Medical Mycology 2001, **39**, 423–428 Accepted 27 November 2000

Levels of specific antigen (gp43), specific antibodies, and antigen–antibody complexes in saliva and serum of paracoccidioidomycosis patients

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The present study analyses human immunoglobulin G (IgG) antibodies directed against the *Paracoccidioides brasiliensis* exoantigen, gp43, as well as the presence of gp43–IgG immune complexes (ICs) in 31 samples of saliva and serum from 19 patients with paracoccidioidomycosis (PCM) and 12 normal donors. Additional analysis of secretory IgA (sIgA) was performed on the same saliva samples. Consistent with previous findings, a significant increased specific IgG level was observed in PCM patients' saliva and serum ($P<0{\cdot}05$). The analysis of serum gp43 and gp43–IgG IC demonstrated a higher level in patients with PCM ($P<0{\cdot}05$); however, this difference was not statistically significant with regard to gp43 and gp43–IgG in saliva when compared to the healthy donors. A high level of sIgA in saliva of PCM patients compared to that of normal donors was also observed ($P<0{\cdot}05$). Patients exhibiting low levels of serum IgG but with high titres of IC were observed, thus strengthening the idea of the necessity to use more than one marker for diagnosis and treatment monitoring of PCM. This is the first report of sIgA in PCM patients' saliva and may be indicative of a protective role in neutralizing antigens on mucosal surfaces.

Keywords gp43, immune complexes, *Paracoccidioides brasiliensis*, paracoccidioidomycosis

Introduction

Paracoccidioidomycosis (PCM) is one of the most important systemic mycoses in Latin America. It is especially prevalent in Brazil. The causal agent is *Paracoccidioides brasiliensis*, a thermally dimorphic human pathogen [1]. The disease is thought to be acquired when airborne propagules produced by *P. brasiliensis* saprobic phase are inhaled into the host's lungs, where they convert to the yeast form [2]. The infection can cause overt disease involving many body sites including the lymph nodes, the skin and the oral and rectal mucosa [3]. PCM can be diagnosed by direct observation of the characteristic multiple-budding yeast forms in clinical specimens or by culture [4]. More frequently, diagnosis is inferred from indirect evidence obtained via serological procedures, mainly immunodiffusion and immunoenzymatic assays [5,6]. *P. brasiliensis* exoantigens have been widely used to detect specific antibodies *in vitro* [6]. Puccia *et al.* [7] purified the specific *P. brasiliensis* antigen gp43, a glycoprotein with a molecular mass of 43 kDa. In all cases of PCM studied, anti-gp43 antibodies [8], as well as specific IgG, IgM and IgA antibodies to gp43 have been detected [9]. The classical enzyme immunoassay (ELISA) and capture enzyme immunoassay (EIA) both detect specific anti-gp43 antibodies. However, the capture method using monoclonal antibody against gp43 proved to be a significantly more sensitive and specific test [10]. Both techniques were capable of distinguishing anti-*P. brasiliensis* antibodies from those formed in response to other

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fungal infections and from immunoglobulins obtained from healthy controls [10].

Decreases in the antibody response to gp43 during therapy indicate that gp43 is a good marker for follow-up of patients with acute or chronic paracoccidioidomycosis [11].

The production of anti-gp43 antibodies is related to the severity of the disease [12], as are levels of circulating antigen [13] and circulating immune complexes (IC) [14]. There is, however, a lack of available data about soluble antigens, IC and secretory immunoglobulin A (sIgA) antibodies in PCM patients' saliva. The present research was directed toward analysing gp43, gp43–IgG IC and sIgA directed against *P. brasiliensis* exoantigens in saliva of patients with PCM. Additionally, we determined levels of anti-*P. brasiliensis* IgG in sera and saliva and of gp43 and IC in patients' sera.

Material and methods

This study was approved by the Internal Scientific Commission and the Bioethics in Research Committee of the State University of Londrina, Paraná, Brazil.

