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Sensitive Immunoradiometric Assay for the Detection of *Paracoccidioides brasiliensis* Antigens in Human Sera

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In the present study we report the standardization of an immunoradiometric assay (IRMA) for detection of *Paracoccidioides brasiliensis* circulating antigens that could be useful in the diagnosis and prognosis of paracoccidioidomycosis. For this purpose we studied the reactivities of *P. brasiliensis* and other mycotic antigens with rabbit polyclonal anti-*P. brasiliensis* antibodies (immunoglobulin G) in order to evaluate the sensitivity and specificity of an IRMA for detecting *P. brasiliensis* antigens. The results were compared with those obtained by the double immunodiffusion test, the standard technique for the serodiagnosis of paracoccidioidomycosis. By using the immunoglobulin G fraction of rabbit antisera (900 ng per well), it was possible to detect up to 3.6 ng (0.12 μg/ml) of cellular antigen and 360 ng (12 μg/ml) of metabolic antigen in contrast to the double immunodiffusion test that could detect only 12 μg (1.2 mg/ml) of both antigens. IRMA was shown to be feasible and very sensitive and may therefore help, together with clinical data, in establishing early diagnosis and assessing disease activity. It could also allow the study of relationships between *P. brasiliensis* circulating antigens and host defense mechanisms during the disease.

Paracoccidioidomycosis is a chronic disease caused by the dimorphic fungus *Paracoccidioides brasiliensis*. It is endemic in South and Central America, with the great majority of cases reported in Brazil. The primary pulmonary infection is generally inapparent, and the disease can then disseminate to any organ or system. Involvement of mucous membrane, cutaneous tissue, and lymph nodes is common.

The standardization and purification of *P. brasiliensis* antigens for diagnosis has been very actively researched for several decades (1–3, 6, 12, 14, 15, 23). However, despite the fact that the detection of antigen can provide more-accurate information on mycotic disease activity (11, 17, 20–22), immunological methods for detecting *P. brasiliensis* antigens are still lacking.

Recently, during a serological follow-up of patients treated for paracoccidioidomycosis, we noted that it takes up to 7 years for the serum antibody reaction to turn negative in 75% of successfully treated patients (5). This fact demonstrated the need for alternative methods to assess disease activity, particularly to detect new outbreaks in patients with past history of the disease.

In the present study we report the standardization of an immunoradiometric assay (IRMA) for detection of *P. brasiliensis* circulating antigens that could be useful in the diagnosis and prognosis of paracoccidioidomycosis.

MATERIALS AND METHODS

***P. brasiliensis* antigens.** Yeastlike forms of *P. brasiliensis* strain 192 (obtained from the fungal culture collection of the Department of Microbiology, University of São Paulo, São Paulo, Brazil) were maintained on Sabouraud's glucose agar slants (Difco, Detroit, Mich.) at 35 to 37°C. The following two antigen preparations were used: (i) metabolic antigen obtained from yeast culture concentrated filtrate and (ii) cellular antigen extracted from yeastlike forms. The metabolic antigen was prepared by ultrafiltration (molecular exclusion, 10 kDa; PM 10 membrane [Amicon Corporation, Lexington, Mass.]) of supernatants of 30-day-old *P. brasiliensis* cultures in Sabouraud's glucose liquid medium, as previously described (5). Cellular antigen was obtained by harvesting yeastlike cells during their logarithmic growth phase (10 to 13 days; Sabouraud's glucose agar medium) by centrifugation at 3,000 × *g* for 15 min at 4°C, suspension in a solution consisting of 0.15 M phosphate-buffered saline (PBS) (pH 7.2), 1.0 mM phenylmethylsulfonyl fluoride, and 1 mM *N-p*-tosyl-L-lysine chloromethyl ketone (PBS-PMSF-TLCK), and freeze-drying. The cells were then lyophilized and resuspended in 1:100 (wt/vol) sterilized PBS-PMSF-TLCK, and the solubilization of antigens was done by continuously stirring the solution in the presence of sterile 2-mm-diameter glass beads at 4°C during 24 h. The extract was then centrifuged at 3,000 × *g* for 15 min at 4°C, and the supernatant was finally centrifuged at 12,100 × *g* for 30 min at 4°C and filtered by passage through wet Millipore membranes (pore size, 0.45 and 0.22 μm) for lipid removal and sterilization. This crude cellular soluble antigen was concentrated to 12 mg of protein per ml in an Amicon stir-cell concentrator, using a PM 10 membrane. The protein concentration was measured by the method of Johnstone and Thorpe (10), using PBS-PMSF-TLCK as a blank.

