blastomycosis, and aspergillosis.

The purpose of this study was to investigate the preparation and serodiagnostic use of a cytoplasmic antigen derived from the yeast phase of *P. brasiliensis*.

(This work was presented in part previously [K. L. McGowan and H. R. Buckley, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, F54, p. 391].)

MATERIALS AND METHODS

Culture methods. *P. brasiliensis* (strain MTC) was obtained from A. Restrepo, Corporación de Investigaciones Biológicas, Medellin, Colombia. The mycelial culture of this isolate was converted to the yeast form at 37°C on McVeigh-Morton agar (4). Every third day, blind subcultures were done onto a fresh McVeigh-Morton agar slant until typical pasty growth was seen. The entire growth from two agar slants was used to inoculate 1,000-ml flasks containing 400 ml of McVeigh-Morton broth. Cultures were incubated at 37°C on a Gyrotory Shaker (New Brunswick Scientific Co., Inc., Edison, N.J.) at 250 rpm. The cells were harvested by centrifugation after 3 weeks of growth and suspended in 0.01 M phosphate buffer (pH 7.4) containing 1.0 mM phenylmethylsulfonyl fluoride. The cell slurry was stored at −80°C until used for preparation of extracts.

Antigen preparation. (i) Preparation of cytoplasmic extract. The yeast cell cytoplasmic extract was prepared by mechanical disruption for 4 min in a Braun homogenizer. Samples were examined microscopically and showed ≥90% breakage. The slurry was centrifuged at 10,000 × *g* for 60 min, after which the supernatant was removed and placed on ice. The cell pellets were rewashed with 0.01 M phosphate buffer (pH 7.4) and centrifuged as described above, and then the wash and initial supernatants were combined. The resultant extract was filtered over siliconized glass wool to remove lipid particles and then centrifuged at 80,000 × *g* for 60 min.

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For each milliliter of supernatant, 50 μl of freshly prepared 10% streptomycin sulfate was added to remove ribosomal proteins. The resultant precipitate was removed by centrifugation at 10,000 × *g* for 15 min, and the supernatant antigens were dialyzed overnight against 0.01 M phosphate buffer (pH 7.4). The antigens were concentrated to a final concentration of 6.0 to 6.2 mg of protein per ml in an Amicon stir-cell concentrator, using a PM 10 membrane. A 1 mM concentration of the protease inhibitor phenylmethylsulfonyl fluoride was maintained throughout the entire procedure. The concentrated antigens were then frozen in samples at −80°C or lyophilized in 500-μl aliquots until used. The reactivity of the cytoplasmic extract prepared from different batches of cells was titrated against a reference serum from a case of disseminated paracoccidioidomycosis on immunodiffusion plates and crossed immunoelectrophoresis and was found to be reproducible.

Reference culture filtrate antigen was supplied by A. Restrepo.

(ii) Concanavalin A affinity chromatography. For fractionation purposes, the cytoplasmic extract was passed through concanavalin A (Con A)-Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) affinity columns. The loading buffer was 0.05 M Tris-hydrochloride (pH 7.4) with 0.02% (wt/vol) NaN_3, 0.001 M $MnCl_2$, 0.001 M $MgCl_2$, and 0.001 M $CaCl_2$. The running buffer was 0.05 M Tris-hydrochloride (pH 7.4) without additional ions. The nonbinding material was pooled, dialyzed against 0.01 M phosphate buffer (pH 7.4), lyophilized, and reconstituted to the original starting volume. The Con A-bound material was eluted with 0.05 M α-methyl mannoside in 0.05 M Tris-hydrochloride (pH 7.4). This fraction was then dialyzed, lyophilized, and reconstituted to 1/10 the original starting volume.

A total of 3.8 mg of protein antigen in a total volume of 1.5 ml was placed on a 7.5-ml Con A-Sepharose column. The efficiency of recovery obtained with the column was 74%. The Con A-adherent fraction represented 1.9% of the total protein mixture loaded on the column.

