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Diagnosis of Neuroparacoccidioidomycosis by Detection of Circulating Antigen and Antibody in Cerebrospinal Fluid

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Neuroparacoccidioidomycosis (neuroPCM) is the central nervous system infection by the fungus *Paracoccidioides brasiliensis*. Its diagnosis is a difficult task that depends on neuroimaging techniques such as computed tomography and magnetic resonance imaging. However, the detection of circulating *P. brasiliensis* antigens in body fluids by inhibition enzyme-linked immunosorbent assay (inh-ELISA) has provided encouraging results. In this study, 14 cerebrospinal fluid (CSF) and 11 serum samples of patients with neuroPCM were analyzed by inh-ELISA for detection of circulating glycoprotein antigens of 43 kDa (gp43) and 70 kDa (gp70). Circulating gp43 and gp70 antigens were detected in all CSF samples from patients with neuroPCM at mean concentrations of 19.3 and 6.8 μg/ml, respectively. In addition, both gp43 and gp70 antigens were detected in 10 of 11 serum samples analyzed at mean concentrations of 4.6 and 4.0 μg/ml, respectively. By immunodiffusion test, CSF samples were determined to be negative in 13 of 14 samples. The detection of anti-gp43 and anti-gp70 antibodies by conventional ELISA showed positive results for all CSF samples, with titers ranging from 1:50 to 1:51,200. Therefore, the high sensitivity of the inh-ELISA technique in detecting gp43 and gp70 antigens in the CSF of neuroPCM patients strongly indicates that this assay can be considered as a powerful diagnostic tool. In addition, the finding of anti-gp43 and anti-gp70 antibodies in CSF samples by conventional ELISA also seems to be a promising diagnostic method for this special modality of PCM.

Paracoccidioidomycosis (PCM), a disease caused by *Paracoccidioides brasiliensis*, particularly affects the poorer portions of the population in Latin American countries, with a high incidence in Brazil, where the disease is endemic. PCM predominantly affects 30- to 50-year-old adult males. It is uncommon in childhood and relatively rare near puberty (3, 9). There are a wide spectrum of clinical manifestations, ranging from mild pulmonary lesions to severe disseminated forms involving many organs, especially the mucosae, skin, lymph nodes, adrenals, and the central nervous system (CNS) (9). The primary site of infection, which is often not apparent, is assumed to be the lung. After being inhaled, *P. brasiliensis* causes a benign and transient pulmonary infection that may be reactivated to cause chronic disease (5, 11, 12). The involvement of the CNS may occur more frequently than has been reported (21).

The involvement of the CNS in PCM, neuroparacoccidioidomycosis (neuroPCM), is secondary to the hematogenous dissemination of the fungus. NeuroPCM usually occurs as a manifestation of widely disseminated disease but, occasionally, CNS may be the sole location of the infection. Whenever the CNS is involved, neuro-radiological methods such as computed tomography (CT) and magnetic resonance imaging (MRI) are needed for the identification of expansive lesions. NeuroPCM is usually represented by multiple or solitary parenchymal lesions that lead to sensory or motor deficits, seizures, changes in mental status, and intracranial hypertension (10, 22). However, the definitive diagnosis of neuroPCM may only be obtained after visualization of the fungus in biopsy material, fungal isolation by culture, or by serological methods.

Considering the morbidity associated with the invasive neurologic procedures, clinicians are not prone to recommend aspiration or biopsy of CNS lesions. Therefore, sensitive and specific immunodiagnostic assays to determine the presence of *P. brasiliensis* in cerebrospinal fluid (CSF) specimens are indispensable. Recently, Marques da Silva et al. (15–17) described an antigen detection assay (the inhibition enzyme-linked immunosorbent assay [inh-ELISA]) for the gp43 and gp70 molecules of *P. brasiliensis* that has a good potential for the diagnosis and follow up of patients with PCM. The detection of *P. brasiliensis* antigens in body fluids might facilitate the early diagnosis of PCM, including patients with cerebral lesions.

In the present study, gp43 and gp70 antigens of *P. brasiliensis* were detected in CSF and serum samples from patients with neuroPCM by using an inh-ELISA, and the results were compared to those obtained for anti-*P. brasiliensis* antibodies detected by immunodiffusion (ID) and conventional ELISA tests.

