Rev. Inst. Med. trop. São Paulo
36 (3): 217-223, maio-junho, 1994

EXPERIMENTAL PARACOCCIDIOIDOMYCOSIS IN HAMSTER: TRANSMISSION ELECTRON MICROSCOPY OF INOCULATION SITE LESION

K. I. R. COELHO (1), K. TAKEO, M. YAMAGUCHI (2), A. SANO, N. KURITA, S. YOSHIDA, K. NISHIMURA & M. MIYAJI

SUMMARY

Interaction between *Paracoccidioides brasiliensis* (Pb) and inflammatory cells in hamster testis was studied sequentially by transmission electron microscopy. In early lesions (six hours after inoculation), polymorphonuclear neutrophils (PMNs) were the major and mononuclear cells and eosinophils were the minor constituents of the inflammatory cells. PMNs were later replaced by mononuclear cells. Viable Pb cells were phagocytosed or surrounded by inflammatory cells. Preserved Pb cells usually had broad host-parasite interphases, whereas dying ones had narrow interphases. The outer layer of the fungus wall was sometimes broken by PMN in some focal points, broken pieces being peeled off and phagocytosed. Small Pb cells were uninuclear, and were often related to broad interphase. Large Pb cells were multinucleated with irregularly shaped wall, and sometimes had lomasome and/or myelin like structures. Different interaction patterns of Pb with inflammatory cells may be due to functionally different host cell flow to the inoculation site or due to the age of Pb cells or both.

KEYWORDS: Paracoccidioidomycosis; Intratesticular Inoculation; Transmission Electron Microscopy.

INTRODUCTION

Established and active lesions of paracoccidioidomycosis in humans and experimental animals are characterized by chronic granulomatous inflammation with suppurative center containing yeast form of *Paracoccidioides brasiliensis* (Pb) [17]. Polymorphonuclear neutrophils (PMNs) represent the first inflammatory response to the invading Pb cells, and play an important role on the modulation of the disease [7, 11].

In vitro incubation of Pb with human [8, 9, 10] or animal [3, 13] phagocytes has been used to test phagocytic and killing capacity of those cells. Activity of PMNs may be modulated, and their digestive capacity is known to increase by previous contact of the host animal with the Pb antigen. Among murine PMNs, collected from peripheral blood, elicited intraperitoneally with thioglycollate, or with the antigen in Pb sensitized and non-sensitized mice, antigen-elicited peritoneal PMNs from Pb sensitized mice have been reported to have the most potent fungicidal activity [13]. The proportion of ghost cells increases as a function of incubation time and increased number of leukocytes [8]. Interestingly, PMNs

Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, 1-8-1 Inohana, Chuo-Ku, Chiba-Shi, Chiba 260, JAPAN
(1) Department of Pathology, Faculty of Medicine, UNESP, 18618-000, Botucatu, São Paulo, Brazil.
(2) Institute of Basic Medicine, Jikei Medical University, 3-25-8 Nishi-Shinbashi, Minato-Ku, Tokyo, 105 JAPAN
Address for correspondence: K. I. R. COELHO, Department of Pathology, Faculty of Medicine, UNESP, 18618-000 Botucatu, São Paulo, Brazil, Fax (0149) - 21-23-48

from human paracoccidioidomycotic patients have low ability to digest Pb cells *in vitro*, although they are able to phagocytose the latter [9].

Phagocytosis is evaluated as an important step to kill Pb cells but it may not be essential, specially when the fungal cells are too big for inflammatory cells. Under this condition frustrated phagocytosis may occur with gross extracellular release of lysosomal contents. In human [17] and animal [11] paracoccidioidomycotic lesions, PMNs are often observable surrounding instead of phagocytosing Pb cells.

Ultrastructural studies of Pb cultured as yeast or mycelial forms have been done for about three decades [4, 5, 14] but only a few ultrastructural studies have been published on infected human [7] and animal [11] tissues. In vivo model for studying the fungal and inflammatory-cell relationship is the main point of the present paper, using intratesticularly inoculated hamster.

