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Monoclonal Antibody Capture Enzyme Immunoassay for Detection of *Paracoccidioides brasiliensis* Antibodies in Paracoccidioidomycosis

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Four murine monoclonal antibodies (MAbs 17C, 21A, 21F, and 32B) raised against the 43-kDa glycoprotein of *Paracoccidioides brasiliensis* were tested in a capture enzyme immunoassay (EIA) for the detection of specific human anti-gp43 immunoglobulin G in patients with paracoccidioidomycosis (PCM). All MAbs reacted similarly in the assay. These MAbs, which detected anti-gp43 at levels of as low as 500 pg/ml, were demonstrated to specifically recognize at least two different epitopes in gp43 binding assays. Specific antibodies in the sera of patients with active PCM were detected at dilutions of as high as 1:819,200, and the reactivities of patient sera, as measured by optical densities, were found to be significantly higher than those of control sera. The comparison between classical ELISA and our capture enzyme immunoassay showed that both sensitivity and specificity were greatly improved by the latter. These MAbs represent the first specific reagents to *P. brasiliensis* described for use in serological tests for PCM.

Almost all South and Central American countries have large regions where paracoccidioidomycosis (PCM) is endemic. The disease, a systemic mycosis, is caused by the thermally dimorphic fungus *Paracoccidioides brasiliensis*. Although neither the habitat nor the exact mode of transmission to humans is exactly known, the infection is believed to occur by inhalation of airborne propagules present in the environment. The infection is thought to take place in the lungs and eventually disseminate via the bloodstream and/or the lymphatics to all parts of the body (12). Two main clinical forms of the disease are recognized: the acute or subacute form (juvenile type) and the chronic form (adult type) (12). The acute form affects both sexes and involves mainly the reticuloendothelial system, whereas the chronic form, which is prevalent in adult males, is a chronic disease with predominant pulmonary and/or mucocutaneous involvement. A definitive diagnosis of PCM includes direct observation of the characteristic multiple-budding cells in biological fluids and tissue sections or isolation of the fungus from clinical materials. For cases in which *P. brasiliensis* is not observed through direct examination, several serological tests have been used to detect antibodies against the fungus in order to establish the diagnosis. Among others, the most common serological assays are immunodiffusion (7), counterimmunoelectrophoresis (10), and complement fixation (11), but indirect immunofluorescence (13), enzyme-linked immunosorbent assay (ELISA) (5, 9, 15), magnetic-ELISA (6), passive hemagglutination (19), and immunoblotting (3, 8) are also used by some laboratories. To date, no serological method for the diagnosis of PCM has been made available commercially. Several fungi present antigenic similarities, resulting in serological cross-reactions, mainly *P. brasiliensis*, *Loboa loboi*, and *Histoplasma capsulatum* (5, 8, 9, 15). Immunoenzymatic assays of sera from patients with PCM have been tested by various groups (8, 9, 15), who are in agreement that the observed cross-reactions pose difficulties for the diagnosis of the mycosis. Puccia et al. (18) purified one specific antigen of *P. brasiliensis*, a glycoprotein of 43 kDa (gp43), which was shown to be specific in tests such as immunodiffusion but not in ELISA. Those investigators showed that more than 85% of the reactions of sera from patients with PCM with gp43 involved peptidic epitopes. They also found that cross-reactions with sera from patients with Jorge Lobo's disease and sera from patients with histoplasmosis by ELISA were predominantly attributed to periodate-sensitive carbohydrate epitopes containing galactosyl residues.

In the study presented here we describe a capture enzyme immunoassay (EIA) technique in which monoclonal antibodies (MAbs) raised against gp43 were used for the first time as specific reagents in the diagnosis of PCM and as a means to control the cross-reactions seen with heterologous patient sera.

MATERIALS AND METHODS

Serum specimens. Individual serum specimens from 30 patients with PCM were selected on the basis of a positive direct examination of histopathological or other biological fluids (30 serum specimens) plus immunodiffusion tests (28 positive and 2 negative serum specimens). Heterologous sera (8 from patients with histoplasmosis, 10 from patients with Jorge Lobo's disease, 9 from patients with aspergillosis, and 10 from patients with candidiasis; all were selected after positive cultures) and 25 serum specimens from healthy individuals were also tested. All patient sera came from Hospital São Paulo, Escola Paulista de Medicina. All serum specimens were divided into aliquots and were stored at −20°C.

