

A COMPARATIVE STUDY OF THE IMMUNOANTIGENICITY OF EIGHT PARACOCCIDIOIDES BRASILIENSIS ISOLATES

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S U M M A R Y

The present study was carried out to investigate differences in the immuno-antigenicity of 8 *P. brasiliensis* isolates from 3 endemic areas (Botucatu: Pb 1, 2 and 3; São Paulo: Pb 18, 192 and 265; Venezuela: Pb 9 and 73). The antigenic reactivity of each isolate in the indirect fluorescent test (II) and in the immunodiffusion test (ID) was studied using a panel of 20 positive control sera for paracoccidioidomycosis. The humoral (measured by immunodiffusion) and the cellular (measured by the footpad test) immune response inducing capacity of each isolate was assessed by the immunization of mice. It was observed: 1. Pb 265 and Pb 9 showed the highest reactivity in II; 2. Pb 192 and Pb 73 were the most reactive in ID; 3. These results demonstrate differences in antigenicity among these isolates; 4. Pb 18 exhibited weak cellular immune response inducing power and great capacity of evoking anti *P. brasiliensis* antibody production in the immunized mice showing dissociation of its immunogenicity. These differences may indicate the occurrence of strains of the fungus or may reflect modification of the parasite in the host or during culturing.

KEY WORDS: *Paracoccidioides brasiliensis* — Immunoantigenicity of isolates.

I N T R O D U C T I O N

Interest in the immunological diagnosis of paracoccidioidomycosis has led to the development of many serological techniques using several types of antigens, especially extracts and filtrates, prepared both from the mycelial and the yeast-like phase of *Paracoccidioides brasiliensis* (*P. brasiliensis*)³. Different isolates of the fungus were soon found to have variable antigenicity, and therefore it was proposed that a pool of several samples should be used in the preparation of the antigens for the serodiagnostic reactions³.

Immunochemical studies have disclosed the existence of 25 antigen fractions of *P. brasiliensis* which vary among the different fungal isolates^{15,16}. On the other hand, morphological, biochemical, metabolic and pathogenic variations among *P. brasiliensis* samples have not been extensively studied¹⁵. We recently isolate several *P. brasiliensis* samples from the secretions of patients with paracoccidioidomycosis from the endemic area of Botucatu, but could not determine with certainty whether these were different strains or isolates of the same strain affecting several patients. To contribute to the study of the immunoantigenic differences among *P. brasiliensis* isolates, we carried out the present study to determine possible

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antigenic and immunogenic differences among 8 *P. brasiliensis* samples collected from different endemic areas.

MATERIAL AND METHODS

EXPERIMENTAL DESIGN — We studied three *P. brasiliensis* samples isolated from patients from the endemic area of Botucatu (Bt 1, Bt 2 and Bt3), three samples from the Collection of fungi of the Department of Microbiology, Faculty of Medicine, University of São Paulo (Pb 18, Pb 192 and Pb 265), and two samples from the Venezuelan Institute of Scientific Investigation, Caracas (Pb 9 and Pb 73). Experiments were carried out on these 8 samples with the following objectives: 1. To test the antigenicity of each sample by indirect immunofluorescence and by double immunodiffusion on agar gel for the serodiagnosis of paracoccidioidomycosis: 2. To test the immunogenicity of each sample by determining the immune humoral and cellular response induced in specifically immunized mice.

INDIRECT IMMUNOFLUORESCENCE TEST — The test was performed by the technique described by FRANCO et al.⁴ and PETANA & WANKE¹⁰. Suspensions the 8 *P. brasiliensis* samples were used separately as antigenic substrate after culturing in FAVA NETTO'S² medium for 14 days at 37°C.

Each sample was tested against a panel of 20 positive standard sera and 20 negative standard sera for paracoccidioidomycosis. The diagnosis of paracoccidioidomycosis was confirmed by histopathology and/or cytopathology. The standard sera were initially tested at 1:32 dilution in phosphate-buffered saline (PBS), pH 7.2. The fluoresceinated conjugate used was total human anti-Ig serum labelled with fluorescein isothiocyanate (Bio-Mérieux-Laboratory).