MATERIALS AND METHODS

Patients and samples. CSF and serum samples were obtained from 14 patients with neuroPCM whose selection was based on the clinical, serological and tomographic findings. The patients enrolled in the present study were taken from Hospital São Paulo, São Paulo Federal University, and Hospital das Clínicas da Universidade Estadual de Campinas, Campinas, São Paulo, Brazil. All patients were male with a mean age of 45 years. The diagnosis of systemic PCM was established in all of the patients based on the visualization and/or culture of the fungus in clinical specimens. The neurological involvement was ascertained based on clinical and tomographic findings, as well as on the clinical response to

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TABLE 1. Serological results of 14 patients with neuroPCM and control groups evaluated by inh-ELISA, ID, and ELISA

Subject	CSF antigen concn (μg/ml)[a]		CSF titer (ELISA)[b]		Serum antigen concn (μg/ml)[a]		Antibody titer (ID)	
	gp43	gp70	Anti-gp43	Anti-gp70	gp43	gp70	CSF	Serum
Case 1	19.5	6.75	+ (1:1,600)	+ (1:1,600)	NA[d]	NA	NR[c]	NA
Case 2	24.0	6.75	+ (1:400)	+ (1:50)	NA	NA	NR	NA
Case 3	0.46	0.43	+ (1:200)	+ (1:50)	NA	NA	NR	NA
Case 4	3.75	1.83	+ (1:50)	+ (1:50)	2.85	1.03	NR	NR
Case 5	21.0	6.75	+ (1:6,400)	+ (1:400)	5.27	1.49	NR	1:8
Case 6	18.0	4.16	+ (1:200)	+ (1:50)	5.27	2.49	NR	1:4
Case 7	22.5	3.39	+ (1:50)	+ (1:50)	0	1.03	NR	NA
Case 8	13.5	2.67	+ (1:50)	+ (1:50)	1.21	0	NR	NA
Case 9	16.5	8.25	+ (1:50)	+ (1:50)	4.16	1.77	NR	+NDS[e]
Case 10	19.5	7.5	+ (1:200)	+ (1:50)	1.03	11.25	NR	+NDS
Case 11	30.0	16.5	+ (1:200)	+ (1:50)	4.16	1.68	NR	NR
Case 12	30.0	13.5	+ (1:50)	+ (1:50)	7.12	2.49	NR	NR
Case 13	25.5	5.64	+ (1:50)	+ (1:50)	12.75	8.25	NR	NR
Case 14	25.5	6.38	+ (1:51,200)	+ (1:50)	6.75	11.25	1:16	1:16
Mean	19.3	6.8			4.6	4.0		
Control group A[g]	0	0	(−)[f]	(−)				
Control group B[h]					0	0	NR	NR

[a] Antigen detection by inh-ELISA.
[b] Antibody detection by conventional ELISA.
[c] NR, nonreactive.
[d] NA, not available.
[e] +NDS, positive with nondiluted sera.
[f] (−), negative.
[g] Ten CSF samples from noninfectious neurologic diseases were negative for antigens and antibodies (control patients).
[h] Thirty serum samples from healthy volunteers were negative for antigens and antibodies (control patients).

antifungal treatment. All patients had simultaneous involvement of other organs from which clinical specimens confirmed the diagnosis of PCM. A lumbar puncture was performed in all cases as part of the routine medical assistance at those institutions. In addition, serum samples from 11 patients were also requested to investigate the etiology of the neuroinfection. Control groups included 10 CSF samples from patients with noninfectious neurologic diseases and 30 serum samples from healthy volunteers (blood donors). The study was evaluated and approved by the Ethics Committee of our institution.

Fungal isolates, antigen preparations, and gp70/gp43 purifications. *P. brasiliensis* B-339 (ATCC 200273) and Pb 113 were obtained from the culture collection of the Disciplina de Biologia Celular, Universidade Federal de São Paulo. The isolates were transformed to the yeast phase, and exoantigen was produced according to the method of Camargo et al. (7, 8). Gp43 was purified from the Pb B-339 crude exoantigen as described elsewhere (23), and gp70 was purified from Pb 113 cytoplasmic antigen as described previously (17). Protein content was determined by the Bradford method (4).