ID test. The ID test was performed in 1% Noble agar (Difco Laboratories, Detroit, Mich.) with borate buffer (pH 8.6) (0.015 M $N_2B_4O_7$ and 0.05 M H_3BO_3) on glass plates (2 by 2 in [5.08 by 5.08 cm]) (Eastman Kodak, Rochester, N.Y.). A basic immunodiffusion pattern consisting of a central well surrounded by six lateral wells was used. For control purposes, a reference serum known to produce a precipitin band with the culture filtrate antigen of Restrepo was run with each batch of sera tested. A 25-μl portion of a solution of antigen (3.1 mg of protein per ml) was placed in the center well, and patient sera were added to the six lateral wells. The immunodiffusion plates were placed in a humid chamber at room temperature. Reactions were examined by transmitted light at both 24 and 48 h. The plates were washed in a solution containing 0.4% NaCl and 0.4% $N_2B_4O_7$ for 24 h and then pressed, dried, and stained with Coomassie blue R250 (0.1% [wt/vol] in 10% [vol/vol] CH_3COOH, 10% [vol/vol] CH_3OH, and 80% [vol/vol] distilled water). The plates were then destained in a solution of 10% (vol/vol) CH_3COOH, 10% (vol/vol) CH_3OH, and 80% (vol/vol) distilled water, and precipitin bands were recorded.

Enzyme-linked immunosorbent assay (ELISA). The ELISA procedure described by Voller et al. (7) was used with some modifications. Duplicate wells of polyvinyl flat-bottomed microtiter plates (Costar, Cambridge, Mass.) were coated with 100 ng of protein antigen in a carbonate-bicarbonate coating buffer (pH 9.6). After overnight incubation at 4°C, the wells were washed twice with phosphate-buffered saline–0.05% Tween 20 (PBS-T). Then 100 μl of 3% Hammarsten casein (Gallard-Schlesinger, Carle Place, N.Y.) in 0.85% NaCl (pH 7.6) was added to the wells. Plates were incubated overnight at 4°C. Each well was washed three times with PBS-T, and the plates were stored at −80°C until used. Sera to be tested were diluted in PBS-T at dilutions ranging from 1:10 to 1:40,960, and a 50-μl sample of each dilution was placed in duplicate wells. After a 90-min incubation at 37°C, the plates were washed as described above, and 50 μl of a 1:1,000 dilution of peroxidase-conjugated goat anti-human immunoglobulin M (IgM), IgA, IgG (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) was added. Incubation was for 90 min at 37°C, and excess conjugate was removed by washing. A 200-μl sample of a 0.1-mg/ml solution of 2,2′-azino-di(3-ethyl-benzthiazoline sulfonate) (Sigma Chemical Co., St. Louis, Mo.) in 0.1 M citrate buffer (pH 4.0) containing 0.06% H_2O_2 was then added. Plates were incubated at room temperature for 30 min, and the absorbance was measured at 410 nm in a microplate colorimeter reader (Dynatech Laboratories, Alexandria, Va.). Net absorbance was calculated by subtracting the mean absorbance of blank wells from the mean absorbance of wells coated with antigen. An absorbance level of ≥0.100 was recorded as a positive result. Each specimen was tested in triplicate.

Patient sera. Sera from culture-proven human cases of aspergillosis (14 sera), blastomycosis (14 sera), candidiasis (40 sera), coccidioidomycosis (32 sera), cryptococcosis (9 sera), histoplasmosis (58 sera), other miscellaneous fungal diseases (10 sera), and paracoccidioidomycosis (218 sera from 139 cases) were used in both the ID and ELISA testing. Of the 139 patients with paracoccidioidomycosis, all but two were male. Their ages ranged between 11 and 67 years, and the sera included both acute and convalescent cases. In addition, sera from 50 normal controls, 30 of which were from within the endemic zone, were tested. All sera had been frozen at −20°C before testing. Sera were provided by R. Cox, San Antonio Chest Hospital, San Antonio, Tex.; A. Di Salvo, South Carolina Public Health Laboratories, Columbia, S.C.; L. Kaufman, Division of Mycotic Diseases, Centers for Disease Control, Atlanta, Ga.; P. Negroni, Facultad de Medicine, Centro de Micologia, Buenos Aires, Argentina; A. Restrepo, Corporación de Investigaciones Biológicas, Medellin, Colombia; and the Immunoserology Laboratory, Temple University Hospital, Philadelphia, Pa.