Serum and saliva samples

Serum and saliva samples were obtained from 19 patients with the chronic form of PCM. Of these patients, ten had unifocal lesions (five oropharyngeal including laryngeal, four pulmonary and one intestinal) and nine had multifocal lesions. All were receiving antifungal treatment (3 for less than 1 year, 4 for less than 2 years, and 12 for more than 2 years) from the Clinical Hospital of the State University of Londrina. Sera and saliva from 12 healthy control subjects were obtained from blood donors at the Hematology Institute of Londrina. Both groups consisted entirely of persons between 30 and 59 years of age. Informed consent was obtained from all subjects. Saliva was collected without exogenous stimulation, then clarified by centrifugation at 400*g* for 1 min. All serum and saliva specimens were divided into aliquots and stored at −20 °C.

P. brasiliensis *exoantigen*

A lyophilized exoantigen was prepared from a yeast-phase culture of *P. brasiliensis* strain B-339 according to Camargo *et al*. [6].

ELISA for antibodies

P. brasiliensis exoantigen (1 mg ml^{-1} dry weight) was diluted in carbonate bicarbonate buffer (Na_2CO_3 1·59 g, $NaHCO_3$ 2·93 g, distilled water qsp 1000 ml, pH 9·6), then used to coat immunoplates (100 µl $well^{-1}$) for 1 h at 37 °C and then overnight at 4 °C. The plates were washed with phosphate-buffered saline containing 0·05% Tween 20 (PBS-T), blocked with PBS-T 5% skim milk for 1 h at 37 °C, and then incubated for 1 h with sera (1/400) or saliva (1/10) diluted in PBS-T. The threshold was previously determined by titration of positive and negative serum samples. The plates were washed with PBS-T (5×) and incubated with mouse anti-human IgG labelled with peroxidase (100 µl $well^{-1}$, 1/1700 dilution) and 100 µl $well^{-1}$ of substrate solution was added (5 mg ortho-phenylenediamine, 10 ml of 0·1 M citrate buffer, pH 4·5 and 10 µl H_2O_2). The reaction was stopped with 50 µl of 4 N H_2SO_4 per well and the absorbance was read in a Multiskan EX reader (Labsystems, Helsinki, Finland) at 492 nm. For salivary sIgA determination after incubation with saliva samples, mouse anti-human secretory component (1/10 000; Sigma Chemical Co., St. Louis, Missouri, USA) and anti-mouse IgG labelled with peroxidase were employed.

Monoclonal antibodies anti-gp43

Anti-gp43 monoclonal antibodies (Mab) [10] (provided by Dr José D. Lopes, Department of Immunology, Federal University of São Paulo, SP, Brazil) were purified from ascitic fluids in a Sepharose-protein G column (Sigma). The protein concentration was estimated using the Folin phenol method [15].

gp43 purification

Purified gp43 was obtained by affinity chromatography of the crude exoantigen of *P. brasiliensis* B-339 on Affigel-10 (Bio-Rad Laboratories, Hercules, CA, USA) with anti-gp43 Mab as a specific ligand. The gp43 was eluted with 0·1 M glycine–HCl, pH 2·8, and immediately neutralized with 2 M Tris, pH 9·0. Fractions (1·0 ml) were collected and read in a spectrophotometer at 280 nm. The fractions with the highest absorbance were mixed. The resulting pool was dialysed against 0·15 M PBS, and the protein concentration was determined.

Preparation of anti-gp43 rabbit IgG

The purified *P. brasiliensis* gp43 (200 µg) was subcutaneously injected into rabbits. The first immunization was performed with complete Freund's adjuvant (CFA; 800 µl CFA and 800 µl gp43) and the second and third with incomplete Freund's adjuvant (IFA) at successive intervals of two weeks. After bleeding, the hyperimmune serum was passed through a Sepharose-protein G column and the protein concentration was determined using the Folin phenol method with bovine serum albumin as a standard.