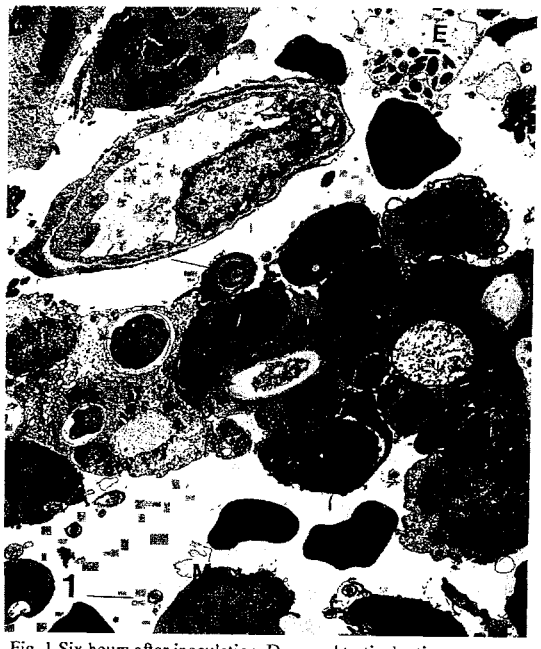

Fig. 1 Six hours after inoculation. Damaged testicular tissue represent the background. One alive and several dead Pb cells are phagocytosed and involved by PMNs. One small living Pb cell in PMN is surrounded by a visible space between the host cell and the parasite (arrow). The rest are dead cells (arrow heads) in very close contact with PMNs. One macrophage (M) and one eosinophil (E) are also seen (5,000 x).
The bar in all the figures indicates 2 μm.

MATERIAL AND METHODS

Animals

Twenty eight adult male Syrian hamsters of about two months of age were used.

Inoculum

Pb strain 18 from the Department of Immunology, University of São Paulo, Brazil was cultured as yeast like form on brain-heart-infusion agar slants at 37°C for seven days. Cells were harvested and suspended in a sterile phosphate buffered saline (PBS), pH 7.4, centrifuged for five minutes at 1,500 rpm, resuspended and washed twice in PBS, and sonicated once for one minute at 70 cpm. Viability, estimated by fluorescein-diacetate technique, was of 78%. Concentration was adjusted to $1x10^7$ cells 0.2 ml^{-1} which was the inoculum size per animal.

Experimental procedure

All animals were inoculated intratesticularly (the right testis) under superficial ether anesthesia. Four animals were sacrificed by bleeding through aorta sectioning under ether anesthesia, six, 12, 24 hours, two and three days after inoculation. Two or three animals were sacrificed five, seven and 14 days after inoculation. Only the right testis was collected from each animal, and tissues containing inoculation site lesion were taken and fixed immediately in 2.5% phosphate buffered glutaraldehyde at pH 7.2, for at least four hours. Then the material was washed with buffer and post fixed in 1% osmium tetroxide for two hours, washed, and immersed in 5% uranyl for four to 12 hours, dehydrated with ethanol and embedded in araldite. Ultrathin sections were cut and stained with uranyl acetate and lead cytrate. Samples were examined under Hitachi H-700 H or Japan Electron Optic Laboratory JEM 1200 transmission electron microscope.

RESULTS

Host-parasite interactions

In the initial inoculation site lesion (six hours after inoculation) alive and dead Pb cells were observed, mixed with damaged testicular tissues. They were involved in inflammatory cells, i. e., PMNs besides a small number of mononuclear cells and eosinophils (Figs. 1-2). Small Pb cells were phagocytosed by PMNs or macrophages, and large ones were surrounded by a team of inflammatory cells (Fig. 1). From 24 hours after inoculation on, PMNs were gradually replaced by

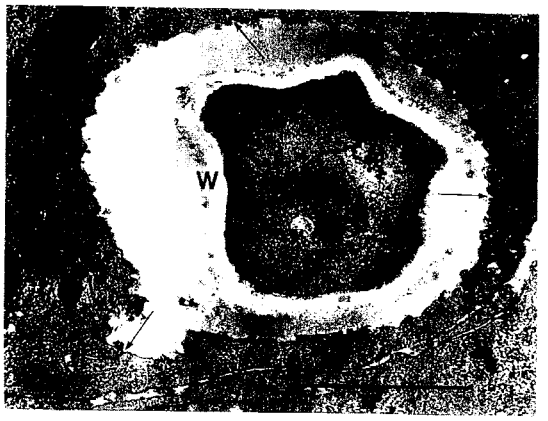

Fig. 2 - Six hours after inoculation. Living Pb cell with two layered cell wall (W). The outer layer is fibrilar and more electron-dense, and the inner layer is homogeneous and less electron-dense. The plasma membrane is thin, regular and electron-dense with some corrugated segments. A few mitochondria (M) and glycogen particles (G) are seen. Note the wide host-parasite interphase (I) which appears mostly empty. The inner limit is the outer layer of the fungal wall (W) and the outer limit is a continuous surrounding plasma membranes of inflammatory cells with intimate intercellular connection (arrows) (mag. 20,000 x).