Antigenic preparations. (i) gp43 antigen purification. *P. brasiliensis* B-339, grown in liquid medium similar to the Universal Beer Agar (GIBCO Laboratories), was used as a source of antigen. *P. brasiliensis* yeast cells from Sabouraud agar slants were grown in 50 ml of this medium for 3 days at 35°C with shaking. In order to obtain larger antigenic volumes, these cultures served as inocula for the final 500-ml cultures

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which were incubated at 35°C for 7 days with shaking. The supernatants of 550-ml cultures killed with merthiolate (0.2 g/liter) were collected following paper filtration, concentrated under vacuum at 45°C, and dialyzed against three changes of 5 liters each of distilled water. Purification of gp43 was performed by affinity chromatography in Affi-gel (Bio-Rad) coupled with anti-gp43 MAb 17C columns (see below). gp43 was eluted with 50 mM citrate buffer (pH 2.8) and was concentrated in an Amicon 10K cell. Antigen purification was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then silver staining (1).

(ii) Heterologous antigen preparations. *H. capsulatum* and *Aspergillus fumigatus* were cultivated similarly at room temperature for 7 days. Exoantigens were obtained as described above for *P. brasiliensis*. Antigens from *Candida albicans* were prepared as follows. Cells grown in Sabouraud broth at room temperature for 7 days were harvested and washed twice in phosphate-buffered saline (PBS). Phenylmethylsulfonyl fluoride (1 mM; Sigma) was added to the cells, which were put in an ice bath and then sonicated in a Thornton-Inpec-Electrônica S.A. (São Paulo) device with 20 pulses of 1 min each at 90 cycles per second at 5-min intervals. The sonicated product was then centrifuged at 10,000 × *g* for 30 min at 4°C, and the supernatant was used as the antigen. The protein contents of all antigen preparations were measured by the method described by Bradford (4).

Hyperimmune polyclonal antibodies. Hyperimmune anti-gp43 sera were obtained by immunization of rabbits with gp43 as described previously (18). Anti-gp43 immunoglobulin G (IgG) was obtained by affinity chromatography of the hyperimmune sera passed through a protein A-Sepharose column (Pharmacia) after dilution (vol/vol) with Tris-HCl buffer (pH 8.2). The column was washed with the same buffer until the optical density at 280 nm was close to zero. Elution was then carried out with 0.1 M citrate buffer (pH 2.8) and then immediate neutralization with 1 M Tris (pH 9.0).

Anti-gp43 MAbs. MAbs were previously produced in our laboratory by Puccia and Travassos (17). Briefly, BALB/c mice were immunized with 50 μg of purified gp43 per ml every 15 days; the gp43 was incorporated in complete Freund's adjuvant for the first injection and in incomplete Freund's adjuvant for the subsequent injections. Fusion of spleen cells from BALB/c mice with SP-2.Ag14 myeloma cells was performed as described by Lopes and Alves (14). For the large-scale production of MAbs, hybridoma cells (2×10^6 cells per mouse) were injected intraperitoneally into BALB/c mice primed with Pristane (Sigma). MAbs were purified from ascites in Sepharose-protein A columns. MAb concentrations were estimated spectrophotometrically at 280 nm. From the 13 stable clones of MAbs obtained, four produced stronger reactions against the purified antigen and were selected for the present study: 17C, 32B, 21A, and 21F.

Epitope characterization of anti-gp43 MAbs. MAb 17C was conjugated with peroxidase type VI (Sigma) (2). EIA plates (Costar) were coated overnight with 2 μg of purified gp43 per ml in PBS (50 μg per well) at 4°C. The remaining binding sites were blocked with PBS–1% bovine serum albumin for 1 h at room temperature. The wells were then incubated with both MAb 17C–peroxidase (1 μg/ml) and MAbs 17C, 32B, 21A, and 21F, which were all used as inhibitors at 10, 1, 0.1, and 0.01 μg/ml, at 37°C for 2 h and were washed three times with PBS–0.5% gelatin–0.05% Tween 20. Reactions were developed by adding 50 μl of *o*-phenylenediamine hydrochloride solution (Sigma) in 0.1 M citrate-phosphate buffer (pH 5.0) and were blocked with 4 N H_2SO_4 (50 μl per well). The optical densities were measured in a Multiskan MCC/340 II EIA reader at 492 nm.