***Histoplasma capsulatum* antigen.** *H. capsulatum*, strain B 679, kindly supplied by L. Ajello from the Communicable Disease Center, Atlanta, Ga., was maintained on Smith's asparagine agar slants at room temperature in the dark. To produce antigen, the cells were grown on Smith's asparagine liquid medium at room temperature for approximately 5 months when thimerosal (1:10,000 [wt/vol]) was added. The mycelium was separated from the culture by passage through filter paper (Whatman no. 1) followed by centrifugation at 3,000 × *g* for 15 min at 4°C. The supernatant was concentrated 20 times by dialysis against Aquacide III (flake polyethylene glycol [Calbiochem/Hoechst, La Jolla, Calif.]) followed by precipitation with cold ethanol overnight at

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−20°C. The protein content was measured by the method of Johnstone and Thorpe (10), using identically processed noninoculated medium as a blank.

***Aspergillus fumigatus* antigen.** Four strains of *A. fumigatus* were isolated from patients with fungus balls and maintained on Sabouraud's glucose agar slants (Difco) at room temperature in our laboratory. The inoculum was prepared from a 4-day-old inoculated Sabouraud's glucose liquid medium. Each strain was grown for a period of 4 weeks. Thimerosal (1:10,000 [wt/vol]) was then added, and the four strains were pooled. The subsequent procedures were similar to those used for the production of *H. capsulatum* antigen.

***Candida albicans* antigen.** *C. albicans* antigen was obtained from serotype A and B strains (kindly supplied by E. Drouhet, Institut Pasteur, Paris, France). The yeast cells were grown in Sabouraud's liquid medium at 37°C for 20 days, and the cells were killed by the addition of thimerosal (1:10,000 [wt/vol]). After 48 h, the yeast cells were separated from the supernatant by centrifugation at 3,000 × *g* for 15 min at 4°C. The subsequent steps were done following the protocol already described for production of *H. capsulatum* antigen.

Rabbit antiserum. Rabbit antiserum to *P. brasiliensis* cellular antigen was raised by injecting rabbits intracutaneously with about 1.5 mg of antigen protein emulsified in Freund's complete adjuvant (Sigma, St. Louis, Mo.). Six weeks later, the rabbits were injected intracutaneously with the same amount of antigen in Freund's incomplete adjuvant. Animals were bled 5 and 7 days after the second injection, and sera were tested by the double immunodiffusion test (DID).

Isolation and labeling of IgG from rabbit antiserum. The procedure for purification of antibodies involves a combination of salt precipitation and ion-exchange chromatography. Briefly, a saturated solution of ammonium sulfate was added, in a final concentration of 33%, to a rabbit anti-*P. brasiliensis* antiserum, previously diluted 1:1 (vol/vol) in PBS, with constant stirring and incubated for 30 min at room temperature. The supernatant was discarded by centrifugation at 30,000 × *g* for 30 min at 25°C, the pellet was redissolved in distilled water, and the resulting solution was dialyzed against 0.07 M sodium phosphate, pH 6.3, for further purification. The ion exchanger (DEAE-cellulose; LKB, Bromma, Sweden) was equilibrated with the phosphate buffer, and immunoglobulin G (IgG) eluted from the starting buffer in a single asymmetrical peak. The protein yield was calculated from the extinction coefficient by A_{280}. The polyclonal antibodies were labeled with $Na^{125}I$ (Amersham Laboratories) using Iodogen (Pierce Chemical Company, Rockford, Ill.) by the method of Fraker et al. (8).

DID. The DIDs were performed in agarose gel (1% in PBS [wt/vol]) by applying 10 μl of the rabbit antiserum or the reference human serum (5, 6) in the central well and by applying serial dilutions of the antigens, in twofold geometric steps, in adjacent wells. This technique was used to assess the antigenicity of the fungous extract, to test the antiserum produced, and to study the antigenic relationship between cellular and metabolic *P. brasiliensis* preparations. All the antigens used were positive in DID against their homologous sera and negative against their heterologous sera using an already described criterion (5, 6). Polyclonal IgG from rabbits and from paracoccidioidomycosis patients were found to react with both cellular and metabolic *P. brasiliensis* antigens.

