Rev. Inst. Med. trop. São Paulo
34 (6): 511-516, novembro-dezembro, 1992

BIOCHEMICAL ANALYSIS OF THE METHYLIC ANTIGEN OF Paracoccidioides brasiliensis

Júnia Soares HAMDAN, Maria Aparecida de RESENDE, Sarah Piancastelli FRANZOT & Eduardo Osório CISALPINO

SUMMARY

Yeast forms of five strains of **Paracoccidioides brasiliensis** (SN, 2, 18, 192 and JT-1) were cultured in a synthetic medium for obtaining methylic antigens. These antigens were lyophilized and studied for each strain, to determine their partial biochemical composition, through measurements of total lipid, protein and carbohydrate contents. Lipids of methylic antigens were purified and analysed for sterols, phospholipids, glycolipids, lipoproteins, and partial characterization of sterols. Significant differences were found among antigenic preparations derived from distinct **P. brasiliensis** strains, in relation to the quantitative determinations. On the other hand, sterol analysis revealed the presence of ergosterol, lanosterol and squalene in all samples. The diversity verified in the biochemical characteristics of antigens derived from different **P. brasiliensis** strains, confirm the need of using a pool of fungal samples in order to produce antigen preparations for serological procedures without hampering their sensitivity.

KEY WORDS: Paracoccidioides brasiliensis; Antigens; Methylic antigens; Biochemical analysis.

INTRODUCTION

Paracoccidioidomycosis is an infection caused by the dimorphic fungus, **Paracoccidioides brasiliensis**. This disease occurs only in Latin America where it is the predominant deep mycosis and constitutes an important diagnosis for consideration in patients from endemic areas who have pulmonary or sistemic disease compatible with fungal infection[14,29,35].

The definitive etiological diagnosis is given through microscopy, from clinical specimens and/or by obtaining colonies of the microrganism, through cultivation of these materials. However, in many cases it is difficult to demonstrate the fungus by those methods, therefore, diagnosis has to be done by serological methods[6,10,11].

Several serological techniques have been used to diagnose this mycosis. Different preparations of various somatic and metabolic antigens, derived from the mycelial or yeast phases of **P. brasiliensis**, have been used in these techniques [6, 8, 10]. The serological technique is undoubtedly very valuable for the prognostic supervision of the treatment, early detection of relapses and criteria for cure [8, 11, 29]. However, diagnosis is highly restricted mainly due to the high frequency of cross reactions observed. These cross reactions have been extensively reported, even with the most sensitive techniques being unespecific. This is undoubtedly due to the complex biochemical characteristics of the antigens normally used. The **P. brasiliensis** cells contain a so called "antigenic mosaic" and most of the antigens expressed are similar to those produced by other fungi, particularly **Histoplasma capsulatum**[5,6,17,27] and **Blastomyces dermatitidis**[6,11]. The complexity of antigens is clearly seen by the presence of several precipitation lines in the double agar gel difusion reaction [10,11,30,32].

To improve the serological diagnosis, antigens have been characterized biochemically, fractioned and purified [1,3,27,33]. The most promising results, as to acquisition of purified antigens, refer to a glycoprotein of 43 kDa, excreted by **P. brasiliensis** in liquid medium. This antigen has been used in immunodiffusion tests with highly specific results[4,22,23,26,34]. However, it is produced in small amounts, and the methodology involved

Address for correspondence: Dra. Júnia Soares Hamdan, Departamento de Microbiologia; Instituto de Ciências Biológicas; Universidade Federal de Minas Gerais; Avenida Antônio Carlos, 6627; Caixa Postal 2486; CEP 31.270-901 Belo Horizonte, Minas Gerais, Brasil.

may not be generally adaptable for routine laboratory use[4,25].

This work studied the characterization and partial fractionation of the methylic antigens of 5 **P. brasiliensis** strains. This antigen comprises a methanolic extract of lyophilized yeasts and has been used in Minas Gerais State, Brazil, with good results in complement-fixation reaction for diagnosis and supervision of the treatment of patients with paracoccidioidomycosis[8,9,17]. Moreover, investigations about this antigen are justified by the facility of its obtention in great amounts and the lower costs involved.