The sera that were positive at this dilution were then titrated at twofold dilutions in each of the antigenic substrates.

DOUBLE IMMUNODIFFUSION ON AGAR GEL — The test was performed by the technique described by PERAÇOLI et al.⁹. We used the soluble antigen of each sample prepared from yeast like forms from 14-day cultures. The fungi were washed 3 times with saline and

resuspended in an equal volume of saline. The suspension was sonicated 20 times in a Thornton cell disruptor for 20 min at 60-70 cycles/sec and centrifuged at 12,000 rpm for 20 min at 4°C. The supernatant (AgSPb) was sterilized by passing through Millipore prefilters and filters 0.45 and 0.22 μ thick. Protein was measured in each sample by the method of LOWRY et al.⁶ (Table 1).

TABLE 1

Determination of the antigenic dilution of each soluble antigen from 8 *P. brasiliensis* isolates to be used in immunodiffusion (ID): protein content; titers of a positive control serum for paracoccidioidomycosis given by 4 twofold dilutions of each antigen. The chosen dilutions are demarcated by parallel lines

P. brasiliensis isolates	Protein content (mg/ml)	Serum titers * in ID in 4 antigenic dilutions			
		Undiluted	1:2	1:4	1:8
Pb 18	4.25	32	32	8	Neg
Pb 192	4.30	64	32	8	?
Pb 9	2.5	8	4	Neg	Neg
Pb 265	2.0	4	8	4	8
Pb 73	4.0	64	32	4	Undiluted
Bt 1	2.0	4	8	8	Undiluted
Bt 2	4.0	4	8	4	Undiluted
Bt 3	5.0	Undiluted	4	4	4

* Titers expressed as the highest serum dilution reciprocal giving positive result.

To determine the dilution to be employed in the reaction, each antigen was initially tested at 4 twofold-dilutions against a positive control serum for paracoccidioidomycosis. The dilution was selected taking into consideration the serum titer, the sharpness of the precipitation lines, and the protein measurement (Table 1).

Each antigen was then tested against the panel of positive and negative sera for paracoccidioidomycosis as used in the immunofluorescence test. The standard sera were initially tested undiluted, and the positive sera were then titrated at twofold-dilutions in saline.

MOUSE IMUNIZATION — Groups of 10 Swiss white out-bred male mice aged 28 days were immunized with antigens from each of the 8 *P. brasiliensis* samples. The animals were immunized with dead yeast-like forms according to the procedure of RIFKIND et al.¹³, i.e weekly intradermal injection for 4 weeks.

One week after the last immunizing dose, the anti-*P. brasiliensis* immune response of each animal was tested. Delayed hypersensitivity was evaluated by the footpad test⁷, and humoral immunity by the double immunodiffusion test on agar gel.⁹

The soluble antigen used to measure the immune response was a pool of the antigens of the 8 *P. brasiliensis* samples. To select the footpad test, the pool was initially tested at 3 twofold-dilutions on groups of 5 mice with and without previous specific immunization (Table 2). The selected antigenic dilution was 1:2, since this dilution was not toxic and yielded the highest indices in the immunized mice.

TABLE 2

Footpad test mean indices of immunized and non-immunized mice tested with 3 twofold dilutions of a pool of soluble antigens from 8 *P. brasiliensis* isolates. Titers in the immunodiffusion test (ID) of a positive control serum for paracoccidioidomycosis given by these three dilutions

Antigenic dilution	Footpad test		Serum titers* in ID
	Animal (n=5)	Mean indices ± Standard deviation	
Undiluted	Immunized	1.7 ± 1.3	4
	Non-immunized	1.2 ± 0.5	
1:2	Immunized	1.5 ± 0.9	4
	Non-immunized	0.2 ± 0.4	
1:4	Immunized	1.0 ± 1.0	Undiluted
	Non-immunized	0.4 ± 0.7	

* Titers expressed as the highest serum dilution reciprocal giving positive result.