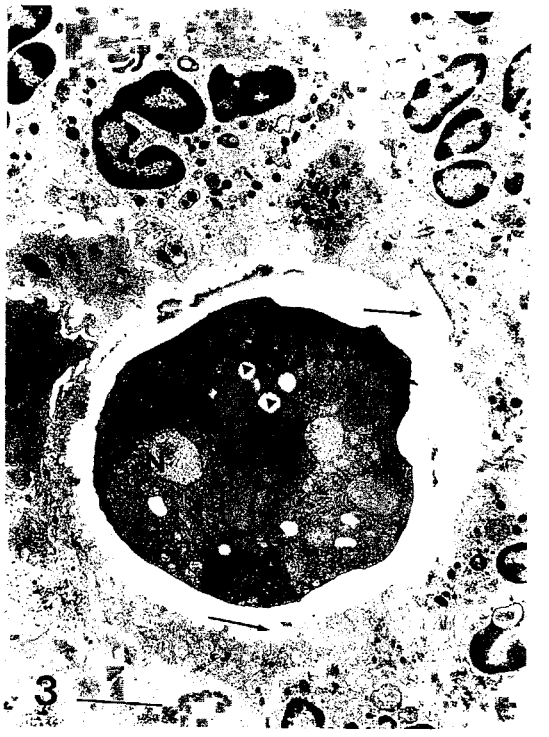

Fig. 3 - Two days after inoculation. Pb cell circumscribed by a team of PMNs. Note the cell wall of irregular thickness, some parts of the outer layer being broken to pieces, peeled off and separately phagocytosed (arrows). Fungal protoplasm is preserved, multinucleated (N) with glycogen particles (G) and some lipid droplets ( ).
The host-parasite interphase is irregular with areas where contact between fungus and PMNs is very close. Inflammatory cells have loose intercellular connection and interphase is open to extracellular milieu (mag. 7,200 x).

mononuclear cells, and later by epithelioid and a few giant cells. Dead fungi were involved in PMNs or macrophages but most of living Pb cells were in contact with PMNs during all the experimental period.

Contacts between inflammatory cells and Pb were very close or separated by a host-parasite interphase; this interphase was usually an irregular space that was enclosed or open to extracellular milieu. It was enclosed when its inner limit was represented by a regular and well preserved fungal cell wall surface and other limit represented by a continuous inflammatory cells cytoplasmic membrane (fig. 2); in this case this space was empty or contained some amorphus, fibrillar and/or vesicular material. The interphase was open when the surrounding inflammatory cells intercellular junctions were loose, leaving a channel between the fungus containing space and extracellular milieu (fig. 3). This latter type of host-parasite interaction was associated with a very well preserved fungal cell or with a fungal cell which outer layer of its wall was broken and torn to pieces; sometimes these pieces were peeled off and phagocytosed by inflammatory cells leaving an irregular cell wall surface and only continuous inner layer in contact with fungal cell plasma membrane (Fig. 3).

Protoplasms of some fungi with broken cell wall were still preserved (Fig. 3); others were in different stages of degeneration beginning with vacuolar changes (Fig. 4) and ending with granular amorphus transformation. When protoplasm was totally destroyed inflammatory cells as PMNs or macrophages sometimes invaded the empty cell wall.

Fungal cell morphology

Pb cells were always present at the inoculation site lesion and, their number was higher during the first hours up to one day after inoculation and gradually decreased towards the end of experiment. They were represented by living, dying and dead cells with variable sizes.

Small living cells had two layered thick and regular cell wall with fibrilar and more electrondense outer layer and homogeneous and less electrondense inner layer; plasma membrane was usually thin, electrondense and regular. Subcellular structures were represented by a