MAb production. Monoclonal antibody (MAb) anti-gp43 was a gift from R. Puccia (23), and MAb anti-gp70 was prepared as described by Mattos Grosso et al. (18).

Pretreatment of CSF and sera for inh-ELISA. Aliquots of CSF and serum (200 μl) were mixed with an equal volume of 0.1 M EDTA (Sigma; pH 7.2) and boiled at 100°C for 5 min. After cooling, tubes were centrifuged at 13,000 × *g* for 30 min, and the supernatants were used for the test.

Detection of circulating gp43 and gp70 antigens by inh-ELISA. inh-ELISA was performed as previously described (13, 15). First, a standard inhibition curve was prepared by adding known concentrations of gp43 or gp70 to a pool of normal human serum or CSF controls in different plates (inhibition standards) (13, 15). The inhibition reaction occurred when constant aliquots of MAbs anti-gp43 or anti-gp70 were mixed with the inhibition standards, PCM patient sera, or CSF samples and normal human serum and CSF control samples. Samples were then plated on previously blocked microtiter plate (inhibition plate) and incubated overnight at 4°C. Reaction plate was coated with gp43 or gp70 and incubated overnight at 4°C. Free sites on plastic were blocked with 5% skim milk in phosphate-buffered saline (PBS)–Tween 20, and samples from each well in the inhibition plate (containing a mixture of MAb [anti-gp43 or anti-gp70] bound to circulating antigen and free MAb) were transferred to the respective wells in the reaction plate. The plate was washed, probed with goat anti-mouse immunoglobulin G-peroxidase conjugate, and developed with a chromogenic substrate as previously described (13, 15). Optical density (OD) readings at 492 nm were then plotted on a standard curve constructed from the data derived from MAb titration with the inhibition standards. The antigen concentrations in the patient's CSF and serum samples were calculated with a regression model constructed with the reciprocal values of fixed concentrations of gp43 or gp70 and the OD values. All tests were made in duplicates. The cutoff point was established by the receiver operator characteristic (ROC) curve.

ID test. ID tests were performed with CSF and serum samples at the moment of diagnosis of neuroPCM as previously described (7).

ELISA. Microtiter plates (Costar) were coated with 100 μl of purified gp43 or gp70 (500 ng/well), diluted in 0.06 M carbonate-bicarbonate buffer (pH 9.6), and incubated overnight at 4°C. The plates were washed three times with PBS-Tween 20 (0.05%) and free sites were blocked with 5% skim milk in PBS-Tween 20 (200 μl/well) for 2 h at 37°C. After three washes, 100 μl of CSF samples (1:50 to 1:51,200 dilution) of each patient was added to each well, and the plates were incubated at 37°C for 1 h. Plates were then washed again; 100 μl of peroxidase-labeled goat anti-human immunoglobulin G (1:1,000; Sigma) was added to each well, and the plates were incubated for 1 h at 37°C. After three washes, the reaction was developed by the addition 100 μl of a mixture of *o*-phenylenediamine (0.2 mg/ml; Sigma) and 0.05% (vol/vol) H_2O_2. After 5 min of incubation in the dark, the reaction was stopped with 50 μl of 4 N H_2SO_2. The OD was measured at 492 nm by using an ELISA Microplate reader (Titertek Multiskan MCC/340).

Statistical analysis. Inhibition standard curves were performed in duplicates for at least four independent assays. The data were statistically analyzed by the Stata 7.0 version for Windows 98/95/NT (Stata Corp., College Station, TX), and the specificity and sensitivity were determined by using the ROC.

RESULTS

Detection of *P. brasiliensis* gp43 and gp70 antigens in CSF by inh-ELISA. A standard inhibition curve was prepared, and the cutoff values of 0.23 μg/ml for gp43 and 0.21 μg/ml for gp70 were determined by using the ROC curve. CSF samples with concentrations above these values were considered as positive. gp43 and gp70 were detected in all 14 CSF samples (100%). The mean antigen concentration for gp43 (19.3 μg/ml) was found to be significantly higher than that for gp70 (6.8 μg/ml [*P*

< 0.001]). CSF samples from individuals with diseases other than PCM had undetectable antigen levels (Table 1).