Two-site IRMA. The IRMA used was adapted from that described by Zavala et al. (24), for the immunodiagnosis of malaria in the invertebrate (*Anopheles*) host. Wells of flexible polyvinylchloride microtiter plates (Dynatech Laboratories, Alexandria, Va.) were incubated overnight at 25°C with a 30-μg/ml solution (30 μl per well) of purified rabbit anti-*P. brasiliensis* IgG antibodies in PBS. The wells were washed three times with PBS and incubated overnight with 150 μl of PBS containing 1% BSA (bovine serum albumin; Sigma) and 0.5 M ethanolamine (Merck, Schuchardt, Germany) at pH 8.6 (PBS-BSA-ETH). The plates could be frozen at −20°C for at least 2 weeks with no change in the results (results not shown). Antigens diluted in a mixture of 6 volumes of a pool of normal human sera and 1 volume of PBS-BSA-ETH containing 0.5% Tween 20 and 0.1% Nonidet P-40 (NP-40) (PBS–BSA–ETH–Tween 20–NP-40) were tested in duplicate. After centrifugation at 8,000 × *g* for 5 min, 30 μl was placed in each well. After incubation for 2 h at room temperature, each well was washed three times with PBS–BSA–ETH–TWEEN 20–NP-40. Then 30 μl (10^6 cpm) of ^{125}I-labeled rabbit IgG anti-*P. brasiliensis* diluted in PBS–BSA–ETH–TWEEN 20–NP-40 was added to each well with 1% goat normal serum to avoid nonspecific binding. After 1 h at room temperature and three washes with PBS–BSA–ETH–TWEEN 20–NP-40, the wells were dried and radioactivity was measured with a gamma counter (Gamma 4000; Beckman). The average counts per minute of 16 undiluted normal human sera ± 2 standard deviations was defined as the normal value.

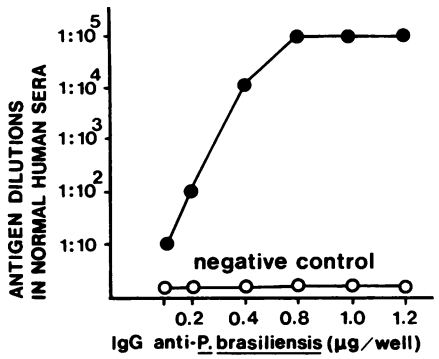

FIG. 1. Determination of optimal antibody concentration to be used in the IRMA for *P. brasiliensis* antigen detection. Each point represents the lowest antigen concentration that can be detected with each concentration of IgG coated to the polyvinylchloride wells.

RESULTS

Binding of cellular *P. brasiliensis* antigen to anti-*P. brasiliensis* polyclonal antibodies. The antigen detection curve with different anti-*P. brasiliensis* antibody concentrations is shown is Fig. 1. The lowest amount of polyclonal antibodies giving positive results with the highest antigen dilution ($1:10^5$, corresponding to 3.6 ng of protein per well) was about 900 ng per well. This concentration of polyclonal antibodies was therefore chosen as the working concentration for subsequent experiments. Negative results were obtained with normal human sera without fungal antigens at all antibody concentrations.

Sensitivity and specificity of IRMA. As shown in Fig. 2, polyclonal IgG raised against yeastlike forms of *P. brasiliensis* (cellular antigen) also binds to the metabolic antigen preparation. Indeed, using a standard patient serum, known

