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PARACOCCIDIOIDES BRASILIENSIS. A MYCOLOGIC AND IMMUNOCHEMICAL STUDY OF A SAMPLE ISOLATED FROM AN ARMADILLO (*DASIPUS NOVENCINCTUS*)

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SUMMARY

A sample of *P. brasiliensis* isolated from the spleen and the liver of an armadillo *(Dasipus novencinctus)* has been analysed under a mycological and immunochemical viewpoint. The armadillo was captured in an area of Tucuruí (State of Pará, Brazil), the animal being already established as an enzootic reservoir of *P. brasiliensis* at that region of the country.

This sample maintained in the fungal collection of the Tropical Medicine Institute of São Paulo (Brazil) numbered 135, has got all the characteristics of *P. brasiliensis*, with a strong antigenic power and low virulence for guinea-pigs and Wistar rats.

The specific exoantigen of *P. brasiliensis* - the glycoprotein with a molecular weight of 43 kDa - was easily demonstrated with double immunodiffusion, immunoelectrophoresis, SDS-PAGE and immunobloting techniques.

KEYWORDS: *Paracoccidioides brasiliensis*; *Dasipus novencinctus*.

INTRODUCTION

Wild armadillos captured in the area of the Tucuruí hydroelectric plant and identified as *Dasipus novencinctus* had been identified by NAIFF et al. [10] as possible reservoirs of *P. brasiliensis* in that region, suggesting the occurrence of enzootic paracoccidioidomycosis infection in the State of Pará. These investigators inoculated a ground spleen and liver homogenate from these armadillos into hamsters by the intraperitoneal and intradermal routes and isolated *P. brasiliensis* samples from four of the twenty armadillos studied. *P. brasiliensis* was identified by culture and by histopathological examination of the hamsters' viscera. These armadillos were captured in a high, firm-earth forest region with extensive areas of secondary vegetation, and in open areas with forest islands. The soil of this region is acid and poor in nutrients. The region is crossed by the Trans-Amazon Highway and is mainly inhabited by settlers from the States of Maranhão and of the Brazilian Northeast. Extensive areas are being deforested for agriculture and cattle raising. The armadillos with *P. brasiliensis*-positive cultures were apparently healthy. [10]

A sample of a dimorphic fungus identified as

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Paracoccidioides brasiliensis was isolated from liver and spleen homogenate from an armadillo *(Dasipus novencinctus)* captured in the Tucuruí region (State of Pará, Brasil) and were taken from Manaus, Amazonas, to the Discipline of Infectious and Parasitic Diseases of the School of Medicine, Botucatu, UNESP.

The objective of the present investigation was to perform a mycologic and immunochemical study of this *P. brasiliensis* sample maintained in the Instituto Nacional de Pesquisas da Amazônia, Brazil (IM3319) and in the Tropical Medicine Institute of São Paulo (Brazil) numbered 135.

MATERIAL AND METHODS

Fungus culture

The sample PbIM3319 was cultured on agar-Sabouraud at 25°C for 20 days to obtain the mycelial phase (M), and on agar-Fava Netto medium at 37°C to obtain the yeast phase (Y). The micromorphology of both forms was studied under the common light microscope in fresh preparations stained with lactophenol blue-cotton.

Inoculations

Male guinea pigs weighing 200-250g were inoculated intratesticularly with a suspension of the M phase of the sample PbIM3319, in the amount of 0.25ml (scale 4 of McFarland). Thirty days after inoculation, the animals were sacrificed and their testicles removed for histopathological examination.

Young male albino Wistar rats were inoculated with a yeast-like suspension of the sample PbIM3319 containing 3 x 10^7 cells/ml. A 0.5 ml amount of this suspension was inoculated intracardially into each animal. Fourteen days after inoculation, the animals were sacrificed and their liver, spleen and kidneys were removed for histopathological examination after hematoxylin-eosin and Grocott staining.