Sensitivity, specificity, predictive value, and efficiency of the results of our serological tests were calculated (3).

RESULTS

A total of 445 sera were examined, 392 by both ELISA and ID and an additional 53 by ID alone. A list of the heterologous sera tested by each method is shown in Table 1. To date, six batches of the cytoplasmic antigen have been prepared, and all have been found to be equally reactive by ID and ELISA when tested against representative sera from each group listed in Table 1.

The cytoplasmic antigen demonstrated a sensitivity of 97% by detecting antibodies in 212 of 218 sera from 139 cases of paracoccidioidomycosis when tested by ID. The majority of sera tested produced multiple (>2) precipitin bands. The six sera that yielded negative results were tested in the field and could not be retested by ID or tested at a later date by ELISA. For comparison, when 136 of the sera from patients with paracoccidioidomycosis were tested by ID with the Restrepo antigen, 118 were positive, resulting in a sensitivity of 87%. When 177 sera from patients with fungal diseases other than paracoccidioidomycosis were tested by ID with

TABLE 1. Number of sera from various diseases tested by ID and ELISA with a cytoplasmic antigen of *P. brasiliensis* (MTC)

Disease	No. of sera tested by ID	No. of sera tested by ELISA	Total no. of sera examined with both tests
Aspergillosis	14	14	14
Blastomycosis	14	14	14
Candidiasis	40	40	40
Coccidioidomycosis	32	32	32
Cryptococcosis	9	9	9
Histoplasmosis	58	47	58
Miscellaneous fungal and bacterial diseases[a]	10	10	10
Paracoccidioidomycosis	218	176	218
Normal controls[b]	50	50	50

[a] Includes infections with *Absidia* sp., *Acremonium* sp., *Nocardia* sp., *Penicillium* sp., and *Rhodotorula* sp.

[b] This includes 30 sera obtained from within the endemic zone that are age- and sex-matched with patients having paracoccidioidomycosis.

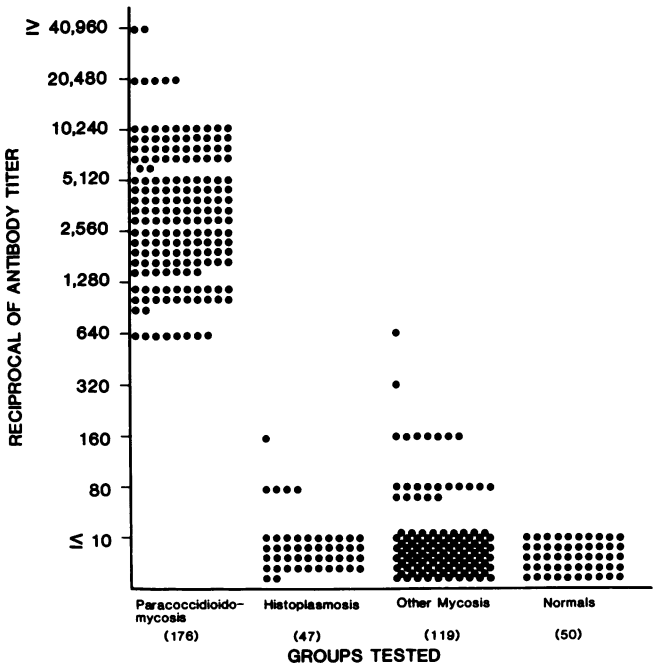

FIG. 1. ELISA antibody titers of 392 sera tested with a cytoplasmic antigen of *P. brasiliensis* (MTC). Each point represents the test result of a single serum specimen. The titers are expressed as the reciprocal of the highest dilution of serum which gave positive results (optical density at 410 nm ≥ 0.100) for antibodies to the cytoplasmic antigen. Groups tested included 50 normal sera, 47 sera from cases of histoplasmosis, 176 sera from cases of paracoccidioidomycosis, and 119 sera from cases of other mycotic infections.