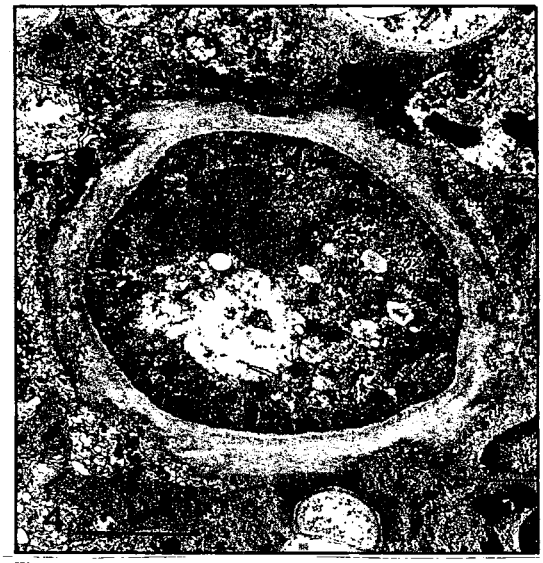

Fig. 4 - Seven days after inoculation. Pb cell with a partially disrupted protoplasm showing three nuclei (N), peripherally placed mitochondria, scattered glycogen particles (arrows) and the ill-stained plasma membrane. Cell wall is irregular in thickness with broken points, and is in a very close contact with inflammatory cells (Mag. 12,000 x).

single nucleus, few mitochondria, endoplasmic reticulum and a few lipid droplets.

Large fungi had two or occasionally three layered cell wall in close contact with plasma membrane; their subcellular structures were represented by multiple nuclei with nucleoli, mitochondria, endoplasmic reticulum with or without attached ribosomes and a variable amount of lipid droplets. Glycogen particles were in most of cases dispersed or in small groups at random (Figs. 2, 4, 5), but sometimes assembled in large groups at the center of protoplasm or in contact with the plasma membrane (Fig. 3). Small Pb cells were not rich in glycogen particles. Dividing Pb cells could not be observed, but sometimes chromatic material was arranged as grumous structures.

Similarly to other fungi, the Pb plasma membrane was a thin trilaminated structure with an electron-lucent middle layer between two electron-dense layers. However, the Pb plasma membrane showed local irregularity in thickness. Some budding points were in close contact with this thickened segments. Sometimes the irregularity corresponded to plasma membrane corrugation (Fig. 2) or protrusion of small vesicles which appeared to be secreted from the plasma membrane (Fig. 5), a lomasoma like structure. Moreover intracytoplasmic membrane system were seen with variable shape as reduplication of the plasma membrane or as a concentric membrane arrangements similar to myelin figures. Interestingly, these membrane systems were never observed in small, young Pb cells.

DICUSSION

The purpose of this experiment was to study the interaction of inflammatory cells with the fungus and its morphology.

It is known that after PMN influx as the first response of animal tissues to invading microorganisms, granulomatous lesion develops when the invaders are not digested. Granulomatous lesion may develop early when an individual has abnormal PMN function [9].

Based on this knowledge, *in vitro* investigations have been carried out in order to study the interaction between PMNs and Pb by co-culturing them. According

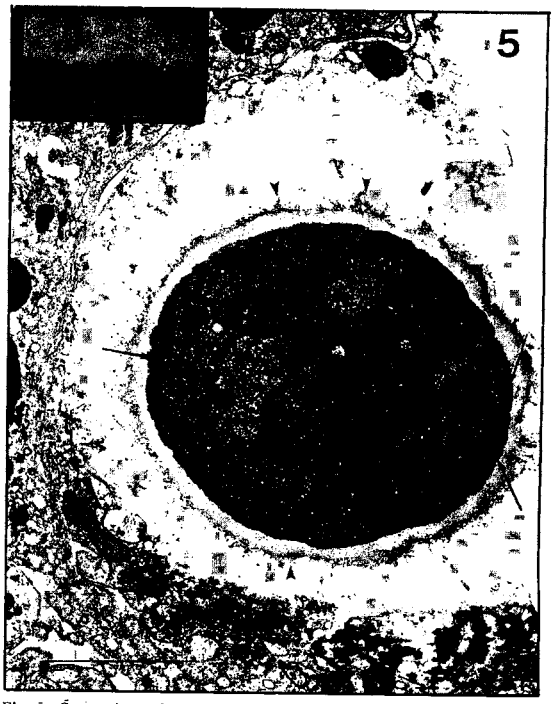

Fig. 5 - Seven days after inoculation. Living Pb cell showing the cell wall with fibrillar and electron-dense outer layer; inner layer is homogeneous and more electron-lucent. Note wide interphase containing fibrillar (arrow heads) structures extending from the outer layer of the cell wall. From space to space the plasma membrane presents bunchs of vesicle like structures protruding from its outer surface towards the cell wall (arrows). Cytoplasmic structures, such as nuclei (N) and mitochondria (M), are well preserved (mag. 15,000 x). (Inset) Vesicle like structures (mag. 52.500 x).

to those assays different proportions of Pb are phagocytosed and killed by inflammatory cells within few hours as measured by different methods including TEM.