MATERIAL AND METHODS

Microrganisms: The **P. brasiliensis** samples used in this study comprised 4 strains (SN, 2, 18 and 192) obtained from the collection of the Faculty of Medicine of the University of São Paulo and maintained in laboratory for a long time, as well as a recent isolate of this fungus (JT-1), identified in the Laboratory of Mycology, Federal University of Minas Gerais, in January, 1990.

Cultivation: The 5 samples were cultivated in yeast phase (36°C), in solid synthetic medium of **McVeigh & Morton**[28]. Subcultures done in 4 or 5 day intervals provided cells in exponential growth phase[20,21]. These cells were carefully collected using a loop, transferred to the centrifuge, washed three times with a sterile saline solution and, then, frozen and lyophilized.

Production of the methylic antigens: The lyophilized masses of the 5 **P. brasiliensis** samples were put in dark flasks, wrapped in aluminium foil and submitted to chemical extraction with methylic alcohol, for 10 days, at room temperature, at a proportion of 1 g of lyophilized fungus to 100 mL of methanol[9]. After this period, the suspension was filtered and the filtrates comprised the antigens SN, 2, 18, 192 and JT-1.

Partial characterization of the methylic antigens: Dry weight, through evaporation in vacuum, lipid content, as described below and protein[19] and carbohydrate[31] contents of the total methylic antigens, obtained for the 5 samples, were measured. With exception of dry weight, all other biochemical measurements were done in antigen preparations previously lyophilized. The results were expressed in μg of substratum/mL of antigen.

Purification and lipid analysis: The lipids of the methylic antigens were purified according to the technique of LETTERS[18] modified by HUNTER & ROSE[15]. The lipid extract obtained was lyophilized for biochemical measurements. Total lipid contents were determined by dry weight. Phospholipids were estimated by determination of phosphorus[7]. Glycolipids were measured by the anthrone technique[31] and lipoproteins by the method described by LOWRY et al.[19]. Sterol analysis was done after saponification[2] of the antigens. Sterol content was determined by the method of MOORE & BAUMANN[24]. Characterization of the non-saponifiable material was done by the absorption profile analysis at the range of the ultraviolet in spectrophotometer[16] and by thin layer chromatography, as described previously[12,13].

Statistical methods: The results of all measurements show the mean and standard deviation of at least three experiments carried out for each antigen sample (SN, 2, 18, 192 and JT-1). The "t" Student test was used to verify the difference in the biochemical composition of antigens derived from the different **P. brasiliensis** strains.

RESULTS AND DISCUSSION

Partial biochemical characterization of the methylic antigens: Table 1 shows the measurements of the total methylic antigens of 5 **P. brasiliensis** strains. Analysis of these data show, in the five preparations analysed, an absolute predominance of lipids in relation to proteins and carbohydrates. This result was expected considering that the antigen was obtained in organic solvent (methanol) and was similar to that ones reported in a previous study with the same preparations[8]. This latter studied the characterization of the antigens obtained from a pool of **P. brasilienses** strains. However, a higher lipid content was observed when the cells were cultivated in McVeigh & Morton medium. Besides, the author observed that these measurements were different when compared to the ones obtained with methylic antigens derived from the same samples cultivated in other media.

Comparing the five antigen preparations, statistical analysis showed that the antigen (Ag) 192

Table 1
Dry weight, total lipid, protein and carbohydrate contents (μg/mL) of five **P. brasiliensis** methylic antigens preparations derived from SN, 2, 18, 192 and JT-1 strains.