Antigen preparation

The sample PbIM3319 was cultured on agar-NGTA (1.6% neopeptone, 1% glucose, 0.01% thiamine, 0.02% asparagine, and 1.5% agar) at 37°C for 7 days. A suspension of yeast-like cells was later prepared in 0.85% saline, according to scale 5 of McFarland. A 5ml amount of the suspension was inoculated into 250ml of liquid NGTA medium and maintained at 37°C for 10 days, under constant shaking. After the growth period, the cultures were killed by the addition of thimerosal at a final concentration of 1:5000 and filtered through Whatman nº 1 filter paper. The filtrate was dialyzed, concentrated 6-fold by evaporation and maintained at 4°C until the time for use, representing the antigen of this sample, that was submitted to protein measurement by the method of LOWRY et al. [9]

The antigen obtained under the same experimental conditions from sample 113 of *P. brasiliensis* (Pb113) maintained in the Fungal Collection, Tropical Medicine Institute of São Paulo, Brazil, was used as reference.

Preparation of purified human anti - P. brasiliensis *IgG*

Derivation of human anti-*P.brasiliensis* IgG sera from 20 patients with paracoccidioidomycosis confirmed by direct examination and by serology were pooled and the IgG fraction of this pool was obtained by precipitation with 40% ammonium sulfate. The mixture was maintained under constant shaking for 30 minutes in an ice bath and then centrifuged at 3000 rpm for 30 minutes. The precipitate was resuspended in an equal volume of phosphate buffer, pH 7.2 (PBS). The solution was dialysed against PBS for 72 hours and then submitted to chromatography on DEAE-Sephadex A-25 (Pharmacia Fine Chemicals, Uppsala, Sweden). The purified fraction was evaluated by OUCHTERLONY double immunodiffusion [12], using the above cited reference antigen (Pb113).

*Preparation of rabbit anti-*P. brasiliensis *serum*

A crude antigen was obtained from the culture filtrate of sample Pb113 in the above cited NGTA medium, at 37°C, 20 days culture, under constant shaking. This antigen was later precipitated by 70% ethanol and inoculated subcutaneously into male New Zealand rabbits weighing 3 kg, by the method of GARCIA et al. [5].

Immunochemical tests:

Ouchterlony double immunodiffusion (DID)

The antigen was evaluated by DID in the presence of sera from patients with paracoccidioidomycosis, purified human anti-*P.brasiliensis* IgG, rabbit anti-*P. brasiliensis* serum, and an anti-gp 43 serum kindly provided by Dr. Zoilo P. Camargo, Escola Paulista de Medicina, São Paulo, Brazil. The antigen was also evaluated in the presence of rabbit anti-*Histoplasma capsulatum* [5], anti-*Aspergillus fumigatus* [14] and anti-

Candida albicans [11] sera to determine possible cross-reactivity.

Immunoelectrophoresis (IEP)

The antigen was analysed by IEP by the technique of SIQUEIRA [15], in the presence of rabbit anti-*P. brasiliensis* serum and purified human anti-*P. brasiliensis* IgG, for observation of the formation of the E arc of YARZÁBAL [18].

SDS-PAGE

Antigens from culture filtrates of both PbIM3319 and Pb113 samples were submitted to 12.5% polyacrylamide gel electrophoresis by the method of LAEMMLI [8]. A 300-µl amount of each antigen was precipitated with 2ml of a 10% trichloroacetic acid solution and centrifuged at 3000 rpm for 30 minutes [2]. The precipitate was resuspended in 2ml acetone and again centrifuged at 3000 rpm for 30 minutes. This last precipitate was diluted in 200µl of buffer solution and 40µl of the sample was submitted to SDS-PAGE for 5 hours at 100V. The antigen fractions were developed by silver nitrate staining [1].

Immunoblotting

The protein fractions obtained after electrophoretic separation (SDS-PAGE) were transferred to nitrocellulose paper (Millipore HATF-151-50) according to the method of TOWBIN & GORDON [16] and incubated with rabbit anti-*P. brasiliensis* serum and purified human anti-*P. brasiliensis* IgG. After incubation, peroxidase-labelled anti-IgG (Sigma, St. Louis, MO) was added at 1:5000 dilution. The reaction was developed with 3',3'-diaminobenzidine (Sigma, St. Louis, MO) in 0.1 M Tris-HCl buffer, pH 7.5.