In our model six hours after Pb inoculation, besides a number of PMNs a few mononuclear cells and eosinophils were also seen mixed with disrupted testicular tissue and several living, dying and dead Pb cells. Fungal cells were phagocytosed or circumscribed by PMNs and preserved or in different stages of digestion. The viability of inoculum was 78%, but the proportion of dead cells found in early lesions appeared to be higher than 22%. These data indicate that killing of Pb cells by PMNs started early after inoculation as observed *in vitro* assays. *In vitro* studies comparing PMNs collected from sensitized mice peripheral blood and thioglycollate - induced peritoneal exsudate from non sensitized animals, the killing capacity of the former was higher and appeared to be mediated by toxic products liberated during the oxidative metabolism of antigen elicited PMNs from sensitized mice interacting with Pb [13].

Inflammatory cell migration, induced by different subcellular fractions of Pb inoculated into the peritoneal cavity of rats, have shown that lipid and polysacharide Pb fractions were responsible for cellular influx four hours after challenge [1]. In this experiment the early cellular increase was due to PMNs with maximum peak between one and two days. They gradually decreased up to three days and there was concomitant mononuclear cells increase. This sequence might be due to the activation of complement system and subsequent chemotaxis for PMNs; another suggestion was the possibility of macrophages being initially stimulated by above mentioned fractions, producing chemotactic factors for PMNs [2]. If so, a few mononuclear cells present at the beginning of our experiment might be responsible for PMNs exudation in the lesion a few hours after inoculation. Inflammatory cells were always present, phagocytosing or involving fungal cells but something lead to different interactions between them. Sometimes a wide host-parasite interphase appeared to be enclosed surrounding preserved fungal cell. Othertimes the interphase had an irregular amplitude, was open to extracellular milieu, the inflammatory cells been often in close contact with the fungal cell wall; this wall was sometimes broken to pieces and peeled off by splitting at the middle of its thickness. After this damage, inner layer and plasma membrane, although morphologically preserved seemed to be unable to keep the fungal life. This sequence was suggested by the presence of Pb cells with partial destruction of their outer wall layer and protoplasms in different steps of aspects: sub-cellular structures still maintained, partial destruction with vacuolar degeneration, total destruction and transformation to ghost cells. Intercommunication of the space around the outer wall layer with extracellular milieu was described for lobomycosis and its role on antifungal drug effectiveness discussed [16]. Through this channel foreign substances may arrive from or the fungal metabolic products scape to the interstice. PMNs may be in different stages of activation as shown *in vitro* experiment. On the other hand smaller Pb cells usually have thicker cell wall and are more often preserved and related to wider host-parasite interphase; larger Pb cells have thinner cell wall, more often broken and with irregular thickness. PMNs in contact with different strains of Pb with variable virulence tested in vitro have shown different digestive ability [9] and virulence have been related to the cell wall biochemical composition [15].

The preserved protoplasm in early stage of cell wall damage may correspond to an aged, less resistant Pb cell to the PMNs digestive actions. Based on above data the authors suggest that as the fungal protoplasm degeneration progresses the interphase disappears and fungal and inflammatory cells contact becomes very close.

Pb cells were always present from the beginning up to 14 days after inoculation. Most of sub-cellular structures of preserved Pb cells were similar to what have been described by other authors but some details are worthy of comments. Most of cell walls were seen with two layers and the middle layer possibly correponded to the splitting point when they were destroyed. Large amount of glycogen has been described for small fungi by other authors [4, 5] but ours showed little or none. Living fungi presented regular cell wall and regular or irregular plasma membrane, sometimes with thickened segments related to budding points; budding sites *in vitro* have been related to cell wall changes [4] which was sometimes seen in our samples. Vesicular structures protruding from the plasma membrane towards the cell wall were similar to what has been described as lomasome which function is not known yet [5] but they were present from space to space in preserved fungal cells. Intracytoplasmic membrane system often seen as myelin like structures were usually close to the plasma membrane as described for *Arthrinium aureum* [12]. Similar structures have been described in bacteria as mesosoma and originating from invagination and reinvagination of

the plasma membrane; in fungal cells it has been related to septum formation in mycelial form. It also seems to take part in cell wall development and some relationship between mesosome and mitochondria has been described.

From the present study we can say that Pb cells are directly or indirectly chemotactic for PMNs which actively phagocyte or involve the invaders; digestive activity may be observed by intrastructural changes since the beginning of this experiment starting with breaking of the cell wall. However not all phagocytosed Pb cells are digested and the survivors are possibly responsible for the following steps of chronic disease observed in hamsters inoculated as in this experiment [11].