Antigen	PSAG		PSLIPAG		Proteins		Carbohydrates	
	$\overline{X}$	s	$\overline{X}$	s	$\overline{X}$	s	$\overline{X}$	s
SN	3089	083	1022	039	235	2.5	177	4.7
02	3133	148	1000	050	207	5.7	197	4.7
18	2825	143	0933	045	145	7.3	143	7.2
192	3578[1]	171	1156	059	240[3]	0.0	177	4.7
JT-1	3200	160	1320[2]	052	227	5.3	353[4]	9.4

PSAG = dry weight of the antigen
PSLIPAG = dry weight of the antigen lipid

$\overline{X}$ = average of at least three experiments
s = standard deviation

1 = measurement significantly higher than SN, 2 and 18 ($p < 0.05$)
2 = content significantly higher than SN,2 and 18 ($p < 0.05$)
3 = content significantly higher ($p < 0.05$)
4 = content significantly higher ($p < 0.05$)

had a higher dry weight than the others but was not significantly different ($p > 0.05$) from JT-1. On the other hand, the Ag JT-1 showed a higher lipid content than the others, but was not different from Ag 192. The latter showed a higher protein content than the other preparations and JT-1 had a higher carbohydrate content than all the other antigens. Coincidently, as to the biochemical composition of the different **P. brasiliensis** strains, the 192 and JT-1 yeasts showed higher lipid contents than the others, and JT-1 showed the highest carbohydrate content of them all. This correlation between biochemical composition of the strain and antigen composition could not be established for protein content[12].

Partial biochemical characterization of the lipids of the methylic antigens: Table 2 shows the measurements done in lipids of the 5 antigen preparations. Phospholipids are not shown since they were not detected in any of the antigens by the technique used. On the other hand, all preparations contained sterols, glycolipids and lipoproteins. Statistical analysis of these data revealed that the antigen 192 contained a higher amount of glycolipids and sterols than the other preparations, whereas the antigen JT-1 showed the highest lipoprotein content.

Qualitative analysis of the sterols detected, through chromatography (Figure 1) and spectrophotometry (Figure 2), the presence of ergosterol, lanosterol and squalene. Since all antigens showed the same pattern, only the Ag SN was reported. The detection of three sterol types agrees with what has been reported on the presence of these compounds in **P. brasiliensis** strains[13].

Table 2
Total glycolipids, lipoproteins and sterol* contents (μg/mL) determined in lipid extracts of five **P. brasiliensis** methylic antigens preparations, derived from SN, 2, 18, 192 and JT-1 strains.

Antigen	Glycolipids		Lipoproteins		Sterols	
	$\overline{X}$	s	$\overline{X}$	s	$\overline{X}$	s
SN	16.0	0.82	19.2	0.11	46	2.5
02	13.5	0.80	20.0	0.14	38	1.8
18	15.0	0.90	18.0	0.00	34	1.7
192	40.0[1]	0.00	19.2	0.21	72[1]	0.0
JT-1	14.2	0.62	39.0[2]	0.00	34	0.0

* The phospholipids total content is not shown in this Table because they were not detected in the samples, by the employed technique.

$\overline{X}$ = average of at least three experiments
s = standard deviation
1 = content significantly higher ($p < 0.05$)
2 = content significantly higher ($p < 0.05$)

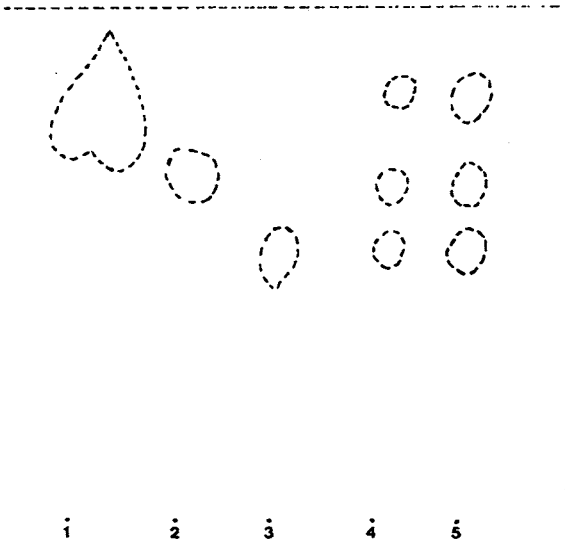

Figure 1 - Chromatogram of sterol standards and of non saponifiable lipids of *P. brasiliensis* methylic antigen (sample SN). 1. Squalene, 2. Lanosterol, 3. Ergosterol, 4. Antigen (SN), 5. Antigen (SN)