Specificities of anti-gp43 MAbs. The specificities of the anti-gp43 MAbs were determined by immunoblotting assays with the *P. brasiliensis*, *H. capsulatum*, *A. fumigatus*, and *C. albicans* antigens as described previously (3, 7). Briefly, 50 μg of protein of each antigen was submitted to electrophoresis by SDS-PAGE, with subsequent transfer to a nitrocellulose membrane (Bio-Rad). After blocking for 1 h with PBS containing 0.1% Tween 20 (PBS-T) and 5% nonfat dry milk (PBS-T-M), the membranes were sliced and incubated at 37°C with MAbs dissolved in 10 μg of antibody per ml in PBS-T containing 0.25% gelatin (Difco) (PBS-T-G) for 1 h, washed with PBS-T, and incubated again with goat anti-mouse IgG-peroxidase (Bio-Rad) at a 1:3,000 dilution in PBS-T-G. After washes with PBS-T, the strips were incubated in 15 mg of 4-chloro-1-naphthol (Sigma) in 25 ml of 50 mM Tris (pH 6.8)–50 μl of 30% H_2O_2. The reaction was interrupted after 5 min with distilled water.

Inhibition of EIA reactions by Sugars. D-(+)-galactose, α-methylmannopyranoside, mannose, and *N*-acetylglucosamine (Sigma) at 25 mM (final concentration in PBS [pH 7.2]) were sugars used as inhibitors in the assay. EIA was performed following the protocol described below for the capture EIA.

MAb effectiveness in binding gp43. Wells of microtiter plates (Costar) were incubated with MAbs (20 μg/ml diluted in PBS-T-G; 100 μl per well) for 1 h at 37°C. The remaining binding sites were blocked with PBS-T-M for 2 h at 37°C. The wells were washed three times with PBS-T and were incubated with gp43 at different concentrations (1,000 ng/ml to 250 pg/ml) for 1 h at 37°C. To assess the binding of gp43 to the MAb-coated plate, 100 μl of rabbit polyclonal anti-gp43 IgG (10 μg of PBS-T-G per ml) was added to each well. Plates were incubated for 1 h at 37°C and were then washed three times with PBS-T. Goat anti-rabbit IgG (100 μl) labeled with peroxidase and diluted 1:1,000 (Sigma) in PBS-T-G was added to each well. After incubation for 1 h at 37°C and three washes with PBS-T, 100 μl of substrate solution (5 mg of *o*-phenylenediamine in 25 ml of 0.1 M citrate-phosphate buffer [pH 5.0] plus 10 μl of 30% H_2O_2) was added to each well, and the reaction was interrupted by the addition of 50 μl of 2 N H_2SO_4. The optical densities were read in a Multiskan MCC/340 II EIA reader at 492 nm.

ELISA. Enzyme-linked immunosorbent assay (ELISA) was performed as described previously (5) by using the purified gp43 as the coating antigen (2.5 μg/ml).

Capture EIA. To optimize the EIA system for the specific and sensitive detection of human antibodies, combinations of a capture antibody and a detector antibody were examined by checkerboard titration. Four MAbs (MAbs 17C, 21A, 32B, and 21F) were tested for their effectiveness in capturing gp43 and detecting human anti-gp43 IgG. Capture MAbs were screened at four different concentrations (10, 15, 20, and 30 μg/ml). This led to standardization of the capture assay. In our system MAb 17C at 20 μg/ml was used thereafter. With the standardized assay, immunoplates (Costar) were coated with 20 μg of MAb 17C per ml (100 μl per well) in 0.1 M carbonate buffer (pH 9.6) for 1 h at 37°C. The plates were washed three times with PBS-T, the remaining sites were blocked with PBS-T-M for 2 h at 37°C, and the blocking agent was removed and incubated with gp43 (300 ng of PBS per ml; 100 μl per well) for 1 h at 37°C. After washing three times with PBS-T, human serum specimens (dilutions ranging from 1:800 to 1:819,200 in PBS-T-G) were added and the plates were incubated for 1 h at 37°C. The wells were washed as described above, treated for 1 h at 37°C with goat anti-human IgG-peroxidase (dilution, 1:1,000;