RESULTS

Mycologic examination

Cultures of the sample PbIM3319 incubated at 25°C and 37°C showed a characteristic thermal dimorphism (Fig. 1). Direct examination of the yeast-like culture revealed elements with numerous oval buds (Fig. 2).

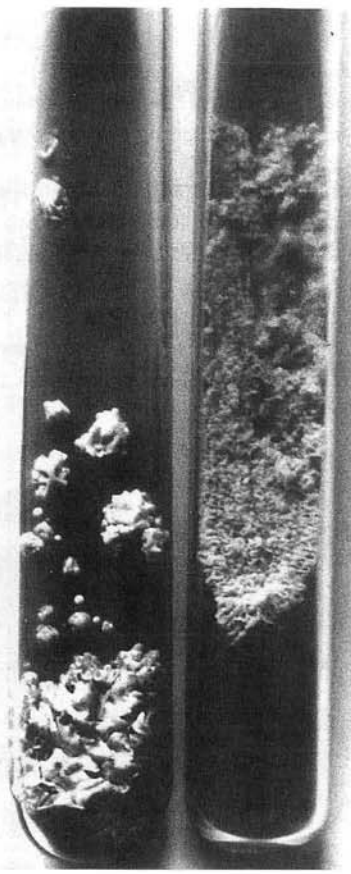

Fig. 1 - Cultures of *Paracoccidioides brasiliensis* (PbIM3319). M phase on agar-Sabouraud at 25°C, and Y phase on agar-Fava-Netto medium at 37°C. Incubation period: 20 days.

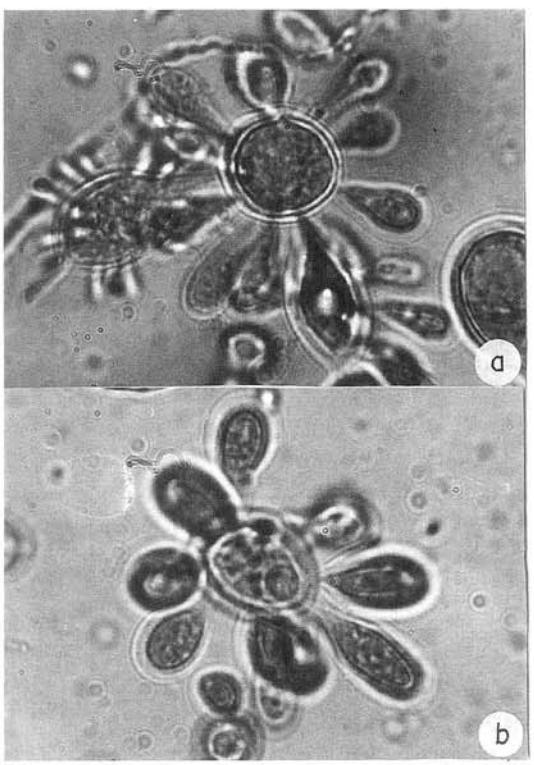

Fig. 2 - Direct examination of *Paracoccidioides brasiliensis* (PbIM3319), yeast-like phase a) and b) 400X. Lactophenol blue-cotton stain.

Histopathology

Inoculation of the M phase of the sample PbI-M3319 into the testicles of guinea pigs did not lead to the development of orchitis and few yeast-like cells were observed by direct examination. Histopathological examination showed the absence of spermatogenesis in the testicles. A granulomatous lesion was observed in the epididymis, essentially formed by confluent accumulations of epithelioid cells at times containing small areas of central necrosis. In these areas, neutrophils and giant cells of the foreign body type were visible in the central portion of the granulomas. A small number of fungi with typical reproduction shapes was also present (Fig. 3).

