ISSN- 0102-9010

EARLY HISTOPATHOLOGICAL CHANGES IN BONE MARROW OF *Paracoccidioides brasiliensis*-INFECTED MICE

Vânia N. Brito[1], Paula C. S. Souto[1], Maria Alice da Cruz-Höfling[2], José Vassallo[3], Lucila C. Ricci[1] and Liana Verinaud[1]

[1]Department of Microbiology and Immunology, [2]Department of Histology and Embryology, Institute of Biology, [3]Department of Pathology, Faculty of Medical Sciences, State University of Campinas (UNICAMP), Campinas, SP, Brazil.

ABSTRACT

The involvement of bone marrow in the pathology of experimental *P. brasiliensis* infection in BALB/c mice was investigated. The histopathological features of bone marrow induced by the fungus were correlated with hematological changes in peripheral blood from 1 to 28 days post-infection. Intense lymphopenia and moderate neutrophilia were detected. The early changes in bone marrow included (i) maturation arrest characterized by an increase in immature blood cell precursors, mainly of granulocytic origin, (ii) intense vascular congestion when compared with the vessels of normal marrow, and (iii) an increased number of megakaryocytes. The normal histological pattern of bone marrow was restored by 28 days post-infection. No histologically recognizable lesion, such as granuloma formation or an abnormal cellular infiltrate, which could indicate the presence of the *P. brasiliensis* in bone marrow, was observed. In addition, special stains were unable to detect the fungus. The mechanisms responsible for the alterations described here are still unclear but are probably related to more general phenomena affecting the host rather than to direct damage of the precursors cells by *P. brasiliensis.*

Key words: *Paracoccidioides brasiliensis*, bone marrow, histopathology, paracoccidioidomycosis

INTRODUCTION

Paracoccidioides brasiliensis, the etiological agent of paracoccidioidomycosis (PCM), is a dimorphic fungus with a mycelial phase at 25ºC and a yeast-like phase at 35-37ºC [1,9]. PCM is the most prevalent human mycosis in Latin America. In Brazil, the disease occurs mainly in the State of São Paulo in the southeast of the country [9].

Despite of the well-known tropism of *P. brasiliensis* for lymphoid tissues [1,8], there are few conclusive reports about the damage of primary lymphoid organs induced by this fungus and their consequences during infection. Recently, we observed thymic fungal invasion and concomitant organ involution in experimentally infected BALB/c mice, an event which correlated with fungal dissemination to other organs [V.N. Brito, Masters Dissertation, State University of Campinas, 2001]. Some authors have also described fungal invasion of bone marrow tissue in acute and chronic disseminated PCM [4,12,13,15,20]. However, the histological alterations described in these studies were not correlated with the development of the disease or with alterations in the cellular immune response which are frequently observed in this disease [3,14,17,18]. Furthermore, no study has yet examined the bone marrow alterations in experimental models of *P. brasiliensis* infection.

In this report, we describe histopathological alterations in bone marrow of experimentally infected mice, and correlate these findings with the hematological changes in peripheral blood obtained 1 to 28 days post-infection (p.i.).

Correspondence to: Dr. Liana Verinaud
Departamento de Microbiologia e Imunologia, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), Campinas, SP, Brasil, Caixa Postal 6109, CEP: 13083-970. Tel: (55) (19) 3788 7911, Fax: (55) (19) 3289 7050,E-mail: verinaud@unicamp.br; vianb@unicamp.br
This work is part of a Masters Dissertation by V. N. B.

MATERIAL AND METHODS

Mice and fungus inoculation

Specific pathogen free male BALB/c mice, 8 weeks old, obtained from the Centro Multi-Institucional de Bioterismo (State University of Campinas, UNICAMP) were used in all

experiments. This strain of mice is relatively resistant to *P. brasiliensis* infection based on the mean survival time and on the capacity to restrain the fungus in compact, well-organized granulomas [2]. The mice were housed in plastic isolators under aseptic conditions with sterile water and food provided *ad libitum*. All procedures were done in accordance with the general guidelines proposed by the Brazilian Council for Animal Experimentation (COBEA). The highly virulent isolate Pb18 of *P. brasiliensis* was used for infection and mice were inoculated intraperitoneally with $5x10^6$ yeasts in 0.5 ml of phosphate-buffered saline (PBS). PBS alone was used to inject uninfected control mice (sham infection). Groups of four mice were sacrificed at 1, 3, 5, 7, 14 and 28 days after inoculation.