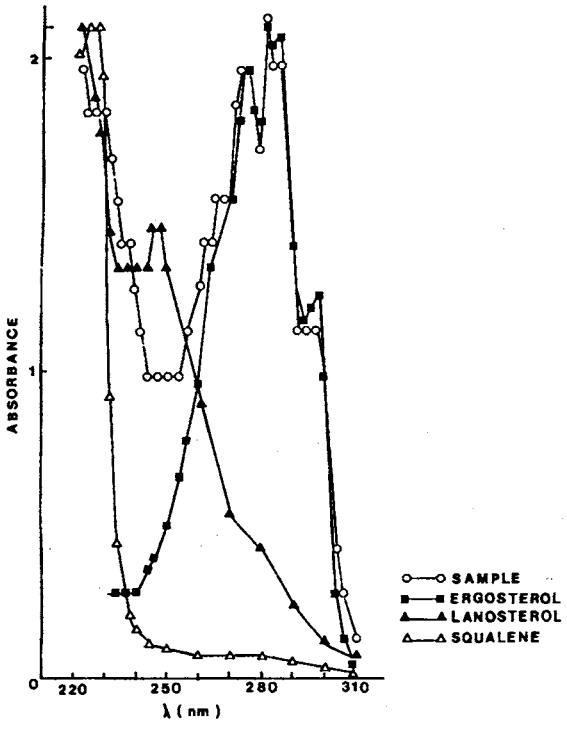

Figure 2 - Absorption spectrum of the sterols of *P. brasiliensis* methylic antigen (sample SN) and of authentic ergosterol, lanosterol and squalene standards

The global results, showing significant biochemical differences in the methylic antigens derived from different **P. brasiliensis** samples, confirm the need of using a pool of fungal strains in order to produce the antigen preparations used in serological procedures without compromising the sensitivity of such reactions[1,8,10,11,32].

Further studies should verify if the purified lipid fraction of this antigen keeps its activity in the reaction of complement-fixation. This could mean the detection of a more specific preparation and consequently would make a better diagnosis possible.

RESUMO

Análise bioquímica de antígeno metílico de **Paracoccidioides brasiliensis**

Cinco amostras de **Paracoccidioides brasiliensis** (SN, 2, 18, 192 e JT-1) em fase leveduriforme foram cultivadas em meio sintético para obtenção de antígenos metílicos. Os antígenos provenientes de cada amostra foram liofilizados e analisados quanto à sua composição bioquímica parcial, através da determinação do conteúdo total de lipídios, proteínas e carboidratos. Os lipídios dos antígenos metílicos foram purificados e analisados quanto ao teor de esterois, fosfolipídios, glicolipídios e lipoproteínas. Esterois foram parcialmente caracterizados. Em relação às medidas quantitativas, foram encontradas diferenças significantes entre as preparações antigênicas provenientes de amostras distintas de **P. brasiliensis**. Por outro lado, a análise dos esterois revelou a presença de ergosterol, lanosterol e esqualeno em todas as preparações. As diferenças verificadas nas características bioquímicas de antígenos derivados de amostras diferentes de **P. brasiliensis** confirmam a necessidade do uso de um pool de cepas para obtenção de preparações antigênicas a serem empregadas em procedimentos sorológicos, sem o que tais reações poderão ter sua sensibilidade comprometida.

ACKNOWLEDGMENTS

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Pro-Reitoria de Pesquisa of Federal University of Minas Gerais (PRPq-UFMG) - Brasil.

REFERENCES

1. BIAGIONI, L.M.V.; SADATSUNE, T.; FRANCO, M.F. & MATTOS, M.C.F.I. - A comparative study of eight **Paracoccidioides brasiliensis** isolates. **Rev. Inst. Med. trop. S. Paulo, 28**:281-286, 1986.

2. BREIVIK, O. N. & OWADES, J. L. - Spectrophotometric semi-microdetermination of ergosterol in yeast. **J. Agric. Food Chem., 5**:360-363, 1957.