Intracardiac inoculation of the yeast-like cell suspension of the sample PbIM3319 into rats did not induce apparent alterations in any of the organs studied, as observed in an evaluation performed after 2 to 3 weeks. Direct examination of these organs did not reveal the presence of *P. brasiliensis*. Histopathological examination of liver sections showed compact epithelioid granulomas in the portal spaces, with clearly visible peripheral fibrosis. Few forms of the fungus were identified in the central portion of the granulomas. Spleen sections revealed intense, nonspecific lymphoid reactivity and marked reactivity of the red pulp, with hemosiderosis. Grocott staining revealed the absence of fungi. Kidney sections showed preserved structure, mild mesangial cell hyperplasia and absence of fungi.

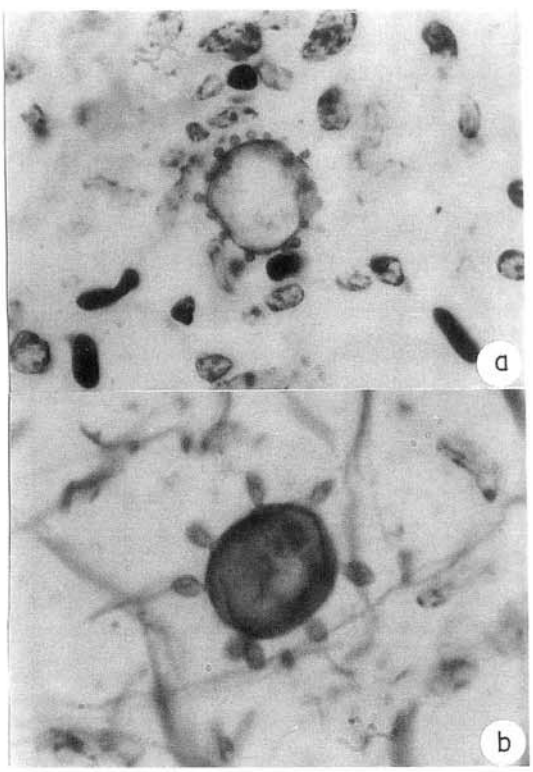

Fig. 3 - Histopathological examination of guinea pig testicle inoculated with *Paracoccidioides brasiliensis* (PbIM3319) showing yeast-like cells with multiple budding. a) 400X; b) 630X. Gomori-Grocott staining.

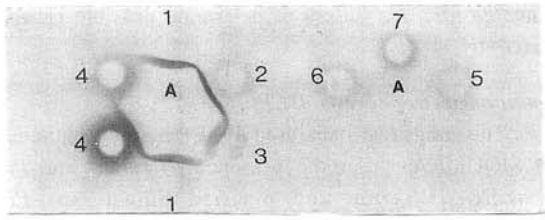

Fig. 4 - Double immunodiffusion test 1) Rabbit anti-gp 43 kDa serum; 2) Rabbit anti-*P. brasiliensis* serum; 3) Purified human anti-*P. brasiliensis* IgG; 4) Sera from patients with paracoccidioidomycosis; 5) Rabbit anti-*H. capsulatum* serum; 6) Rabbit anti-*C. albicans* serum; 7) Rabbit anti-*A. fumigatus* serum; A) PbIM3319 antigen.

Immunochemical tests

The protein content of the antigen, determined by the technique of LOWRY et al. [9], was 6.8 mg/ml.

The immunochemical tests performed permitted us to detect by double immunodiffusion and by immunoelectrophoresis the specific antigen of *P. brasiliensis*, represented by the glycoprotein of molecular weight 43 kDa (Figs. 4 and 5). No cross-reactions were observed by DID in the presence of rabbit anti-*H. capsulatum*, anti-*A. fumigatus* and anti-*C. albicans* sera.