Histopathological study

At sacrifice by cervical dislocation, femures were obtained, cleaned, decalcified in Zenker solution (24 h), washed in tap water, dehydrated in an alcohol series, and embedded in Histosec. Sections 4 µm thick were deparaffinized, re-hydrated, treated with alcoholic iodine solution and sodium thiosulfate, and stained with hematoxylin-eosin (HE) and Grocott methenamine silver using standard procedures.

Two independent observers semi-quantitatively estimated megakaryocyte numbers and abnormal blood cell maturation in three or four sections from three animals for each period of infection. The alterations observed in the granulocytic maturation were scored as slight (+), 20-40%, moderate (++), 40-60% or strong (+++), >60%, according to the percentage of cells with maturation arrest. As for megakaryocyte when there were 10%, 20% or 30% increase in the cell number in the infected mice, respectively.

Immunohistochemistry

Detection of *P. brasiliensis* in bone marrow sections was done by using a human anti-*P. brasiliensis* anti-serum (kindly provided by Dr. H. Blotta, Faculty of Medical Sciences, UNICAMP). Briefly, after pre-absorption with normal serum, sections were incubated with anti-*P. brasiliensis* anti-serum (diluted 1:100 in PBS-1% BSA) overnight at 4ºC. After washing, the sections were overlaid for 1 h with peroxidase-conjugated goat anti-human IgG antibody (diluted 1:200 in PBS-1% BSA). This was followed by incubation with the substrate 3,3-diaminobenzidine (Sigma Chemical Co., St. Louis, Mo, USA) in a solution containing 0.1 M Tris-HCl buffer (pH 7.6), 1% (v/v) normal goat serum, and 0.1% (v/v) 3% hydrogen peroxide.

Hematological procedures

Leukocyte number was determined in standard Neubauer hemocytometers. Leukocyte differential counts were done on May-Grunwald-Giemsa stained smears. In addition, a semiquantitative analysis of blood smears by two independent observers provided an estimate of alterations in the number of platelets.

Statistical analysis

The results are reported as the mean ± SEM, as appropriate. Analysis of variance (ANOVA) followed by Dunnet's multiple co mparison test were used to compare the results. The level for statistical significance was $p<0.05$.

RESULTS

Morphological alterations in bone marrow

Infected mice showed the typical alterations associated with systemic infection. As early as 24 h p.i., there was a discrete increase (+) in the number of immature blood cell precursors and intense vascular congestion in the bone marrow of infected mice when compared with control marrow.

There was a strong increase (+++) in granulocytic precursors in the peritrabecular area between 3 and 5 days p.i., but there were no fat cells; there was also an increase (++) in the number of megakaryocytes. An abnormal cytological maturation in the granulocytic lineage characterized by a high number of large cells with circular nuclei was observed (+++) (Fig. 1).

At 7 days p.i., the hypercellularity intensified and the maturation of the granulocytic lineage was very marked (+++). By the 14th day of infection, the hypercellularity and the number of megakaryocytes had decreased. However, the number of granulocytic cells increased and the arrest of maturation was still evident (+++). By the 28th day p.i., the histological pattern of marrow was restored, and there was a reduction in the hypercellularity which paralleled a decrease in the number of granulocytic cells (+).

No invasion of bone marrow by *P. brasiliensis* or histologically recognizable lesions such as granuloma formation or an abnormal cellular infiltrate, indicative of the presence of yeasts in the tissue, was observed. Neither special staining nor immunohistochemical techniques revealed any fungus in the marrow compartment.

Hematological changes in peripheral blood

Since infection by *P. brasiliensis* induces hypercellularity in bone marrow tissue, the possible alterations in the number of leukocytes in peripheral blood were evaluated.

The total number of leukocytes varied slightly during infection (Fig. 2A). At 24 h p.i., the number of cells in infected mice was lower than in control mice, although the difference was not significant.