To assess cell immunity, 0.05 ml of the pool was injected into the right hind footpad of each animal (test footpad), and 0.05 ml of the diluent was injected into the left hind footpad (control footpad). The volume of the footpads was measured 24 hs later by plethysmography and the indices were expressed by the difference in volume between the test and control footpad.

To select the antigenic dilution to be employed in immunodiffusion, the pool was initially tested against a positive control serum for paracoccidioidomycosis at 3 twofold-dilutions (Table 2). The selected dilution was 1:2.

To evaluate the specific antibody production of the mice, the sera were tested both

qualitatively and quantitatively at twofold-dilutions

RESULTS

INDIRECT IMMUNOFLUORESCENCE — Table 3 shows the titers of the 20 positive standard sera tested against the *P. brasiliensis* isolates. They showed similar antigenicity, except for Pb 265 and Pb 9, which yielded significantly higher titers when Kruskal-Wallis analysis of variance was applied to the data ($H = 29.47$; $p < 0.05$)⁵. The 20 negative standard sera showed no titer in any of the antigenic substrates tested.

TABLE 3

Distribution of 20 sera from patients with paracoccidioidomycosis according to their titers in the indirect immunofluorescence test (II), using 8 *P. brasiliensis* isolates as antigenic substrates

<i>P. brasiliensis</i> isolate	Titers* in II							N.º of sera	Median
	32	64	128	256	512	1024	2048		
Pb 18	1	3	10	4	2	—	—	20	128
Pb 192	1	—	8	9	2	—	—	20	256
Pb 265	—	—	4	7	8	1	—	20	256**
Bt 1	—	2	9	8	1	—	—	20	128
Bt 2	—	2	9	8	1	—	—	20	128
Bt 3	—	3	12	3	1	—	1	20	128
Pb 9	—	1	6	6	7	—	—	20	256**
Pb 73	—	1	8	8	3	—	—	20	256

* Titers expressed as the highest serum dilution reciprocal giving positive result.

** Titers significantly higher ($H = 29.47$; $p < 0.05$).

DOUBLE IMMUNODIFFUSION ON AGAR GEL

— Table 4 shows the titers of the positive standard sera obtained with the antigens of each *P. brasiliensis* isolates. They showed similar antigenicity, except for Pb 192 and Pb 73 which yielded significantly higher titers than Pb 265, Bt 1, Bt 2 and Bt 3, when the data were analyzed by Kruskal Wallis analysis of variance ($H = 55.37$; $p < 0.001$). The negative standard sera showed no titers in any of the antigens tested.

MOUSE IMMUNIZATION WITH *P. brasiliensis*

— The indices of the footpad test for the evaluation of the immune cell response of mice immunized with antigen from each sample of *P. brasiliensis* are shown in Table 5. The behavior of the variation of mean indices expresses the difference among the samples studied, which was tested by Gaussian analysis of va

T A B L E 4

Distribution of 20 sera from patients with paracoccidioidomycosis according to their titers in the immunodiffusion test (ID), using 8 *P. brasiliensis* isolates soluble antigens

P. brasiliensis isolate	Titers* in ID										N.º of sera	Median
	Neg	Undiluted	2	4	8	16	32	64	128	256		
Pb 18	7	2	2	4	2	3	0	0	0	0	20	2
Pb 192	0	0	0	6	4	1	5	0	2	2	20	8-16**
Pb 265	10	4	3	1	0	0	0	2	0	0	20	Neg-Und
Bt 1	8	6	2	1	0	2	0	1	0	0	20	Undiluted
Bt 2	16	0	1	0	1	0	2	0	0	0	20	Neg
Bt 3	13	2	2	1	0	0	2	0	0	0	20	Neg
Pb 9	3	3	2	6	3	0	2	1	0	0	20	4
Pb 73	4	0	0	2	2	3	4	1	2	2	20	16**

* Titers expressed as the highest serum dilution reciprocal giving positive result.

** Titers significantly higher than Pb 265, Bt 1, Bt 2 and Bt 3 (H = 55.37; p < 0.001).

nized animals, respectively. Only Pb 18 showed significantly higher titers than the other samples⁵.