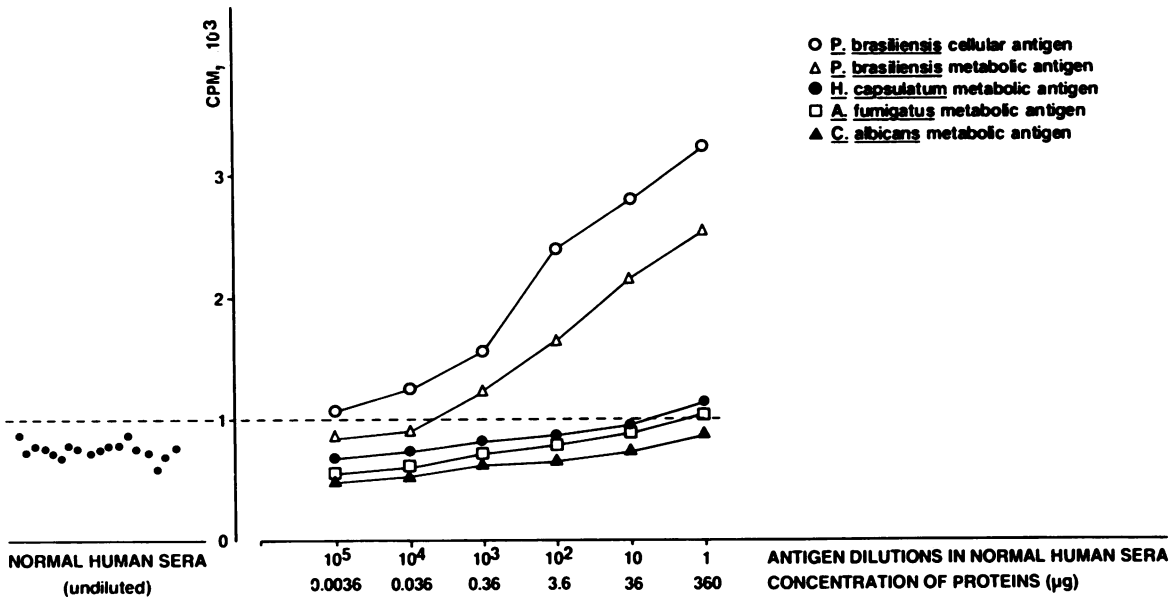

FIG. 2. Reactivity of polyclonal anti-*P. brasiliensis* yeastlike form antibody (IgG) with increasing concentrations of fungal antigens.

to produce three precipitin bands with metabolic antigen, we observed two bands of precipitation common to metabolic and cellular antigens, which were identified as precipitins 1 and 2 (14). As expected, the binding capacity of polyclonal IgG was higher for cellular antigen (limit of sensitivity, 3.6 ng) than for metabolic *P. brasiliensis* antigen (limit of sensitivity, 360 ng). No cross-reaction was observed between *P. brasiliensis* and other fungal antigens except when *H. capsulatum* was tested undiluted (360 μg).

Comparison of the abilities of IRMA and DID to detect *P. brasiliensis* antigens. Serial dilutions of the cellular and metabolic antigen preparations were tested by both IRMA and DID. The IRMA was capable of detecting 3.6 ng (0.12 μg/ml) of cellular *P. brasiliensis* antigen and 360 ng (12 μg/ml) of metabolic antigen (Fig. 2), in contrast to DID for which the limit of resolution was 12 μg (1.2 mg/ml) for both *P. brasiliensis* antigens.

Although variations in counts per minute between different tests were observed, data concerning the prevalence of positive results and sensitivity were reproducible throughout the 11 assays performed.

DISCUSSION

Because other fungal antigens have been shown to present enzymatic activity (18, 19) that could interfere with the enzyme-substrate reaction in the immunoenzymatic assay, we developed an IRMA for the detection of *P. brasiliensis* antigens.