ELISA for antigenemia determination

Immunoplates were coated with 1·2 μg ml^{-1} of rabbit anti-gp43 IgG in carbonate bicarbonate buffer (pH 9·6) for 1 h at 37 °C and then overnight at 4 °C. After blocking and washing, the plates were incubated with sera or saliva (1/10 in PBS), for 1 h at 37 °C and then with anti-gp43 Mab (300 ng ml^{-1}) for 1 h at 37 °C. Plates were washed and incubated with anti-mouse IgG labelled with peroxidase (1/18 000 in PBS). Processing was then done as described in the previous section.

Immune complexes determination

Plates were sensitized with anti-gp43 Mab at a concentration of 20 μg ml^{-1} and then sera or saliva was added, followed by peroxidase conjugated anti-immunoglobulin (sIgA or IgG). Processing was then carried out as described in the previous section.

Statistical analysis

Data were analysed statistically using Statistica for Windows Release 4, 3D, StatSoft, Inc. Tulsa, USA 1993 software; the *t* test for independent samples was considered significant if $P<0·05$.

Results

ELISA analysis of serum and salivary IgG reacting with P. brasiliensis *exoantigens*

The results of ELISA expressed as optical densities (OD) were higher in PCM patients' serum ($0·192 \pm 0·088$) than in normal serum ($0·092 \pm 0·036$), $P<0·05$. ODs were also higher in PCM patients' saliva ($0·165 \pm 0·086$) than in normal saliva ($0·069 \pm 0·014$); $P<0·05$ (Fig. 1). Fourteen of 19 patients had positive serum IgG, while eighteen of 19 had positive salivary IgG.

Antigenemia

Soluble gp43 levels measured as OD in ELISA were significantly increased in patients' sera ($0·84 \pm 0·013$ vs. $0·146 \pm 0·083$ for controls, $P<0·05$) but not in saliva ($0·171 \pm 0·23$ vs. $0·167 \pm 0·021$ for controls, $P>0·05$) (Fig. 2). All the PCM cases exhibited positive antigenemia in serum, but the three cases exhibiting antigens in saliva all had buccal lesions noted prior to treatment, although not during the salivary sample collection.

Circulating immune complexes

Circulating IC in patients' serum, read as OD, were also increased ($0·609 \pm 0·191$) as compared to controls ($0·260 \pm 0·96$), $P<0·05$. Salivary immune complexes were slightly higher in PCM patients ($0·199 \pm 0·249$) than in controls ($0·149 \pm 0·112$), but this difference was not statistically significant ($P>0·05$) (Fig. 3).