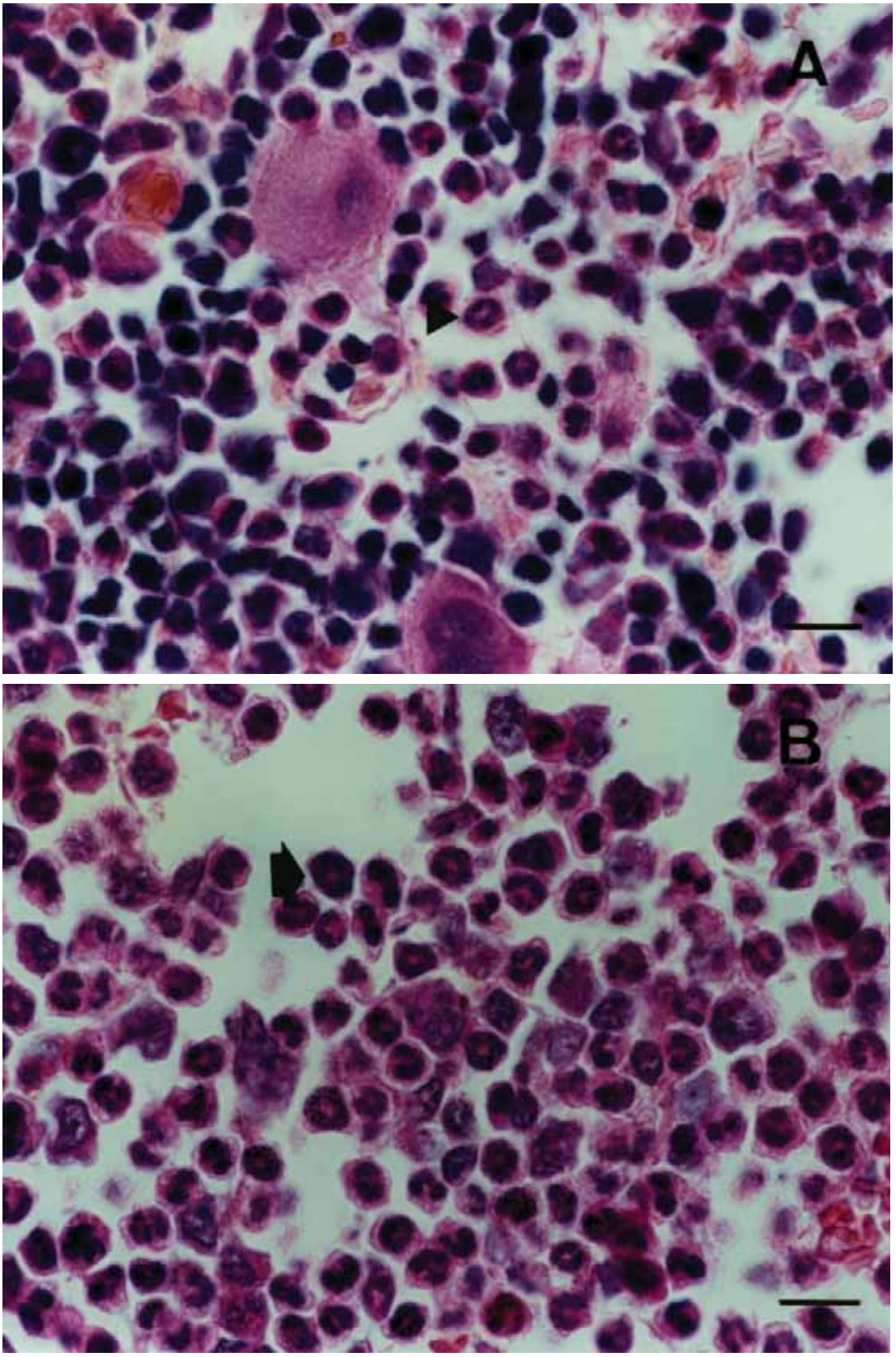

Figure 1. Photomicrograph of a section of bone marrow from a normal (**A**) and *P. brasiliensis*-infected mouse (**B**) 24 h after infection. Delayed maturation is seen in the infected mouse (◗) compared to the normal mouse (▶). HE staining. Bar = 10 µm.

The number of lymphocytes decreased in the early phase of infection but it was restored from the 5th day p.i. onwards (Fig. 2B). The number of monocytes varied considerably during the infection and among the infected mice (Fig. 2C). Differential counts of neutrophils showed increase of these cells during infection, mainly between days 7 and 14 p.i., followed by a marked reduction to normal values between 14 and 28 days p.i. (Fig. 2D). There was no significant change in the number of eosinophils in our experimental model. Platelet counts revealed no consistent changes during this experiment (results not shown).