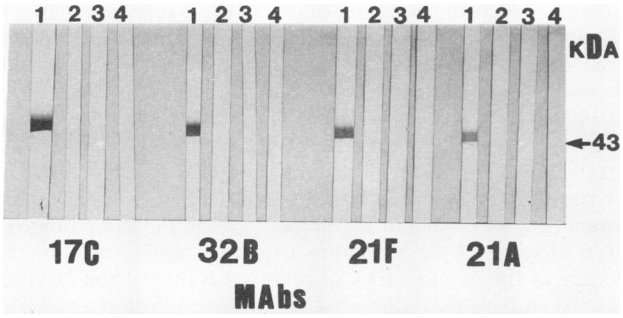

FIG. 1. Immunoblots showing the reactivities of MAbs 17C, 21A, 21F, and 32B against antigens from *P. brasiliensis* (lane 1), *H. capsulatum* (lane 2), *A. fumigatus* (lane 3), and *C. albicans* (lane 4). All MAbs specifically recognized gp43 from the *P. brasiliensis* antigen.

Sigma), and washed again. Color development was performed and optical density readings were taken as described above.

Statistical analysis. All data were analyzed by analysis of variance ($P < 0.05$).

RESULTS

Characterization of anti-gp43 MAbs. From a number of different hybridomas producing antibodies against gp43, four stable clones producing specific antibodies against gp43 at high titers were selected for further studies. By using the Immunoselect kit (GIBCO-BRL, Gaithersburg, Md.) the isotypes of these four MAbs (MAbs 17C, 21A, 32B, and 21F) were determined to be IgG2a,κ (MAb 17C) and IgG2b,κ (MAbs 21A, 32B, and 21F). The specificities of the MAbs for gp43 were checked by ELISA and Western blotting (immunoblotting). By the latter method, all MAbs tested recognized only the 43-kDa component of *P. brasiliensis* B-339 and did not recognize any antigenic component of *H. capsulatum*, *C. albicans*, or *A. fumigatus* (Fig. 1). These MAbs are probably directed against the peptidic epitopes of gp43, since sugars such as D-(+)-galactose, α-methylmannopyranoside, mannose, and *N*-acetylglucosamine were not inhibitory in this assay (data not shown). As seen in inhibition tests, these MAbs recognized different gp43 epitopes; MAbs 32B and 17C seemed to recognize the same or similar epitopes, while MAbs 21F and 21A recognized different ones (Fig. 2). Each MAb was similarly effective in detecting gp43 in the EIA capture format at levels of as low as 500 pg/ml.

Detection of specific human anti-gp43 IgG antibodies. In comparing the antibody capture EIA method with the classical ELISA method (Fig. 3), both methods detected specific anti-gp43 antibodies in all 30 serum specimens from patients with PCM. However, the capture method proved to be a significantly more sensitive and specific test. The arithmetical mean titers by the EIA capture method were approximately 20-fold higher (1:549,093 versus 1:26,960) than those by ELISA in detecting PCM gp43 antibodies. Both techniques were capable of distinguishing PCM antibodies from those formed in response to other fungal infections and from healthy controls.

DISCUSSION

Improved serological tests for PCM are of considerable interest because of the well-known limitations of the presently used serological assays. The most difficult limitation that must be overcome is cross-reactions of the *P. brasiliensis* antigen with antigens from other pathogenic fungi. Recent progress in