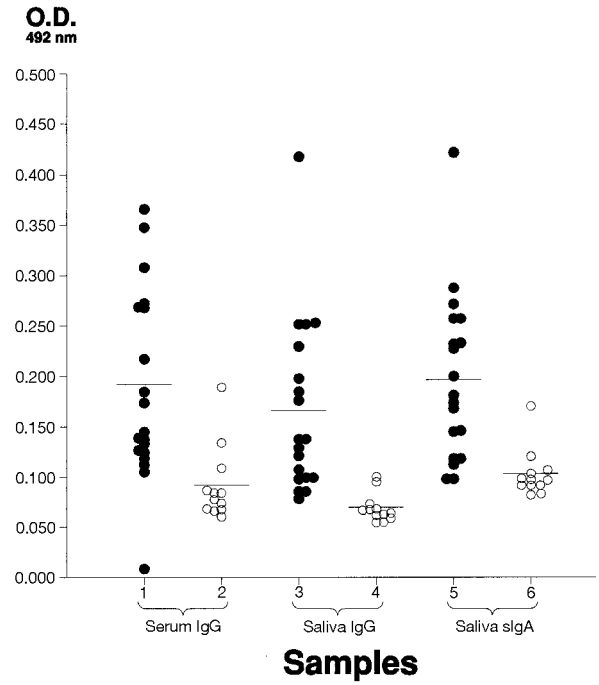

Fig. 1 Levels of specific serum IgG, salivary IgG and secretory IgA (sIgA) antibodies reacting with *Paracoccidioides brasiliensis* exoantigens in paracoccidioidomycosis (PCM) patients (●) and normal donors (○), in optical densities (OD). ELISA plates coated with *P. brasiliensis* exoantigens were incubated with serum (1/400) or saliva (1/10) diluted in phosphate-buffered saline containing 0·05% Tween 20 (PBS-T). Plates were washed, incubated with peroxidase-conjugated anti-human IgG (1/18 000 in PBS-T) for IgG study or with mouse anti-secretory component of IgA (1/10 000) and then with peroxidase-conjugated anti-mouse IgG labelled with peroxidase for sIgA. Orthophenylenediamine solution was added, and the reactions were read at 492 nm. Results are expressed in optical densities (OD) at 492 nm and horizontal bars represent mean values. (1) PCM patients' serum IgG; (2) normal human serum IgG; (3) PCM patients' salivary IgG; (4) normal salivary IgG; (5) PCM patients' salivary sIgA; (6) normal salivary sIgA. (1, 2) $P<0·05$; (3, 4): $P<0·05$; (5, 6): $P<0·05$.

Salivary sIgA

Salivary sIgA values were significantly higher in patients ($0·196 \pm 0·082$) than controls ($0·102 \pm 0·024$), $P<0·001$. OD ranged from 0·097 to 0·420. Fourteen of 19 patients were positive reactors (Fig. 1).

Discussion

As in previous findings [9], our results demonstrate an elevated anti-*P. brasiliensis* IgG antibody level in PCM patients' sera. Sera of 14/19 patients showed positive

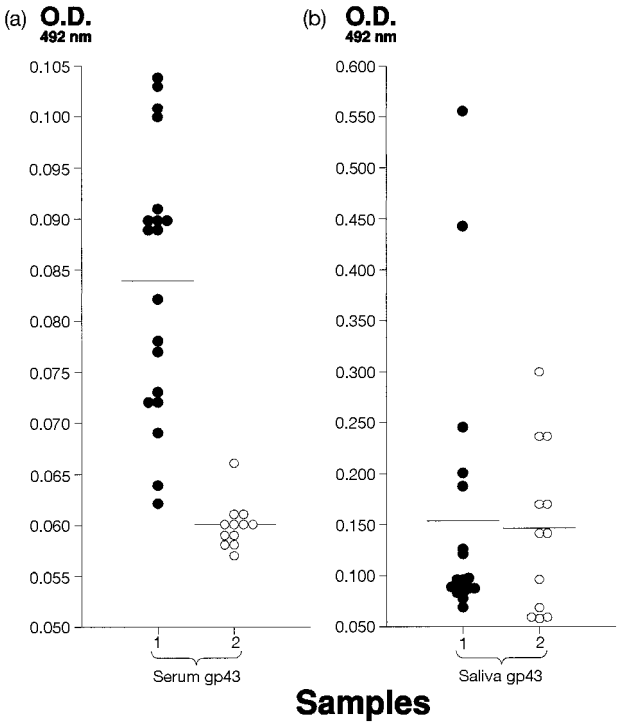

Fig. 2 Levels of serum (a) and salivary (b) free gp43 antigenemia, in PCM patients (●) and normal donors (○), in optical densities (OD). ELISA plates coated with rabbit IgG anti gp43 were incubated with serum or saliva diluted 1/10 in PBS-T. Plates were washed, incubated with mouse monoclonal antibodies (Mab) anti-gp43 and then with peroxidase-conjugated anti-mouse IgG. Orthophenylenediamine solution was added, and the reactions were read at 492 nm. Results are expressed in OD and horizontal bars represent mean values. (a) (1) PCM patients'serum; (2) normal serum ($P<0.05$). (b) (1) PCM patients' saliva; (2) normal saliva ($P>0.05$).