DISCUSSION

In the present study, the antigenicity of 8 *P. brasiliensis* isolates from three different endemic areas was assessed by two serological tests against a panel of 20 positive sera for paracoccidioidomycosis. Pb 265 (São Paulo) and Pb 9 (Venezuela) were more antigenic in the indirect immunofluorescence test, and Pb 192 (São Paulo) and Pb 73 (Venezuela) were more antigenic in double immunodiffusion test on agar gel. These data demonstrate differences in antigenicity among the isolates tested,

T A B L E 5

Footpad test indices of mice immunized with each of 8 *P. brasiliensis* isolates

P. brasiliensis isolate	Footpad test indices										N.º of sera	Mean ± Standard Deviation
	Pb 18	3.0	2.5	1.8	1.7	1.5	1.5	1.4	1.2	1.0		
Pb 192	4.8	3.9	3.6	3.4	3.0	2.6	1.8	1.5	1.3	1.3	10	2.7±1.2
Pb 265	4.4	3.0	3.0	3.0	2.0	1.8	1.4	1.2	1.0	ND*	9	2.3±1.0
Bt 1	3.7	3.2	3.0	3.0	3.0	2.7	2.1	2.0	1.9	1.6	10	2.6±0.6
Bt 2	4.3	4.0	3.6	3.1	3.0	2.9	2.8	2.7	2.5	2.4	10	3.1±0.7
Bt 3	4.1	3.9	3.7	3.1	3.0	2.8	2.8	2.3	2.0	1.4	10	2.9±0.8
Pb 9	3.4	3.4	2.6	2.2	2.2	2.1	2.0	1.5	1.1	1.1	10	2.1±0.3
Pb 73	3.6	3.6	3.0	3.0	2.6	1.9	1.7	1.6	1.4	1.3	10	2.3±0.8

* ND — not done

** Indices significantly lower than Bt 2 and Bt 3 (p < 0.05)

riance⁵. All of the animals immunized with the different samples showed positive cell immune response, with indices varying from 0.5 to 4.8. Indices from Pb 18 were lower than all other isolates, being significantly different from those of Bt 2 and Bt 3 (p < 0.05). The indices of the other isolates did not differ amongst themselves; the highest indices were given by Pb 192, Bt 1, Bt 2 and Bt 3.

Table 6 shows the anti *P. brasiliensis* antibody titers detected by immunodiffusion in the sera of immunized mice. The animals immunized with Pb 265, Bt 1, Bt 3 and Pb 9 did not develop specific antibodies. Pb 192, Pb 73, Bt 2 and Pb 18 induced a humoral response in 30%, 30%, 50% and 80% of the immu-

T A B L E 6

Distribution of sera from mice immunized with antigen of 8 *P. brasiliensis* isolates according to their titers in the immunodiffusion test (ID)

P. brasiliensis isolated	Titers* in ID							N.º of sera	Median
	Neg	Undiluted	2	4	8	16			
Pb 18	2	2	3	1	2	—	10	2**	
Pb 192	7	3	—	—	—	—	10	Neg	
Pb 265	10	—	—	—	—	—	10	Neg	
Bt 1	10	—	—	—	—	—	10	Neg	
Bt 2	5	4	1	—	—	—	10	Neg-Und	
Bt 3	10	—	—	—	—	—	10	Neg	
Pb 9	10	—	—	—	—	—	10	Neg	
Pb 73	6	1	—	—	—	2	9	Neg	

* Titers expressed as the highest serum dilution reciprocal giving positive result.

** Titers significantly higher.

the serological test used. The three isolates from Botucatu gave similar results.