The IRMA described here was shown to be feasible and very sensitive. It allows the estimation of the circulating antigen concentration in human sera by plotting the values obtained against a titration curve of the reference antigen. By using the IgG fraction of a rabbit antiserum in this assay, it was possible to detect 0.12 μg/ml of cellular antigen and 1.2 μg/ml of metabolic antigen. This is in contrast to the sensitivity of DID that could detect either somatic or metabolic antigen only at concentrations higher than 1.2 mg/ml. The yeast cell extract antigen was chosen in preference to the yeast culture concentrated filtrate for antiserum production because (i) the former is free of culture medium, and consequently, an additional stage of purification is not needed, (ii) results obtained with the latter are not always reproducible (1, 12), and (iii) it has already been reported that cytoplasmic antigenic preparations do not present, as do metabolic antigen preparations, the precipitin 3 that cross-reacts with the histoplasmin precipitin M (12). Indeed, our yeast cell extract and metabolic antigens share only *P. brasiliensis*-specific precipitins 1 and 2, as identified by its location by DID (16). This fact can explain the high specificity found in our assay, using antibodies raised by immunization with cellular antigens without impairing the ability of the test in detecting metabolic as well as cellular antigens. The positive criterion used was readings above the mean level plus 2 standard deviations of controls in two tests. Using this criterion, false-positive results could be expected in this test only when 360 μg of *H. capsulatum* antigen was present, but in these cases, the average counts per minute was always close to the borderline region of the reaction. Thus, the assay is 100,000 times more sensitive for *P. brasiliensis* than for *H. capsulatum*. Whether sera from histoplasmosis patients have antigen concentrations so much higher than sera from paracoccidioidomycosis patients has still to be determined. However, if the levels of cross-reacting circulating antigens are higher in histoplasmosis patients, dilution of the tested sera to an antigenic concentration of 36 μg would avoid false-positive results due to cross-reactivity between those antigens. Moreover, since the plates can be stored at −20°C for at least 2 weeks after coating and blocking, the results can be rapidly obtained (4-h period), raising the possibility that this test becomes more useful for diagnosis, particularly for the progressive clinical forms, than the more time-consuming and less-sensitive DID (7).

Recently, Mendes-Giannini et al. (13) identified by using immunoblots a soluble glycoprotein in sera of paracoccidioidomycosis patients. Our preliminary results show that circulating antigens can be detected before treatment of paracoccidioidomycosis patients with the juvenile (2 of 2

patients) and chronic (3 of 3 patients) forms or even during the course of therapy of the chronic form (7 of 16 patients) of the infection (data not shown). The present data and the fact that the detection of circulating antigens can represent the best indicator of recent infection indicate the value of this technique, together with clinical data, in establishing early diagnosis and assessing disease activity.

Finally, we can foresee that the availability of antigenic detection methods in paracoccidioidomycosis will allow the study of relationships between *P. brasiliensis* circulating antigens and host defense mechanisms during the disease (4, 9).