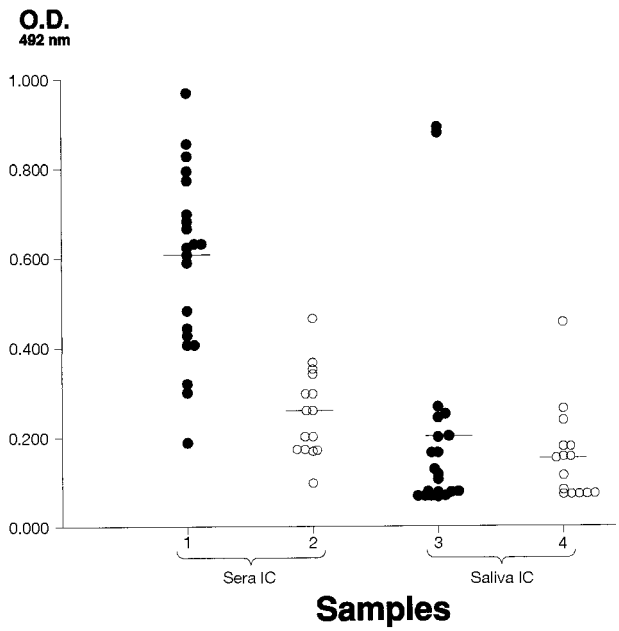

Fig. 3 Levels of serum and salivary immune complexes (IC), in PCM patients (●) and normal donors (○), in optical densities (OD). ELISA plates coated with Mab anti-gp43 were incubated with serum or saliva diluted 1/10 in PBS-T. Plates were washed, incubated with peroxidase-conjugated anti-mouse IgG. Orthophenylenediamine solution was added, and the reactions were read at 492 nm. Results are expressed in OD and horizontal bars represent mean values. (1) PCM patients' serum IC; (2) normal serum IC; (3) PCM patients' salivary IC; (4) normal salivary IC. (1, 2) $P<0{\cdot}05$; (3, 4) $P>0{\cdot}05$.

results by classical ELISA. The high threshold titre could be due to some positive reactors in the normal controls, who were from endemic areas [16]. The serum gp43 level was also significantly different between PCM patients and controls.

Circulating IC, consisting of two polypeptides with apparent masses of 43 and 62 kDa, were previously demonstrated through western blot assay in PCM patients' serum [17]. The present study showed increased amounts of circulating IC containing gp43 molecules in sera. In this study, patients had active chronic type PCM and showed response to treatment, so that serum IgG titres may have decreased with the death of *P. brasiliensis.* This may have liberated soluble Ag and consequently increased the levels of circulating IC.

IC were detected in 17/19 (89%) patients, whereas specific IgG was detected in only 13/19 (68%). In five of the cases with negative IgG, IC levels appeared to be elevated. This suggested that the imunoglobulins produced were complexed with gp43, and that only low levels of free specific IgG were present in serum. It would thus seem prudent to use more than one marker for routine serodiagnosis and monitoring of treatment.

Anemia is a common complication of PCM, and is similar to that seen in other chronic inflammatory conditions. It may be mild to moderate in intensity. Antifungal drugs such as sulphonamides, ketoconazole, and amphotericin B are also associated with anemia [18,19]. Erythrocytes have an important role in binding circulating IC and carrying these complexes to the liver and spleen [20]. The reduction of the level of erythrocytes could hinder the system in removing circulating IC.

The lungs are the usual portal of entry of *P. brasiliensis*. The infecting inoculum could stimulate the mucosally associated lymphoid tissue and induce the production of sIgA to *P. brasiliensis*. sIgA can efficiently eliminate microorganisms in the intestinal lumen or in the mucus-rich environment of other mucosal surfaces [21]. The activated plasmocytes producing the IgA may migrate to other regions such as salivary glands [22].

The saliva has an important role in protecting the mouth against endogenous microorganisms. It inhibits microbial growth and adherence, neutralizes toxins and

agglutinates foreign cells, and flushes them to the gut. Inhibition of adhesion is a major function of sIgA in saliva [23].

Because antigen–sIgA complexes are transported across M-cells, specialized cells in the epithelia over organized mucosa-associated lymphoid tissue in the intestine, the bronchi, and the nasal and oral cavities, it is possible that receptors specific for IgA, Fc α-receptors, on antigen-presenting cells (B-cells or macrophages) may enhance uptake and processing of antigens, thus enhancing the immune response. In this way, reuptake of secreted IgA may be important in maintenance of antigenic stimulation and may contribute to secondary responses [24,25].