Recent studies using serological, immunoelectrophoretic and biochemical techniques have demonstrated important antigenic differences between strains 192 and 265 of *P. brasiliensis*¹⁵ and differences in the biochemical constitution of the cell wall between strains 9 and 73¹⁴. These data agree with our results, which characterize further these samples of *P. brasiliensis* as characterize further these samples of *P. brasiliensis* as real strains. RESTREPO¹² tested, in the double immunodiffusion test, soluble antigens prepared from the two morphological phases of 6 strains of *P. brasiliensis* against panel of 8 positive sera from patients with paracoccidioidomycosis. Most of the strains produced a common antigen, but reactions of partial identity and of nonidentity were recorded for two strains. This tends to indicate that individual isolates, when grown in vitro, vary in the production of antigens, as also revealed in the present study.

Using a methodology similar to ours, POULAIN et al¹¹ demonstrated that not all strains of *Candida albicans* show the same reactivity in the indirect fluorescent test against sera from patients with candidosis. These authors also showed that strains freshly isolated from patients give much stronger reactions than those subcultured for a long time in the laboratory. This finding was not confirmed by the present study, since the three Botucatu samples, which were the most recently isolated ones, did not show greater reactivity in either serological test used.

The immune response inducing capacity of the 8 *P. brasiliensis* isolates was assessed by immunizing groups of mice with particulate antigens. With the exception of Pb 18, all isolates induced similar cellular and humoral anti-*P. brasiliensis* immune response. This study revealed data of great interest. Pb 18 showed weak cell immune response-inducing power and great capacity of evoking anti-*P. brasiliensis* antibody production in the immunized animals. Since the host conditions were constant, this result demonstrates that the fungus itself may be the responsible for the dissociation between the humoral and cellular immune response induced in the animal. This might be one explanation for the frequent detection of patients with paracoccidioidomycosis whose anti-*P. bra-*

siliensis cell immune response is depressed and whose humoral response is maintained or even exacerbated^{1,8}.

Together with PB 192, the three Botucatu isolates yielded the highest indices of cellular immune response in the mice, maybe indicating that for this immunologic parameter the shorter the time of isolation the stronger the immune response inducing capacity of the sample.

When data from the antigenicity study were compared with those from the immunogenicity experiment, it was observed a positive correlation between the high reactivity of Pb 192 and Pb 73 in the immunodiffusion test and their good humoral response inducing capacity in mice, as determined by the same serological technique. This data shows the presence of *P. brasiliensis* components that are both capable of antigenic specificity and of immunogenicity.

On the whole, data from the present study showed differences in the antigenic composition and in the immunogenicity between *P. brasiliensis* isolates. These differences may indicate the occurrence of strains of the fungus or may reflect adaptation of the same parasite in different hosts or even alteration or mutation of the parasite during culturing and maintenance "in vitro", as been showed to *Candida albicans*¹¹.

Further studies should be done to clarify these questions.

RESUMO

Estudo comparativo da imuno-antigenicidade de 8 amostras de *Paracoccidioides brasiliensis*

Para se detectar diferenças imuno-antigênicas entre 8 amostras de *P. brasiliensis* isoladas de diferentes áreas endêmicas (Botucatu: Pb 1, 2 e 3; São Paulo: Pb: 18, 192 e 265; Venezuela: Pb 9 e 73), estudaram-se: 1. A reatividade antigênica de cada amostra nas reações de imunofluorescência indireta (II) e de imunodifusão dupla em gel de ágar (ID) contra painel de 20 soros controles positivos para paracoccidioidomicose; 2. A capacidade de induzir resposta imune humoral (medida por imunodifusão) e celular (medida pelo teste de coxim

plantar) em camundongos imunizados com antígenos de cada amostra. Observamos: 1. As amostras Pb 265 e Pb 9 mostraram-se mais reativas na II; 2. Os antígenos das amostras Pb 192 e Pb 73 foram significativamente mais reativas na ID; 3. Estes dados demonstram diferenças de antigenicidade entre estas amostras; 4. A amostra Pb 18 mostrou baixo poder indutor de resposta imune celular e alta capacidade de indução de resposta imune humoral em camundongos imunizados, revelando dissociação de sua imunogenicidade.

Estas diferenças podem indicar a existência de cepas distintas do fungo ou refletir modificações do parasita no hospedeiro ou durante seu cultivo.

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