3. BURGOS, L. C.; CANO, L. E. & RESTREPO, A. - Purificación de antígenos somáticos del **Paracoccidioides brasiliensis**. Estudio preliminar. **Rev. Inst. Med. trop. S. Paulo, 27**:76-81, 1985.

4. CAMARGO, Z. P.; UNTERKIRCHER, C.; CAMPOY, S. P. & TRAVASSOS, L. R. - Production of **Paracoccidioides brasiliensis** exoantigens for immunodifusion tests. **J. clin. Microbiol., 26**:2147-2151, 1988.

5. CANO, L. E. & RESTREPO, A. - Predictive value of serologic tests in the diagnosis and follow-up of patients with paracoccidioidomycosis. **Rev. Inst. Med. trop. S. Paulo, 29**:276-283, 1987.

6. CASOTTO, M. - Characterization of the cellular antigens of **Paracoccidioides brasiliensis** yeast form. **J. clin. Microbiol., 28**:1188-1193, 1990.

7. CHEN, P. S.; TORIBARA, T. Y. & WARNER, H. - Microdetermination of phosphorus. **Analyt. Chem., 28**:1756-1758, 1956.

8. CISALPINO, P. S. - Sorodiagnóstico da paracoccidioidomicose através da reação de fixação do complemento com antígenos metílicos do **Paracoccidioides brasiliensis**; comparação com as reações de imunofluorescência indireta e imunodifusão em gel de agarose. Belo Horizonte, 1978. (Dissertação de Mestrado - Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais).

9. CISALPINO, E. O.; OLIVEIRA, L. G. & CARDOSO, J. P. - Experiência com um antígeno metílico para o diagnóstico da blastomicose sul-americana. **Arq. Cent. Estud. Fac. Odont. Minas Gerais, 1**:117-121, 1964.

10. FAVA NETTO, C. - Imunologia da Paracoccidioidomicose. **Rev. Inst. Med. trop. S. Paulo, 18**:42-53, 1976.

11. FAVA NETTO, C. - Antígeno polissacarídico de **Paracoccidioides brasiliensis. Interciência, 15**: 209-211, 1990.

12. HAMDAN, J. S. - Frações bioquímicas de **Paracoccidioides brasiliensis** e seu papel na gênese da doença causada por este microrganismo. Estudo experimental em camundongos. Belo Horizonte, 1991. (Tese de Doutoramento - Faculdade de Medicina da Universidade Federal de Minas Gerais).

13. HAMDAN, J. S. & RESENDE, M. A. - Lipid composition and effect of amphotericin B on yeast cells of **Paracoccidioides brasiliensis. Mycopathologia (Den Haag), 102**:97-105, 1988.

14. HAMDAN, J. S. & ROCHA, R. L. - Epidemiologia da paracoccidioidomicose. **An. Fac. Med. Minas Gerais, 36**:52-61, 1987.

15. HUNTER, K. & ROSE, A. H. - Lipid composition of **Saccharomyces cerevisiae** as influenced by growth temperatures. **Biochim. biophys. Acta** (Amst.), 260:639-653, 1972.

16. KIM, S. & KWON-CHUNG, K. J. - Polyene-resistant mutants of **Aspergillus fennelliae**: sterol content and genetics. **Antimicrob. Agents Chemother., 6**:102-113, 1974.

17. LACAZ, C. S.; PORTO, E. & MARTINS, J. E. C. - Paracoccidioidomicose. In: LACAZ, C.S.; PORTO, E. & MARTINS, J.E.C., ed. **Micologia Médica**. São Paulo, Sarvier, 1991. p.248-297.

18. LETTERS, R. - Phospholipids of yeasts. In: MILLS, A. E., ed. **Aspects of yeast metabolism**. Oxford, Blackwell Scientific Publications, 1968. p.303-319.

19. LOWRY, O. H.; ROSENBROUGH, N. J.; FARR, A. & RANDALL, R. J. - Protein measurement with the folin phenol reagent. **J. biol. Chem.**, 193:265-275, 1951.