the cytoplasmic antigen, all 177 yielded negative results. All 50 normal sera were negative, yielding a specificity of 100%. No nonspecific reactions were seen with either the normal sera or sera from patients with other fungal diseases. When the cytoplasmic antigen was used for ID testing, the overall efficiency of the test was 99% and the predictive value positive was 100%.

Table 2 and Fig. 1 show the antibody titers obtained with the 392 sera tested by ELISA with the cytoplasmic antigen. A total of 176 sera from cases of paracoccidioidomycosis were tested, and all gave antibody titers of ≥1:640. A total of 166 sera from patients with fungal diseases other than paracoccidioidomycosis were tested, and with the exception of two cases of blastomycosis, all gave ELISA titers of ≤1:160 (98.8%). Of the 47 sera from patients with histoplasmosis, 1 serum had a titer of 1:160, 4 had a titer of 1:80, and the remaining 42 (89%) had a titer of ≤1:10. The 50 control sera had antibody titers of ≤1:10. For statistical purposes, a titer of ≥1:640 was considered positive. Sera from patients with paracoccidioidomycosis had significantly higher levels of antibody ($P < 0.005$) directed against the cytoplasmic antigen. When ELISA testing was done with this antigen, the sensitivity of the ELISA was 100%, the level of specificity and predictive value positive were both 99%, and the efficiency was 99.7%.

Initial ID testing revealed that the cytoplasmic antigen shared a band of precipitation with the culture filtrate antigen described by Restrepo-Moreno and Schneidau (6). This was identified by Restrepo as the b_1 band seen with their culture filtrate antigen (personal communication). This shared b_1 component can be separated from the whole cytoplasmic preparation by Con A-Sepharose 4B affinity chromatography. After Con A chromatography, the adherent and nonadherent fractions of the column were tested by ID against serum from a case of paracoccidioidomycosis. The cytoplasmic and culture filtrate antigens were placed next to each fraction for comparison (Fig. 2). Figure 2A shows the comparison between the cytoplasmic and culture filtrate antigens. Band 1 is the b_1 band that is shared between the two preparations. Band 2 is a precipitin band present only with the cytoplasmic preparation. The precipitin line for

TABLE 2. Antibody titers obtained with a cytoplasmic antigen of *P. brasiliensis* (MTC) in ELISA testing of 392 human sera

Disease	No. of cases	No. of sera with the following antibody titer determined by ELISA[a]										
		≤10	80	160	320	640	1,280	2,560	5,120	10,240	20,480	≥40,960
Aspergillosis	14	3	4	7								
Blastomycosis	14	12			1	1						
Candidiasis	40	38	2									
Coccidioidomycosis	32	31	1									
Cryptococcosis	9	6	3									
Histoplasmosis	47	42	4	1								
Miscellaneous fungal and bacterial diseases	10	5	5									
Paracoccidioidomycosis	176					8	22	47	50	42	5	2
Normal controls	50	50										

[a] Titers are expressed as the reciprocal of the highest dilution of serum which was positive. An optical density at 410 nm of ≥0.10 was considered positive.