The high level of anti-exoantigen sIgA observed in 14/19 PCM patients' saliva could be due to the migration of activated IgA-producing plasma cells to other mucosal regions such as oral mucosa. Alternatively, it could reflect the existence of focal infections in the buccal mucosa inducing the local production of sIgA. The level of salivary IgG reacting with *P. brasiliensis* exoantigens can also reflect both circulating IgG and IgG produced in response to local stimulation.

Levels of salivary gp43 were elevated, but no statistically significant differences between patients and controls were found. Using the mean plus the standard deviation as the cut-off value for determining normal levels of reaction with anti-gp43 Mab in saliva, we found only three positive cases out of 19. Some positive reactors among the normal controls were from endemic areas and this could have contributed to the high threshold titre [16]. The possibility of cross-reactions with an antigen or antigens from the normal oral microflora cannot be excluded. Additional study is required to determine whether gp43 is dispersed through the oral mucosa or if its presence is due to the release of the *P. brasiliensis* via local infection.

The cases of patients presenting a low level of serum IgG but a high titre of IC reinforce the need, already stressed in the literature, to use more than one marker for diagnosis and treatment monitoring of PCM [26]. The presence of sIgA in patient's saliva suggests a protecting role for neutralizing antigens on mucosal surfaces. This will be the object of future investigations.

Acknowledgements

This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Coordenadoria de Pós-Graduação da Universidade Estadual de Londrina (CPG-UEL). The authors thank Mári Sumigawa Kaminami and Nilson de Jesus Carlos for their excellent technical assistance and Robert Boyle for the careful correction of the English text.

References

1 San-Blas G. Paracoccidioidomycosis and its etiological agent *Paracoccidioides brasiliensis*. *J Med Vet Mycol* 1993; **31:** 99–113.
2 McEwen JG, Garcia AM, Ortiz BL, *et al.* In search of natural habitat of *Paracoccidioides brasiliensis*. *Arch Med Res* 1995; **26:** 305–306.
3 Mendes RP. The Gamut of clinical manifestations. In: Franco M, Lacaz CS, Restrepo-Moreno A, Del-Negro G, eds. *Paracoccidioidomycosis*. 1st edn. Boca Raton, Florida: CRC Press, 1994: 233–258.
4 Manns BJ, Baylis BW, Urbanski SJ, *et al.* Paracoccidioidomycosis: case report and review. *Clin Infect Dis* 1996; **23:** 1026–1032.
5 Mendes-Giannini MJS, Camargo ME, Lacaz CS, Ferreira AW. Immunoenzymatic absorption test for serodiagnosis of paracoccidioidomycosis. *J Clin Microbiol* 1984; **20:** 103–108.
6 Camargo ZP, Unterkircher C, Campoy SP, Travassos LR. Production of *Paracoccidioides brasiliensis* exoantigens for immunodiffusion tests. *J Clin Microbiol* 1988; **26:** 2147–2151.
7 Puccia R, Schenckman S, Gorin PAJ, Travassos LR. Exocellular components of *Paracoccidioides brasiliensis*: identification of a specific antigen. *Infect Immun* 1986; **53:** 199–206.
8 Mendes-Giannini MJ, Bueno JP, Shikanai-Yasuda MA, *et al.* Antibody response to the 43 kDa glycoprotein of *Paracoccidioides brasiliensis* as a marker for the evaluation of patients under treatment. *Am J Trop Med Hyg* 1990; **43:** 200–206.
9 Bueno JP, Mendes-Giannini MJS, Del-Negro GMB, Assis CM, Taniguti CK, Shikanai-Yasuda MA. IgG, IgM and IgA antibody response for the diagnosis and follow-up of paracoccidioidomycosis: comparison of counterimnunoelectrophoresis and complement fixation. *J Med Vet Mycol* 1997; **35:** 213–217.
10 Camargo ZP, Gesztesi JL, Saraiva EC, Taborda CP, Vicentini AP, Lopes JD**.** Monoclonal antibody capture enzyme immunoassay for detection of *Paracoccidioides brasiliensis* antibodies in paracoccidioidomycosis. *J Clin Microbiol* 1994; **32:** 2377–2381.
11 Blotta MHS, Camargo ZP. Immunological response to cell-free antigens of *Paracoccidioides brasiliensis:* relationship with clinical forms of paracoccidioidomycosis. *J Clin Microbiol* 1993; **31**: 671–676.
12 Mendes-Giannini MJS, Bueno JP, Shikanai-Yasuda MA, Ferreira AW, Masuda A. Detection of the 43,000-molecular-weight glycoprotein in sera of patients with paracoccidioidomycosis. *J Clin Microbiol*, 1989; **27:** 2842–2845.
13 Sugizaki MF, Peraçoli MT, Mendes-Giannini MJ, *et al.* Correlation between antigenemia of *Paracoccidioides brasiliensis* and inhibiting effects of plasma in patients with paracoccidioidomycosis. *Med Mycol* 1999; **37**: 277–284.
14 Cherquer-Bou-Habib D, Oliveira-Neto MP, Oliveira-da-Cruz MF, Galvão-Castro B. The possible role of circulating immune complexes in the deficiency of cell-mediated immunity in paracoccidioidomycosis. *Braz J Med Biol Res* 1989; **22**: 205–212.
15 Lowry OH, Rosobrough MJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1971; **246:** 1889–1894.
16 Lacaz CS, Passos-Filho MCR, Fava-Neto C, Macarron B. Contribuição para o estudo da ''blastomicose-infecção''. Inquérito com a paracoccidioidina. Estudo sorológico e clínico-radiológico de paracoccidioidino-positivos. *Rev Inst Med Trop São Paulo* 1959; **1:** 245–259.