Detection of gp43 and gp70 antigens in sera samples by inh-ELISA. A standard inhibition curve was prepared, and the cutoff values of 0.23 μg/ml for gp43 and 0.97 μg/ml for gp70 were determined by using the ROC curve. Samples with concentrations greater than these values were considered positive. gp43 and gp70 antigens were detected in 10 of 11 patient sera. Despite some differences in the mean concentrations of gp43 and gp70 in serum samples, they were not statistically significant ($P = 0.65$). The mean concentration of gp43 was always higher than gp70 in CSF and serum samples.

Detection of antibodies by ID and ELISA. Only 1 CSF sample (7.2%) of 14 was positive by ID using *P. brasiliensis* crude exoantigen, at an 1:16 titer (7.2%). Five of eleven (45.5%) sera were found to be positive by ID, with antibody titers ranging from 1:4 to 1:16; for two of those sera, however, positive reactions were obtained only with undiluted samples. On the other hand, 14 of 14 CSF samples (100%) had anti-gp43 and anti-gp70 antibodies, with titers ranging from 1:50 to 1:51,200 by ELISA (Table 1).

DISCUSSION

Diagnosis of neuroPCM is a difficult task and depends on imaging techniques besides mycologic and serological findings. The reported frequency of CNS involvement in PCM is extremely variable depending on the approach that defines the cases. In necropsy studies, neuroPCM has been found in 9.65 to 27.18% of the PCM cases. Most of the neuroPCM patients present simultaneous involvement of other organs, but isolated CNS involvement may also occur (21). The accurate diagnosis of neuroPCM is obtained by the detection or isolation of the fungus in samples taken from cerebral lesions or in CSF (1, 2, 21, 22). Usually, classical CSF mycologic examinations (microscopy and culture) generate negative results, and tissue samples are requested to check for the presence of fungal components. A recent report by Pereyra (22) evaluated the clinical, radiologic, and laboratory findings of 13 cases of neuroPCM in which the authors detected gp43 in tissue samples of all patients by immunofluorescence (22). However, considering the morbidity related to invasive procedures necessary to obtain a brain biopsy, an accurate serological tool based on specific detection of antigen or antibodies against the fungus is clearly necessary to improve the diagnosis of neuroPCM. In this regard, Almeida et al. detected specific anti-gp43 antibody in eight of nine samples of CSF obtained from patients with neuroPCM (1).

Usually, ID is the method of choice for antibody detection in PCM sera, with a positivity reaching 97.1% by using a 7-day crude exoantigen preparation, in which gp43 is the predominant molecule, representing 80 to 85% of the total antigenic composition (7). An antigen preparation containing large amounts of gp43 is essential for a definitive diagnosis by ID, and its sensitivity can be compared to purified gp43 preparations. Serologic negative results may be observed even when cells of *P. brasiliensis* are found in biological materials; in these cases, they may be related to low-avidity immunoglobulin G2 antibodies directed against carbohydrate epitopes (20). ELISA tests may help in the diagnosis of neuroPCM; however, probably due to the high sensitivity of the method, cross-reactions may occur, mainly with histoplasmosis sera (19). On the other hand, ELISA is very useful for monitoring PCM patients under antimycotic therapy. The detection of circulating *P. brasiliensis* antigens (gp43 and gp70) in body fluids by inh-ELISA has recently shown promising results in patients with PCM (13–17).

In the present study, circulating gp43 and gp70 were detected in all CSF samples from patients with neuroPCM, with mean antigen concentrations of 19.3 and 6.8 μg/ml, respectively. gp43 and gp70 were also detected in 10 (91%) serum samples of 11 samples obtained from 14 patients, with mean antigen concentrations of 4.6 and 4.0 μg/ml, respectively. Moreover, the antigen concentrations were found considerably higher in CSF than in sera, suggesting their local production, and the concentration of gp43 was always higher in relation to gp70.