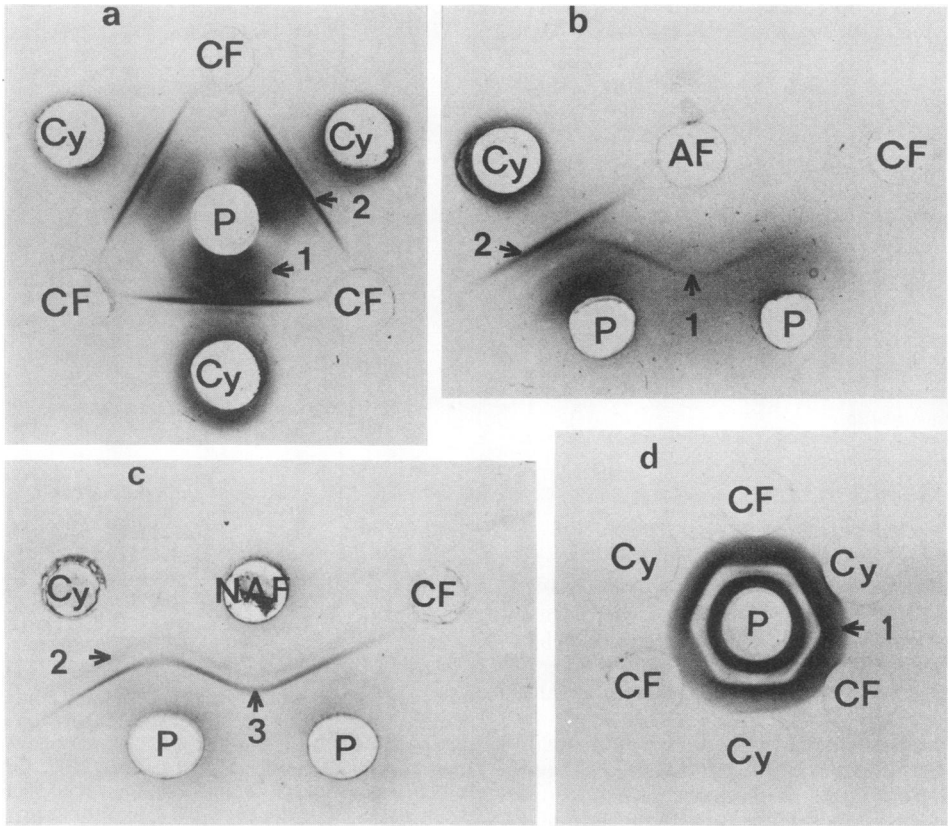

FIG. 2. Comparison of cytoplasmic antigen preparation of *P. brasiliensis* (MTC) with a culture filtrate antigen (Restrepo) before and after affinity chromatography. P, Serum from a patient with paracoccidioidomycosis; CF, culture filtrate antigen of *P. brasiliensis* prepared by the Restrepo method; Cy, cytoplasmic antigen (MTC) prepared as described in the text; AF, Con A-adherent fraction; NAF, Con A-nonadherent fraction. Band 1 represents a band of identity shared between the cytoplasmic and culture filtrate antigens. Bands 2 and 3 represent precipitin bands to the cytoplasmic antigen preparation.

band 2 extends right to the well containing the culture filtrate antigen and shows no identity with the culture filtrate antigen. Thus it is distinct and separate from the culture filtrate antigen. Figure 2B and C show the results obtained on ID when the Con A-adherent and Con A-nonadherent fractions were used. Only band 1 is seen when the culture filtrate and the Con A-adherent fraction of the cytoplasmic antigen are used. Figure 2B shows this shared component as a single band of identity between the two preparations. This is the b_1 band described by Restrepo and Moncada (5). This can also be seen in Fig. 2D, where only the culture filtrate antigen and the Con A-adherent fraction of the cytoplasmic antigen are shown by ID. Again, the precipitin b_1 band is shared between the two preparations. In Fig. 2C the Con A-nonadherent fraction and the culture filtrate antigen of Restrepo are compared, using ID. The Con A-nonadherent fraction is still positive by ID, but the shared b_1 band is not present.