All leukocytes and platelets had a normal morphology as judged from Giemsa-stained sections.

DISCUSSION

The precise mechanism underlying the immunosuppression observed during infection by *P. brasiliensis* is still incompletely understood. A reduction in T cell function [19], the induction of antigen-specific T suppressor cells [6-8], and the preference of the fungus for lymphoid organs [1,8] have been implicated in the suppression of cell-mediated immune responses in humans and in animals. In addition, the fungus may in some way damage primary lymphoid organs (thymus and bone marrow).

We have studied experimental paracoccidioidomycosis using male BALB/c mice infected intraperitoneally with strain 18 of *P. brasiliensis*. These mice develop a limited infection characterized by a granulomatous response in the peritoneal cavity, mainly in the liver and spleen capsule, where epithelioid granulomas can be detected 10 days after injection of fungus. In addition, an intense delayed-type hypersensitivity response was noted throughout the infection as shown by the footpad test. *P. brasiliensis* also invaded the thymic microenvironment which decreased markedly in size and showed structural distortions, including degeneration of the cortical area and a loss of cortico-medullary delimitation in the acute phase of infection [V.N. Brito, Masters' Dissertation, State University of Campinas, 2001].

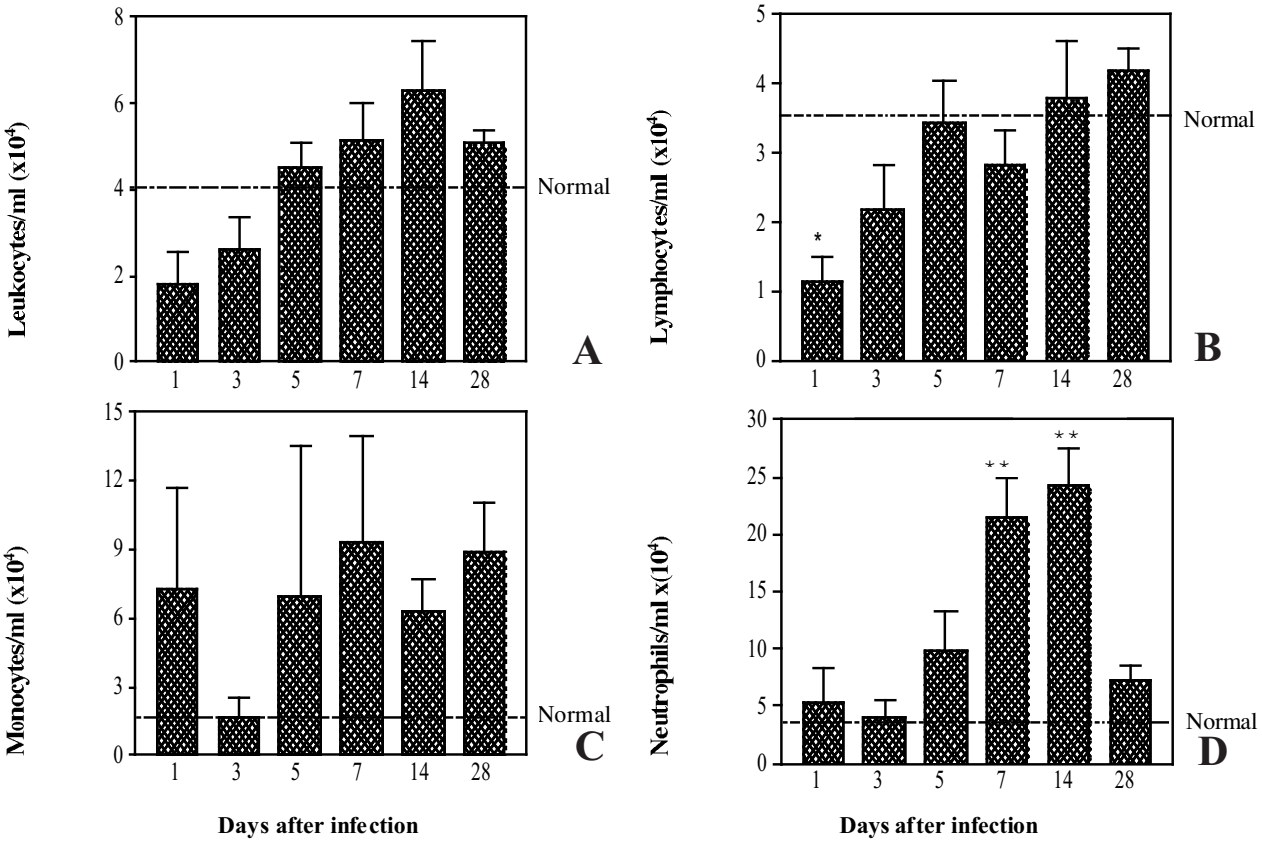

Figure 2. Hematological changes in *P. brasiliensis*-infected mice. Numbers of leukocytes (**A**), lymphocytes (**B**), monocytes (**C**) and neutrophils (**D**). The values represent the mean ± SEM of three experiments for each cell type. * $p<0.05$ and ** $p<0.01$ compared to normal (control) values shown by the dashed lines.

As shown here, infection with *P. brasiliensis* increased the number of immature precursors and caused intense vascular congestion as early as 24 h p.i. An increase in the granulocytic precursors of the peritrabecular area and in megakaryocytes, retarded maturation of the granulocytic lineage, the latter characterized by a high number of large cells with abnormal circular nucleus was observed. The normal histological pattern of the marrow was restored around the 28th day of infection.