Our results are in agreement with those of Almeida et al. (1), who found only one patient (7.1%) to be positive for anti-*P. brasiliensis* antibodies in CSF by ID test in their study. ELISA, a more sensitive test, detected anti-gp43 and anti-gp70 antibodies in all (100%) CSF samples, confirming that ELISA is a better test for that purpose. Thus, search for anti-gp43 and anti-gp70 antibodies in CSF samples by ELISA seems promising as a diagnostic method for neuroPCM.

Our results suggest that monitoring specific antigens of *P. brasiliensis* may be helpful to define the diagnosis of neuroPCM. It was found that both gp43 and gp70 were detectable in almost all of the serum samples, with small differences in their mean antigen concentrations (4.6 and 4.0 μg/ml, respectively). Of note, the CSF gp43 antigen concentrations were substantially higher than the gp70 concentrations (19.3 versus 6.8 [$P < 0.001$]). On the other hand, antibody detection was negative when tested by ID but was positive against both antigens when tested by ELISA, despite being at low titers. These data, from the limited number of patients studied, allow us to assume that the detection of antigen by inh-ELISA or the detection of specific antibody by conventional ELISA may be equally sensitive for identifying neuroPCM. Since antigen values for gp43 were always higher than those found for gp70, assaying only for gp43 may prove sufficient for that purpose.

Detection of antibody by conventional ELISA, as a routine laboratory work, is a less cumbersome and time-consuming procedure than the detection of antigen. Hence, testing CSF samples for antigens would be recommended only when a suspected patient presents either negative or inconsistent results for antibody detection.

In light of the scarce clinical and radiological information available about the enrolled patients, no correlation could be ascertained between antigen levels and severity of the disease. Also, further studies are necessary to validate the potential usefulness of antigen detection for monitoring the patients' response to antifungal therapy.