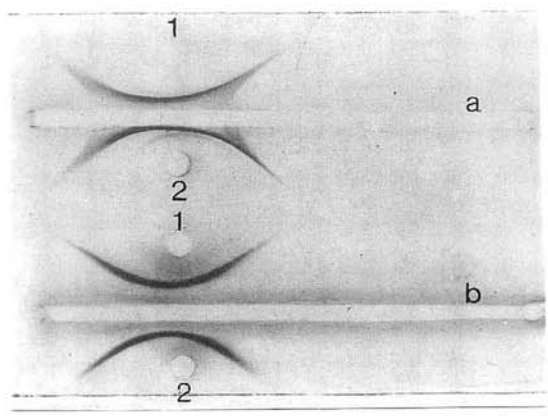

Fig. 5 - Immunoelectrophoresis. 1) *P. brasiliensis* sample 113 antigen; 2) PbIM3319 antigen. a) Purified human anti-*P. brasiliensis* IgG; b) Rabbit anti-*P. brasiliensis* serum.

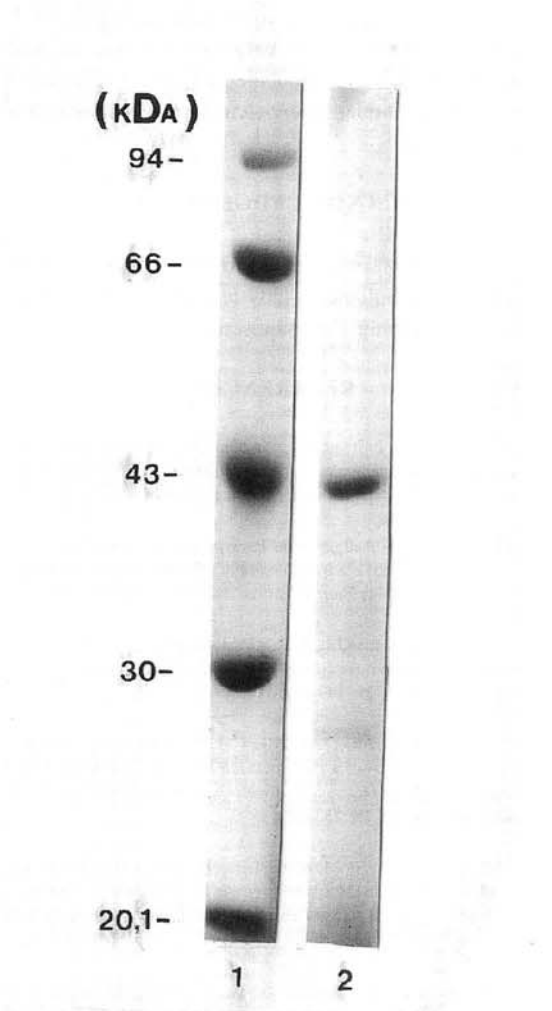

Fig. 6 - SDS-PAGE. 1)Molecular weight standard. 2) PbIM3319 antigen.

SDS-PAGE and immunoblotting also demonstrated the fraction of molecular weight 43 kDa (Fig. 6).

DISCUSSION

PERAÇOLI et al. [13] studied the virulence of the *P. brasiliensis* sample IM3319 and a sample isolated from a patient, maintained in the laboratory for several years (sample 18, Fungal Collection, Tropical Medicine Institute of São Paulo), both inoculated by the testicular route into guinea pigs and by the cardiac route into female hamsters (2 x 10^5 viable cells/ml). These investigators observed greater pathogenicity of the PbIM3319 in terms of parameters such as animal survival, histopathological examination, production of antibodies and recovery of fungi from the left lung (hamsters).

This sample IM3319 was also considered by KURITA et al [7] as a *P. brasiliensis,* in a study of a culture medium containing a growth enhancing factor which consisted in an aqueous extract of yeast-like cells of that fungus.

The sample studied in the present investigation is from Botucatu and was isolated from hamsters inoculated with the viscera of an armadillo captured by NAIFF et al. [10] in the region of the Tucurui hydroelectric plant.

The sample PbIM3319, maintained in the Fungal Collection, Tropical Medicine Institute of São Paulo, numbered 135, induced compact granulomas with scarce central necrosis in guinea pig epididymis. There was a small number of fungi of typical shape and reproduction. Giant cells of the foreign body type were visible in the central portion of the granulomas. A reduced number of compact epithelioid granulomas was observed in the liver of Wistar rats inoculated by the cardiac route. Under the present assay conditions, this sample appeared to be of low virulence.