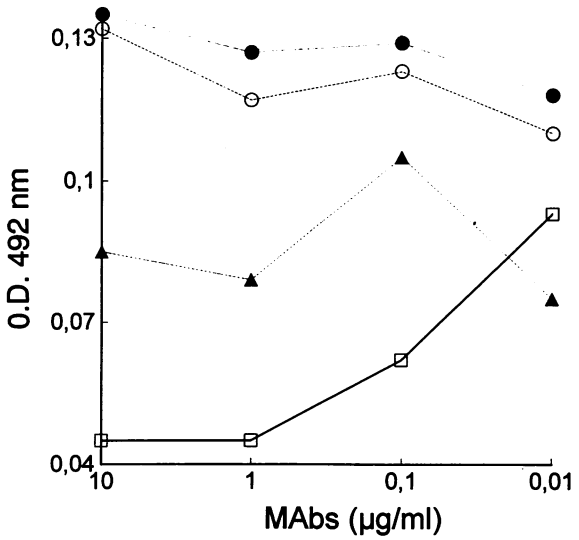

FIG. 2. Epitope characterization of anti-gp43 MAbs by using MAb 17C conjugated with peroxidase (1 µg/ml). Plates coated with 100 ng of gp43 were incubated with MAb 17C conjugated with peroxidase (1 µg/ml) in the presence of various amounts (10, 1, 0.1, and 0.01 µg/ml) of MAbs 21A (●), 21F (○), 32B (▲), and 17C (□). The binding of MAb 17C conjugated with peroxidase was unaffected by MAbs 21A, 21F, and 32B at all concentrations, while the addition of MAb 17C showed a typical inhibition curve. The results are presented as the average of an assay made with triplicates. O.D., optical density.

the purification and characterization of *P. brasiliensis* antigens has prompted the investigation of their application as diagnostic tools, with the gp43 glycoprotein appearing to be the main diagnostic antigen of *P. brasiliensis* (7, 18). This glycoprotein can be purified from culture supernatant by affinity chromatography with Affi-gel 10 (Bio-Rad) coupled to rabbit anti-gp43 IgG or mouse anti-gp43 MAbs. Recently, we confirmed the specificity of gp43 for the diagnosis of PCM by immunodiffusion and Western blotting (7, 8, 20). However, we have also observed that sera from patients with mycoses other than PCM also reacted with the affinity-purified gp43 in ELISAs (20). Puccia and Travassos (16) showed that cross-reactions with sera from patients with histoplasmosis and Jorge Lobo's disease in ELISA were predominantly attributed to periodate-sensitive carbohydrate epitopes containing galactosyl residues. Their studies showed that sera from patients with PCM primarily recognize gp43 peptidic epitopes and are independent of carbohydrate epitopes. However, for some sera from patients with PCM, the carbohydrate epitopes accounted for up to 45% of the total reactivity observed in the ELISA system. These findings were supported by the fact that α-methylmannopyranoside, a galactose analog, was the strongest inhibitor of those reactions.

MAbs are useful tools for diagnostic purposes since they are raised against specific epitopes. The MAbs described herein (MAbs 17C, 21A, 21F, and 32B) are the first species-specific MAbs directed against the *P. brasiliensis* gp43 component. Since these reactions were not inhibited by sugars, it appears that they are directed toward peptidic epitopes of the gp43 molecule. Western blot analyses demonstrated that these MAbs also do not significantly recognize antigenic components of *H. capsulatum*, *A. fumigatus*, or *C. albicans*, pointing to their applicability in immunodiagnostic tests. Although all MAbs were able to detect gp43 at levels as low as 500 pg/ml, MAb