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REFERENCES

1. **Blumer, S. O., M. Jalbert, and L. Kaufman.** 1984. Rapid and reliable method for production of a specific *Paracoccidioides brasiliensis* immunodiffusion test antigen. J. Clin. Microbiol. **19**:404–407.
2. **Burgos, L. C., L. E. Cano, and A. Restrepo.** 1985. Purificacion de antigenos somaticos del *Paracoccidioides brasiliensis*. Estudo preliminar. Rev. Inst. Med. Trop. Sao Paulo **27**:76–81.
3. **Camargo, Z. P., C. Unterkircher, S. P. Campoy, and L. R. Travassos.** 1988. Production of *Paracoccidioides brasiliensis* exoantigens for immunodiffusion tests. J. Clin. Microbiol. **26**: 2147–2151.
4. **Chequer-Bou-Habib, D., M. P. Oliveira-Neto, M. F. Ferreira-da-Cruz, and B. Galvão-Castro.** 1989. The possible role of circulating immune complexes in the deficiency of cell-mediated immunity in paracoccidioidomycosis. Braz. J. Med. Biol. Res. **22**: 205–212.
5. **Ferreira-da-Cruz, M. F., A. C. Francesconi-do-Vale, M. C. D. Espinera, B. Wanke, and B. Galvão-Castro.** 1990. Study of paracoccidioidomycosis antibodies: follow-up of patients during and after treatment. J. Med. Vet. Mycol. **28**:151–157.
6. **Ferreira-da-Cruz, M. F., B. Galvão-Castro, and B. Wanke.** 1985. Produção e padronização dos antigenos de *Paracoccidioides brasiliensis* (Pb), *Histoplasma capsulatum* (Hc) e *Aspergillus fumigatus* (Af) para uso no imunodiagnóstico. Comparação entre as técnicas de imunodifusão e imunoeletroosmoforese. Mem. Inst. Oswaldo Cruz Rio J. **80**:301–305.
7. **Ferreira-da-Cruz, M. F., B. Wanke, and B. Galvão-Castro.** 1987. Prevalence of paracoccidioidomycosis in hospitalized adults in Rio de Janeiro (RJ) Brazil. Mycopathologia **97**:61–64.
8. **Fraker, P. J., and J. C. Speck.** 1978. Protein and cell membrane iodinations with a sparingly soluble chloroamide. Biochem. Biophys. Res. Commun. **80**:849–857.
9. **Franco, M.** 1986. Host-parasite relationships in paracoccidioidomycosis. J. Med. Vet. Mycol. **25**:5–18.
10. **Johnstone, A., and R. Thorpe.** 1988. Immunochemistry in practice, p. 2. Blackwell Scientific Publications, Oxford.
11. **Matthews, R., and J. Burnie.** Diagnosis of systemic candidiasis by an enzyme-linked dot immunobinding assay for a circulating immunodominant 47-kilodalton antigen. J. Clin. Microbiol. **26**: 459–463.
12. **McGowan, K., and H. R. Buckley.** 1985. Preparation and use of cytoplasmic antigens for the serodiagnosis of paracoccidioidomycosis. J. Clin. Microbiol. **22**:39–43.
13. **Mendes-Giannini, M. J. S., J. P. Bueno, M. A. Shikanai-Yasuda, A. Walter-Ferreira, and A. Masuda.** 1990. Detection of the 43,000-molecular-weight glycoprotein in sera of patients with paracoccidioidomycosis. J. Clin. Microbiol. **27**:2842–2845.
14. **Puccia, R., S. Schenkman, P. A. Gorin, and L. R. Travassos.** 1986. Exocellular components of *Paracoccidioides brasiliensis*: identification of a specific antigen. Infect. Immun. **53**:199–206.
15. **Restrepo, A., L. E. Cano, and M. T. Ochoa.** 1985. A yeast-derived antigen from *Paracoccidioides brasiliensis* useful for serologic testing. Sabouraudia J. Med. Vet. Mycol. **23**:23–29.
16. **Restrepo, A., and L. H. Moncada.** 1974. Characterization of the precipitin bands detected in the immunodiffusion test for paracoccidioidomycosis. Appl. Microbiol. **28**:138–144.
17. **Snow, R. M., and W. E. Dismukes.** 1975. Cryptococcal meningitis: diagnostic value of cryptococcal antigen in cerebrospinal fluid. Arch. Intern. Med. **135**:1155–1157.
18. **Tran Van Ky, P., J. Uriel, and F. Rose.** 1970. Caractérisation des types d'activites enzymatiques dans les extraits antigeniques d'*Aspergillus fumigatus* après electrophorese en agarose. Ann. Inst. Pasteur **3**:162–170.
19. **Tran Van Ky, P., T. Vaucelle, S. Andrieu, C. Torck, and F. Floch.** 1969. Caractérisation de complexes enzymes-antienzymes dans les extraits du genre *Candida* après immunoelectrophoregramme en agarose. Mycopathologia **38**:345–357.
20. **Weiner, M. H., and M. Coats-Stephens.** 1979. Immunodiagnosis of systemic aspergillosis. I. Antigenemia detected by radioimmunoassay in experimental infection. J. Lab. Clin. Med. **93**: 111–119.
21. **Weiner, M. H., G. H. Talbot, S. L. Gerson, G. Felice, and P. A. Cassileth.** 1983. Antigen detection in the diagnosis of invasive aspergillosis: utility in controlled, blinded trials. Ann. Intern. Med. **99**:777–782.
22. **Wheat, L. J., R. B. Kohler, and R. P. Tewari.** 1986. Diagnosis of disseminated histoplasmosis by detection of *Histoplasma capsulatum* antigen in serum and urine specimens. N. Engl. J. Med. **314**:83–88.
23. **Yarzabal, L. A., D. Bout, F. Naquira, J. Fruit, and S. Andrieu.** 1977. Identification of the specific antigen of *Paracoccidioides brasiliensis* responsible for immunoelectrophoretic band E. Sabouraudia J. Med. Vet. Mycol. **15**:79–85.
24. **Zavala, F., R. W. Gwadz, F. H. Collins, R. S. Nussenzweig, and V. Nussenzweig.** 1982. Monoclonal antibodies to circumsporozoite proteins identify the species of malaria parasite in infected mosquitoes. Nature (London) **299**:737–738.