The virulence data obtained in the present study seem to disagree from those of PERAÇOLI et al. [13], possibly due to the fact that these investigators used hamsters, animals that are much more sensitive to experimental infection with *P. brasiliensis.*

As to the immunochemical characteristics of the sample, DID revealed positivity with sera from patients with paracoccidioidomycosis and with rabbit anti-*P. brasiliensis* and anti-gp 43 sera. The presence of the E arc was observed by IEP. The specific exoantigen represented by the glycoprotein of molecular weight 43 kDa was detected by SDS-PAGE and immunoblotting. It should be pointed out that when immunoblotting was used the sera recognized, in addition to the 43 kDa fraction, several other fractions, among them two clearly visible ones of approximately 28 and 66 kDa (Fig. 7). Thus, the antigenic activity of this sample was confirmed.

The mycologic and immunochemical studies of samples isolated from soil and from animals is the focal point of our research [4, 6].

Antigenic variability among *P. brasiliensis* isola-

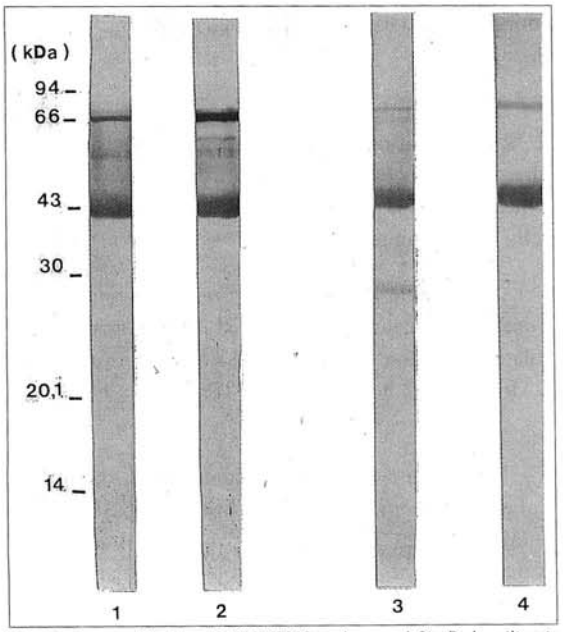

Fig. 7 - Immunoblotting. 1) PbIM3319 antigen and 2) *P. brasiliensis* sample 113 antigen, in the presence of rabbit anti-*P. brasiliensis* serum; 3) PbIM3319 antigen and 4) *P. brasiliensis* sample 113 antigen, in the presence of purified human anti-*P. brasiliensis* IgG.

tes has been demonstrated [3, 17]. Such variability is probably related to the virulence and pathogenicity mecha-nisms of the fungus. More detailed studies on antigenic composition of the sample PbIM3319 and other isolates from the environment should be considered.

RESUMO

Paracoccidioides brasiliensis.* Estudo micológico e imunoquímico de amostra isolada de tatu *(Dasipus novencinctus).

Amostra de *Paracoccidioides brasiliensis* isolada de vísceras (baço e fígado) de um tatu *(Dasipus novencinctus)* foi estudada do ponto de vista micológico e imunoquímico. O tatu havia sido capturado em área da usina hidroelétrica de Tucuruí (Estado do Pará). Este já havia sido considerado como reservatório enzoótico do *Paracoccidioides brasiliensis* naquela região.

Esta amostra, conservada na Micoteca do Instituto de Medicina Tropical de São Paulo sob o número 135, apresenta todas as características de *Paracoccidioides brasiliensis,* com elevado poder antigênico e baixa virulência para cobaios e ratos Wistar.

A demonstração do exo-antígeno específico do *P. brasiliensis*, representado pela glicoproteína de peso molecular 43 kDa, foi evidente através das técnicas de Imunodifusão Dupla, Imunoeletroforese, SDS-PAGE e Imunoblotting.

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