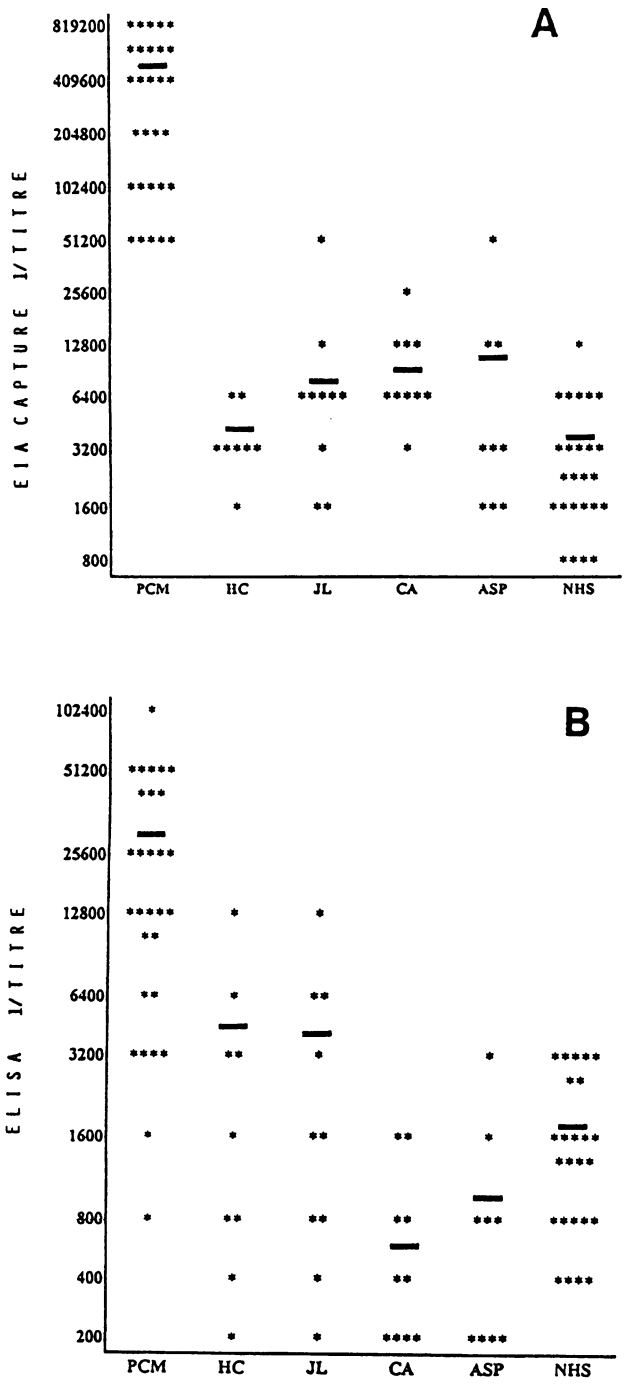

FIG. 3. Results for the different clinical groups for anti-*P. brasiliensis* gp43 IgG by capture EIA (A) and classical ELISA (B). PCM, paracoccidioidomycosis; HC, histoplasmosis; JL, Jorge Lobo's disease; CA, *C. albicans*; ASP, aspergillosis; NHS, normal human sera. Crossbars represent the means of the titers.

17C showed a greater degree of sensitivity in the capture EIA and was selected for use.

In the capture EIA the endpoint of the reactivity of sera from patients with PCM was at a 1:819,200 dilution, with a mean of 1:459,093, while in the heterologous system the maximal reactivity was obtained with the *L. loboi* system (1:51,200), with a mean of 1:10,240. On the other hand, in the classical ELISA the endpoint of reactivity of sera from patients with PCM was at a 1:102,800 dilution, with a mean of 1:26,960, while in the heterologous system the maximal reactivity was obtained with sera from patients with Jorge Lobo's disease and histoplasmosis, with means of 1:3,420 and 1:3,700, respectively. Two of 30 serum specimens from patients with PCM were negative by the immunodiffusion test, but they were positive by the capture EIA (1:51,200); in the classical ELISA their titers were as low as 1:800 and 1:1,600, which is in the same range of those of some of the heterologous sera. These data show the efficacy of the capture EIA in relation to that of the classical ELISA and also to that of the immunodiffusion test. The sensitivity of the capture EIA for PCM was 100%, with 96.7% specificity. The results of the present study of the capture EIA are in agreement those presented in our previous report (3, 8), in which all patients with PCM had anti-gp43 IgG that was detectable by Western blotting.

To date, interpretation of the results of the EIA for PCM by using polyclonal antibodies and unpurified antigens is difficult because of cross-reaction problems. Sera must be previously adsorbed with *H. capsulatum* or *C. albicans* antigens before testing to eliminate those unspecific reactions (5, 15). The capture EIA described herein is a new and sensitive method that can detect minimal amounts of specific antibodies in the sera of patients with PCM. In addition, the supply of MAbs, once established, is unlimited, making quality control of the EIA system easier than that for assays that use polyclonal antibodies. In conclusion, we established a capture EIA system that uses MAbs for the specific detection of anti-*P. brasiliensis* gp43 IgG from the sera of patients with PCM. This procedure can be widely used for diagnostic purposes and can be automated for use in the routine laboratory.