Despite the morphological alterations seen in bone marrow there were no granulomas or abnormal cellular infiltrate. Nevertheless, the fungus could not be demonstrated by special staining techniques. These findings suggest that *P. brasiliensis* was not able to invade the bone marrow in our experimental model. This result differs from that of other studies which have shown bone marrow invasion in human disease [4,12,13,15,20]. Those conflicting findings can be ascribed, at least in part, to the genetic background of the mice which belong to a relatively resistant lineage. BALB/c mice are probably able to restrain fungal dissemination through granulomatous reactions thereby preventing bone marrow invasion. Since bone marrow was protected from fungal invasion for up to 28 days p.i., the morphological alterations seen here probably resulted from the systemic action of cytokines and inflammatory mediators produced in response to the infection.

Our findings in mice agree with clinical reports on the hematological disturbances during infection by *P. brasiliensis*. As in patients with PCM, neutrophilia [4,20] and lymphopenia [4,11,20] were observed. However, eosinophilia, which is seen in humans [4,15,20], was absent. The lymphopenia observed here could be related to the thymic damage that occurs in the initial stages of infection [V.N. Brito, Masters' Dissertation, State University of Campinas, 2001] and could facilitate fungal persistence in the host. On the other hand, neutrophilia could be explained by a massive production of pro-inflammatory cytokines, such as IL-1, TNFα and IL-6, in order to produce a nonspecific resistance to fungal infection. An important role for IL-1 in the regulation of hematopoiesis has been described [5], and increased levels of TNFα, IL-1 and IL-6 were observed in the sera of non-treated disseminated paracoccidioidomycosis patients [16]. In addition, IL-1 can activate neutrophils to kill *P. brasiliensis in vitro* [10].

Despite the increase in megakaryocyte numbers in bone marrow, there were no significant alterations of circulating platelet levels in infected mice.

In conclusion, in this experimental model the bone marrow is apparently not involved in the immunosuppressive phenomena associated with paracoccidioidomycosis. The possibility that bone marrow from highly susceptible mice may be involved in the pathogenesis and clinical manifestations of paracoccidioidomycosis is currently being investigated.

ACKNOWLEDGMENTS

The authors thank Marcos C. Meneghetti for excellent assistance with animal care. V.N.B. was supported by schoolarship from Coordenadoria de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) of the Brazilian Ministry of Education. This work was partially supported by Fundo de Apoio ao Ensino e à Pesquisa da UNICAMP (FAEP/UNICAMP grant number 366/99) and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP grant numbers 93/4994-5 and 98/09660-1).