20. MANOCHA, M. S. - Lipid composition of **Paracoccidioides brasiliensis**: comparison between the yeast and mycelial forms. **Sabouraudia, 18:281-286, 1980.**

21. MANOCHA, M. S.; SAN-BLAS, G. & CENTENO, S. - Lipid composition of **Paracoccidioides brasiliensis**: possible correlation with virulence of different strains. **J. gen. Microbiol., 117**:147-154, 1980.

22. MENDES-GIANNINI, M. J.; BUENO, J. P.; SHIKANAI-YASUDA, M. A.; FERREIRA, A. W. & MASUDA, A. - Detection of the 43,000 molecular-weight glycoprotein in sera of patients with paracoccidioidomycosis. **J. clin. Microbiol., 27**:2842-2845, 1989.

23. MENDES-GIANNINI, M. J.; BUENO, J. P.; SHIKANAI-YASUDA, M. A.; STOLF, M. S.; MASUDA, A.; AMATO-NETO, V. & FERREIRA, A. W. - Antibody response to the 43 kDa glycoprotein of **Paracoccidioides brasiliensis** as a marker for the evaluation of patients under treatment. **Amer. J. trop. Med. Hyg., 43**:200-206, 1990.

24. MOORE, P. R. & BAUMANN, C. A. - Skin sterols. I. Colorimetric determination of cholesterol and other sterols in skin. **J. biol. Chem., 195**:615-621, 1952.

25. MORENO, A. R. - Actualización sobre la paracoccidioidomicosis y su agente etiologico, 1986-1989. **Interciência, 15**:193-199, 1990.

26. PUCCIA, R.; SCHENKMAN, S.; GORIN, P. A. & TRAVASSOS, L. R. - Exocellular components of **Paracoccidioides brasiliensis**: identification of a specific antigen. **Infect. Immun., 53**:199-206, 1986.

27. RESTREPO, A.; CANO, L. E. & OCHOA, M. T. - A yeast derived antigen from **Paracoccidioides brasiliensis** useful for serologic testing. **J. med. vet. Mycol.**, 22:23-29, 1984.

28. RESTREPO, A. & JIMENEZ, B. E. - Growth of **Paracoccidioides brasiliensis** yeast phase on a chemically defined culture medium. **J. clin. Microbiol.**, 12:279-281, 1980.

29. RIPPON, J. W. - Paracoccidioidomycosis. In: RIPPON, J. W., ed. **Medical Mycology. The pathogenic fungi and the pathogenic actinomycetes.** Philadelphia, W. B. Saunders, 1988. p.506-531.

30. SAN-BLAS, G. - Paracoccidioidomicose: a micose sistêmica da América Latina. **Interciência**, 15:191-192, 1990.

31. SCOTT, T. A. & MELVIN, E. H. - Determination of dextran with anthrone. **Analyt. Chem.**, 25:1656-1661, 1953.

32. SCROFERNEKER, M. L.; FAVA NETTO, C. & SCHALCH, A. L. O. - Contribuição ao conhecimento da composição antigênica do **Paracoccidioides brasiliensis.** Estudo de 5 amostras. **Rev. Microbiol.**, 19:293-305, 1988a.

33. SCROFERNEKER, M. L.; FAVA NETTO, C.; SCHALCH, A. L. O. & MOTA NETO, A. A. - Soros hiperimunes anti-**Paracoccidioides brasiliensis. Rev. Microbiol.**, 19:311-316, 1988b.

34. STAMBUK, B. J.; PUCCIA, R.; ALMEIDA, M. L.; TRAVASSOS, L. R. & SCHENKMAN, S. - Secretion of the 43 kDa glycoprotein antigen by **Paracoccidioides brasiliensis. J. med. vet. Mycol.**, 26:367-373, 1988.

35. SUGAR, A. M. - Systemic fungal infections: diagnosis and treatment. I. Paracoccidioidomycosis. **Infect. Dis. Clin. N. Amer.**, 2:913-924, 1988.

Recebido para publicação em 9/03/1992.
Aceito para publicação em 3/09/1992.