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REFERENCES

1. **Ansorge, W.** 1983. Fast visualization of protein bands by impregnation in potassium permaganate and silver nitrate, p. 235–242. *In* D. Stahakos (ed.), Electrophoresis 82. Walter de Gruyter, Berlin.
2. **Avrameas, S., T. Ternynck, and J. L. Guesdon.** 1978. Coupling of enzymes to antibodies and antigens. Scand. J. Immunol. **7**:7–23.
3. **Blotta, M. H. S. L., and Z. P. Camargo.** 1993. Immunological response to cell-free antigens of *Paracoccidioides brasiliensis*: relationship with clinical forms. J. Clin. Microbiol. **31**:671–676.
4. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Anal. Biochem. **72**:248–259.
5. **Camargo, Z. P., J. L. Guesdon, E. Drouhet, and L. Improvisi.** 1984. Enzyme-linked immunosorbent assay (ELISA) in the paracoccidioidomycosis. Comparison with counterimmunoelectrophoresis and erythroimmunoassay. Mycopathologia **88**:31–37.
6. **Camargo, Z. P., J. L. Guesdon, E. Drouhet, and L. Improvisi.** 1984. Magnetic enzyme-linked immunosorbent assay (MELISA) for determination of specific IgG in paracoccidioidomycosis. Sabouraudia J. Med. Vet. Mycol. **22**:291–299.
7. **Camargo, Z. P., C. Unterkircher, S. Campoy, and L. R. Travassos.** 1988. Production of *Paracoccidioides brasiliensis* exoantigens for immunodiffusion tests. J. Clin. Microbiol. **26**:2147–2151.
8. **Camargo, Z. P., C. Unterkircher, and L. R. Travassos.** 1989. Identification of antigenic polipeptides of *Paracoccidioides brasiliensis* by immunoblotting. J. Med. Vet. Mycol. **27**:407–412.
9. **Cano, L. E., Brummer, D. A. Stevens, and A. Restrepo.** 1986. An evaluation of the enzyme-linked immunosorbent assay (ELISA) for quantification of antibodies to *Paracoccidioides brasiliensis*. J. Med. Vet. Mycol. **24**:467–475.

10. **Conti-Diaz, I. A., J. E. Mackinnon, L. Calegari, and S. Casserone.** 1978. Estudio comparativo de la inmunoelectroosmophoresis-inmunodiffusion (IEOF-ID) y de la inmunoelectrophoresis (IEF) en el diagnostico de la paracoccidioidomycosis. Mycopathologia **63:**161–165.
11. **Fava Netto, C.** 1972. The serology of paracoccidioidomycosis: present and future trends. Paracoccidioidomycosis, p. 209–213. Proceedings of the First Pan American Symposium. PAHO-WHO, Medellin, Colombia.
12. **Franco, M.** 1987. Host-parasite relationship in paracoccidioidomycosis. J. Clin. Microbiol. **25:**5–18.
13. **Franco, M., C. Fava Netto, and L. G. Chamma.** 1973. Reação da reação e comparação dos resultados com a reação de fixação do complemento. Rev. Inst. Med. Trop. São Paulo **15:**393–398.
14. **Lopes, J. D., and M. J. M. Alves.** 1983. Production of monoclonal antibodies by somatic cell hybridization, p. 386–400. *In* C. M. Morel (ed.), Genes and parasites. A laboratory manual. Fundação Oswaldo Cruz, Rio de Janeiro, Brazil.
15. **Mendes-Giannini, M. J. S., M. E. Camargo, C. S. Lacaz, and A. W. Pereira.** 1984. Immunoenzymatic adsorption test for serodiagnosis of paracoccidioidomycosis. J. Clin. Microbiol. **29:**103–108.
16. **Puccia, R., and L. R. Travassos.** 1991. 43-Kilodalton glycoprotein from *Paracoccidioides brasiliensis*: immunochemical reactions with sera from patients with paracoccidioidomycosis, histoplasmosis, or Jorge Lobo's disease. J. Clin. Microbiol. **29:**1610–1615.
17. **Puccia, R., and L. R. Travassos.** 1991. The 43-kDa glycoprotein from the human pathogen *Paracoccidioides brasiliensis* and its deglycosylated form: excretion and susceptibility to proteolysis. Arch. Biochem. Biophys. **289:**298–302.
18. **Puccia, R. S., S. Schenckman, P. A. J. Gorin, and L. R. Travassos.** 1986. Exocellular components of *Paracoccidioides brasiliensis*: identification of a specific antigen. Infect. Immun. **53:**199–206.
19. **Taborda, C. P., and Z. P. Camargo.** 1993. Diagnosis of paracoccidioidomycosis by passive haemagglutination assay of antibody using a purified and specific antigen-gp43. J. Med. Vet. Mycol. **31:**155–160.
20. **Unterkircher, C. S.** 1988. Antígenos de *Paracoccidioides brasiliensis* e sua aplicação no diagnóstico sorológico da paracoccidioidomicose. Ph.D. thesis. Escola Paulista de Medicina, São Paulo, São Paulo, Brasil.