REFERENCES

1. Brummer E, Castaneda E, Restrepo A (1993) Paracoccidioidomycosis: an update. *Clin. Microbiol. Rev.* **6,** 89-117.
2. Calich VLG, Singer-Vermes LM, Siqueira AM, Burger E (1985) Susceptibility and resistance of inbred mice to *Paracoccidioides brasiliensis*. *Br. J. Exp. Pathol.* **66,** 585-594.
3. Castaneda E, Brummer E, Pappagianis D, Stevens DA (1988) Impairment of cellular but not humoral responses in chronic pulmonary and disseminated paracoccidioidomycosis in mice. *Infect. Immunol.* **56,** 1771-1777.
4. Castro RM, Bassoi ON, Del Negro G, Faria CV (1958) Dificuldades diagnósticas na blastomicose sul-americana. *Rev. Paulista Med.* **53,** 479-496.
5. Fibbe WE, Willemze R (1991) The role of interleukin-1 in hematopoiesis. *Acta Haematol.* **86,** 148-154.
6. Finkel-Jimenez BE, Murphy JW (1988a) Induction of antigen-specific T suppressor cells by soluble *Paracoccidioides brasiliensis* antigen. *Infect. Immunol.* **56,** 734-743.
7. Finkel-Jimenez BE, Murphy JW (1988b) Characterization of efferent T suppressor cells induced by *Paracoccidioides brasiliensis*-specific afferent T suppressor cells. *Infect. Immunol***. 56,** 744-750.
8. Franco M, Mendes RP, Moscadi-Bacchi M (1989) Paracoccidioidomycosis. *Baillière's Clin. Trop. Med. Comm. Dis.* **4,** 185-220.

9. Goldani LZ, Sugar AM (1995) Paracocci-dioidomycosis and AIDS: an overview. *Clin. Infect. Dis.* **21,** 12 75-1281.
10. Kurita N, Oarada M, Miyaji M, Ito E (2000) Effect of cytokines on antifungal activity of human polymorphonuclear leukocytes against yeast cells of *Paracoccidioides brasiliensis*. *Med. Mycol.* **38,** 177-182.
11. Musatti CC, Rezkallah MT, Mendes E, Mendes NF (1976) *In vivo* and i*n vitro* evaluation of cell mediated immune response in patients with paracocci-dioidomycosis. *Cell Immunol.* **24,** 365-378.
12. Ozaki KS, Munhoz-Junior S, Pinheiro EK, Tadano T, Fontes CJF (1996) Diagnóstico de paracocci-dioidomicose disseminada grave em aspirado de medula óssea: relato de caso. *Rev. Soc. Bras. Med. Trop.* **29,** 263-366.
13. Pimenta de Mello, R (1955) Granuloma blastomicótico de medula óssea. *O Hospital* **48,** 135-138.
14. Roblebo MA, Graybill JR, Ahrens J, Restrepo A, Drutz DJ, Roblebo, M (1982) Host defense against experimental paracoccidioidomycosis. *Am. Rev. Resp. Dis.* **125,** 583-567.
15. Shikanai-Yasuda MA, Higaki Y, Uip ED, Mori NS, Del Negro G, Melo NT, Hutzler RU, Amato Neto V (1992) Comprometimento da medula óssea e eosinofilia na paracoccidioidomicose. *Rev. Inst. Med. Trop. São Paulo* **34,** 85-90.
16. Silva CL, Silva MF, Faccioli LH, Pietro RCL, Cortez SAE, Foss NT (1995) Differential correlation between interleukin patterns in disseminated and chronic human paracoccidioidomycosis. *Clin. Exp. Immunol.* **101,** 314-320.
17. Silva MR, Marques AS, Campos DS, Taboada DC, Soares GH, Brascher HM, Vargens-Neto JR, Cruz MQ, Labarthe NV, Rocha GL, Lima A (1981) Imunologia na paracoccidioidomicose. *Ann. Bras. Dermatol.* **56,** 227-234.
18. Singer-Vermes LM, Caldeira CB, Burger E, Calich VLG (1993) Experimental murine paracocci-dioidomycosis: relationship among the dissemination of infection, humoral and cellular responses. *Clin. Exp. Immunol.* **94,** 75-79.
19. Teixeira HC, Calich VLG, Singer-Vermes LM, D'Imperio-Lima MR, Russo M (1987) Experimental paracoccidioidomycosis: early immunosuppression occurs in susceptible mice after infection with pathogenic fungi. *Braz. J. Med. Biol. Res.* **20,** 367-369.
20. Terra GMF, Rios-Gonçalves AJ, Londero, AT, Braga MP, Ouricuri AL, Mesquita CC, Marinho JCA, Ervilha LM, Vieira ARM, Deker-Macher S, Duarte DMA. (1991) Paracoccidioidomicose em crianças: apresentação de casos. *Sup. Arq. Bras. Med.* **65,** 8-15.

Received: July 10, 2001
Accepted: September 24, 2001