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REFERENCES

1. **Almeida, S. M., F. Queiroz-Telles, E. M. Doi, M. Ono, and L. C. Werneck.** 2002. Anti-gp43 antibodies in the cerebrospinal fluid of patients with central nervous system involvement by paracoccidioidomycosis. Am. J. Clin. Pathol. **118:**864–868.
2. **Almeida, S. M., F. Queiroz-Telles, H. A. G. Teive, C. E. L. Ribeiro, and L. C. Werneck.** 2004. Central nervous system paracoccidioidomycosis: clinical features and laboratory findings. J. Infect. **48:**193–198.
3. **Blotta, M. H. S. L., R. L. Mamoni, S. J. Oliveira, A. S. Nouér, P. M. O. Papaiordanou, A. Gouveia, and Z. P. Camargo.** 1999. Endemic regions of paracoccidioidomycosis in Brazil: a clinical and epidemiologic study of 584 cases in southeast region. Am. J. Trop. Med. Hyg. **61:**390–394.
4. **Bradford, M.** 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
5. **Brummer, E., E. Castañeda, and A. Restrepo.** 1993. Paracoccidioidomycosis: an update. Clin. Microb. Rev. **2:**89–117.
6. **Bueno, J. P., M. J. S. Mendes-Giannini, G. M. Del Negro, G. M. Assis, C. K. Takiguti, and M. A. Shikanai-Yasuda.** 1997. IgG, IgM, and IgA antibody response for the diagnosis and follow-up of paracoccidioidomycosis: comparison of counterimmunoelectrophoresis and complement fixation. J. Med. Vet. Mycol. **35:**213–217.
7. **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.
8. **Camargo, Z. P., R. Berzaghi, C. C. Amaral, and S. H. Marques da Silva.** 2003. Simplified method for producing *Paracoccidioides brasiliensis* exoantigens for use in immunodiffusion tests. Med. Mycol. **41:**539–542.
9. **Del Negro, G., C. S. Lacaz, V. A. Zamith, and A. M. Siqueira.** 1994. General clinical aspects: polar forms of paracoccidioidomycosis, the disease in childhood, p. 225–232. *In* M. Franco, C. S. Lacaz, A. Restrepo-Moreno, and G. Del Negro (ed.), Paracoccidioidomycosis. CRC Press, Inc., Boca Raton, Fla.
10. **Finamor, L. P., C. Muccioli, M. C. Martins, L. V. Rizzo, and R. Belfort, Jr.**2002. Ocular and central nervous system paracoccidioidomycosis in a pregnant woman with acquired immunodeficiency syndrome. Am. J. Ophthalmol. **134:**456–459.
11. **Franco, M., M. R. Montenegro, R. P. Mendes, S. A. Marques, N. L. Dillon, and N. G. S. Mota.** 1987. Paracoccidioidomycosis: a recently proposed classification on its clinical forms. Rev. Soc. Bras. Med. Trop. **20:**129–132.
12. **Franco, M., M. T. S. Peraçoli, A. M. V. C. Soares, M. R. Montenegro, R. P. Mendes, and D. A. Meira.** 1993. Host-parasite relationships in paracoccidioidomycosis. Curr. Trop. Med. Mycol. **5:**115–149.
13. **Gómez, B. L., J. I. Figueroa, A. J. Hamilton, B. Ortiz, M. A. Robledo, R. J. Hay, and A. Restrepo.** 1997. Use of monoclonal antibodies in diagnosis of paracoccidioidomycosis: new strategies for detection of circulating antigens. J. Clin. Microbiol. **35:**3278–3283.
14. **Gómez. B.L., J. I. Figueroa, A. J. Hamilton, B. I. Ortiz, M. A. Tobon, R. J. Hay, and A. Restrepo.** 1998. Antigenemia in patients with paracoccidioidomycosis: detection of the 87-kilodalton determinant during and after antifungal therapy. J. Clin. Microbiol. **36:**3309–3316.
15. **Marques da Silva, S. H., A. L. Colombo, M. H. S. L. Blotta, J. D. Lopes, F. Queiroz-Telles, and Z. P. Camargo.** 2003. Detection of circulating gp43 antigen in serum, cerebrospinal fluid and bronchoalveolar lavage of patients with paracoccidioidomycosis. J. Clin. Microbiol. **41:**3675–3680.
16. **Marques da Silva, S. H., F. Queiroz-Telles, A. L. Colombo, M. H. S. L. Blotta, J. D. Lopes, and Z. P. Camargo.** 2004. Monitoring of paracoccidioidomycosis patients by gp43 antigenemia. J. Clin. Microbiol. **42:**2419–2424.
17. **Marques da Silva, S. H., D. Mattos Grosso, J. D. Lopes, A. L. Colombo, M. H. S. L. Blotta, F. Queiroz-Telles, and Z. P. Camargo.** 2004. Detection of *Paracoccidioides brasiliensis* gp70 circulating antigen and follow up of patients undergoing antimycotic therapy. J. Clin. Microbiol. **42:**4480–4486.
18. **Mattos Grosso, D., S. R. Almeida, M. Mariano, and J. D. Lopes.** 2003. Characterization of gp70 and anti-gp70 monoclonal antibodies in *Paracoccidioides brasiliensis* pathogenesis. Infect. Immun. **71:**6534–6542.
19. **Mendes-Giannini, M. J. S., M. E. Camargo, C. S. Lacaz, and A. W. Ferreira.** 1984. Immunoenzymatic absorption test for serodiagnosis of paracoccidioidomycosis. J. Clin. Microbiol. **20:**103–108.
20. **Neves, A. R., R. L. Mamoni, C. L. Rossi, Z. P. Camargo, and M. H. S. L. Blotta.** 2003. Negative immunodiffusion test results obtained with sera of paracoccidioidomycosis patients may be related to low-avidity immunoglobulin G2 antibodies directed against carbohydrate epitopes. Clin. Diagn. Lab. Immunol. **10:**802–807.
21. **Nóbrega, J. P. S., and A. Spina-França Netto.** 1994. Neuroparacoccidioidomycosis, p. 321–330. *In* M. Franco, C. S. Lacaz, A. Restrepo-Moreno, and G. Del Negro (ed.), Paracoccidioidomycosis. CRC Press, Inc., Boca Raton, Fla.
22. **Pereyra, W. J. F.** 2001. Central nervous system paracoccidioidomycosis: analysis of 13 cases with immunofluorescence research of gp43 antigen. Arq. Neuropsiquiatr. **59:**981–984.
23. **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.