17 Unterkircher CS, Yazaki SC, Shimizu MT, Jorge AOC, Camargo ZP. Specific components found in circulating immune complexes (CIC) in paracoccidioidomycosis. *J Med Vet Mycol* 1996; **34:** 273–277.
18 Martinez R. Other Laboratory tests: hematologic and biochemical changes. In: Franco M, Lacaz CS, Restrepo-Moreno A, Del Negro G, eds. *Paracoccidioidomycosis.* 1st edn. Boca Raton, Florida: CRC Press, 1994: 365–372.
19 Barraviera B, Mendes RP, Pereira PC, Machado JM, Curi PR, Meira DA. Measurement of glucose-6-phosphate dehydrogenase and glutathione reductase activity in patients with paracoccidioidomycosis treated with ketoconazole. *Mycopathologia* 1988; **104**: 87–91.
20 Goldsby RA, Kindt TJ, Osborne BA. *The Complement System. Kuby Immunology.* 4th edn. New York: W.H. Freeman and Company, 2000: 329–350.
21 Tomasi TB, Jr. Mechanisms of immune regulation at mucosal surfaces. *Rev Infect Dis* 1983; **5**: 784–792.
22 Kugler J, Hess M, Haake D. Secretion of salivary immunoglobulin A in relation to age, saliva flow, mood states, secretion of albumin, cortisol, and catecholamines in saliva. *J Clin Immunol* 1992; **12**: 45–49.
23 Tenovuo, J. Antimicrobial function of human saliva – how important is it for oral health? *Acta Odontol Scand* 1998; **56:** 250–256.
24 Kraehenbuhl J-P, Neutra MR. Molecular and cellular basis of immune protection of mucosal surfaces. *Physiol Rev* 1992; **72:** 853–879.
25 Neutra MR, Kraehenbuhl J-P. Mucosal immunization via M cells for production of protective secretory IgA antibodies. *Am J Med Hyg* 1994; **50:** 10–13.
26 Martins R, Marques S, Alves M, Fecchio D, Franco M. Serological follow-up of patients with paracoccidioidomycosis treated with itraconazole using dot blot, ELISA and Western Blot. *Rev Inst Med Trop São Paulo* 1997; **39**: 261–269.