DISCUSSION

Six lots of cytoplasmic antigen were prepared, using the protocol described above and *P. brasiliensis* strain MTC. Results were consistent and reproducible with each of the six lots. The antigen demonstrated a high degree of sensitivity and specificity when used to detect antibody by both ID and ELISA techniques. Numerous authors have observed nonspecific or false-positive reactions when attempting to demonstrate the presence of precipitating antibodies to antigens of *P. brasiliensis* (1, 2, 5). This is probably due to the fact that different antigenic preparations of *P. brasiliensis*, *Histoplasma capsulatum*, *Coccidioides immitis*, and *Aspergillus fumigatus* share antigenic determinants. Blumer et al. (1) used specific reference sera as a control to distinguish specific precipitin bands from nonspecific ones. Since the shared antigens are most likely cell wall components, our use of cytoplasmic material was an attempt to improve the overall specificity of the testing antigen while still maintaining sensitivity. The ID and ELISA results obtained with this antigen (Table 1; see above) indicate that this was possible. In ID tests, no cross-reactions were seen (100% specificity), and in the ELISA assay, only 1 serum of a total of 392 tested gave a false-positive reaction (99% specificity). In the past, when other antigens derived from *P. brasiliensis* were used, sera from patients with histoplasmosis were a consistent source of false-positive reactions (1, 5). These false-positive reactions are a serious problem, since the endemic areas for histoplasmosis and paracoccidioidomycosis do overlap. For this reason, it becomes important that the two diseases can be distinguished serologically, especially by ID testing, since that is the most common method for serodiagnosis used within the endemic area. In our study, 47 sera from patients with histoplasmosis were examined. All 47 of the sera tested gave negative results by ID and ELISA. Forty-two of the 47 sera gave antibody titers $\leq$1:10 on ELISA testing. This was most encouraging, since ELISA, unlike ID testing, is not restricted to detecting only precipitating antibodies, and

false-positive results could be expected with the ELISA technique. Production of this cytoplasmic preparation gave an antigen identical to the b_1 precipitin of the culture filtrate antigen obtained by the Restrepo method (Fig. 2). This fraction can be separated from the whole cytoplasmic preparation by its ability to bind to a Con A-Sepharose 4B column (Fig. 2). Con A binds molecules that contain α-D-mannose, α-D-glucose, and sterically related carbohydrates. Restrepo-Moreno and Schneidau (6) have reported the presence of glucose, galactose, arabinose, and glucosamine in their culture filtrate antigen. This carbohydrate composition would permit the culture filtrate antigen or portions of the antigen to bind to Con A. This allowed us to separate the shared component from our cytoplasmic preparation. Antigen E, a purified antigen described by Yarzabal et al. (8), is believed to be identical to antigen 1 (b_1) described by Restrepo and Moncada (5). Because out cytoplasmic antigen and the Restrepo antigen share the b_1 component, the cytoplasmic antigen may also share components with antigen E.

None of the 218 sera from cases of paracoccidioidomycosis examined in this study demonstrated precipitin bands similar to the b_2 or b_3 bands described by Restrepo and Moncada (5). This probably accounts for the greater specificity seen with our cytoplasmic preparation, since the b_3 precipitin band of the Restrepo antigen is not considered specific for the disease. On ID testing, the b_3 component produces a line of identity with histoplasmin band M (5). This is thought to be the reason that sera from cases of histoplasmosis give false-positive results on ID when tested with the culture filtrate antigen of *P. brasiliensis*. The lack of the b_3 component in our cytoplasmic antigen may explain why we had no false-positive results when 47 sera from patients with histoplasmosis were tested in this study.

Before our studies, the use of a cytoplasmic antigen for the diagnosis of paracoccidioidomycosis had not been investigated. With such a preparation, ID and ELISA results indicate a level of sensitivity and specificity greater than or equal to those reported with other antigenic materials available for serodiagnosis. Because the cytoplasmic antigen can be easily prepared, is reproducible from batch to batch, retains activity and stability even after lyophilization, and appears to be ideal for use in a variety of serological tests, we recommend the use of this material for the serodiagnosis of paracoccidioidomycosis.

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