RESUMO

Paracoccidioidomicose experimental em hamster: microscopia eletrônica de transmissão da lesão do local de inoculação

Estudou-se sequencialmente, à microscopia eletrônica de transmissão, a interação entre *Paracoccidioides brasiliensis* (Pb) e células inflamatórias em hamsters inoculados por via intratesticular. Seis horas após inoculações havia predominância de neutrófilos, estando presentes algumas células mononucleares e eosinófilos. Os neutrófilos foram progressivamente substituídos por células mononucleares. Fungos viáveis apresentavam-se fagocitados ou circunscritos por células inflamatórias, geralmente com ampla interface hospedeiro-parasita. Fungos mortos ou degenerados eram acompanhados de interfase estreita. A camada externa da parede do Pb era às vezes quebrada quando em contacto com neutrófilos, em vários pontos, sendo os fragmentos dessa parede descamados e fagocitados. Células fúngicas pequenas com um único núcleo se relacionavam com ampla interfase enquanto as células maiores e multinucleadas apresentavam paredes irregulares, por vezes, contendo lomasoma e/ou estrutura semelhante à mielina. Diferentes padrões de interação do Pb com células do hospedeiro podem ser decorrentes do afluxo de células inflamatórias funcionalmente diferentes ao local de inoculação ou à idade dos fungos ou ambos os fatores.

ACKNOWLEDGEMENT

During the course of these studies Dra. K. I. R. Coelho was supported by the Japanese Ministry of Education, Science and Culture as a guest professor-foreign researcher at Chiba University Research Center for Pathogenic Fungi and Microbial Toxicoses.

REFERENCES

1. ALVES, L. M. C.; FIGUEIREDO, F.; BRANDÃO FILHO, S. L.; TINCANI, I. & SILVA, C. L. - The role of fractions from *Paracoccidioides brasiliensis* in the genesis of inflammatory response. **Mycopathologia** (Den Haag), **97**: 3-7, 1987.

2. CALICH, V. L. G. & BURGER, E. - PMN chemotactic factor produced by glass-adherent cells in the acute inflammation by *Paracoccidioides brasiliensis*. **Brit. J. exp. Path., 66**: 57-65, 1985.

3. CANO, L. E.; ARANGO, R.; SALAZAR, M. E. et al. - Killing of *Paracoccidioides brasiliensis* by pulmonary macrophages and the effect of cytokines. **J. med. vet. Mycol., 30**: 161-168, 1992.

4. CARBONELL, L. M. - Cell wall changes during the budding process of *Paracoccidioides brasiliensis* and *Blastomyces dermatitidis*. **J. Bact., 94**: 213-223, 1967.

5. CARBONELL, L. M. & GIL, F. - Ultraestrutura del *Paracoccidioides brasiliensis*. In: DEL NEGRO, G; LACAZ, C. da S. & FIORILLO, A. M., ed. - *Paracoccidioidomicose. Blastomicose sul americana*. São Paulo, SARVIER-EDUSP, 1982. p. 23-24.

6. FAVA NETTO, C.; BRITO, T. & LACAZ, C. da S. - Experimental South American blastomycosis of the guinea-pig. An immunologic and pathologic study. **Path. et Microbiol.** (Basel), **24**: 192-206, 1961.

7. FURTADO, J. S.; BRITO, T. & FREYMULLER, E. - The structure and reproduction of *Paracoccidioides brasiliensis* in human tissue. **Sabouraudia, 23**: 245-251, 1967.

8. GOIHMAN-YAHR, M.; ESSENFELD-YAHR, E.; ALBORNOZ, M. C. et al. - New method for estimating digestion of *Paracoccidioides brasiliensis* by phagocytic cells *in vitro*. **J. clin. Microbiol., 10**: 365-370, 1979.

9. GOIHMAN-YAHR, M.; ESSENFELD-YAHR, E.; ALBORNOZ, M. C. et al. - Defect of *in vitro* digestive ability of polymorphonuclear leukocytes in paracoccidioidomycosis. **Infect. Immun., 28**: 557-566, 1980.

10. GOIHMAN-YAHR, M.; ROTHENBERG, A.; ROSQUETE, R. et al. - A novel method for estimating killing ability and digestion of *Paracoccidioides brasiliensis* by phagocytic cells *in vitro*. **Sabouraudia, 23**: 245-251, 1985.

11. IABUKI, K. & MONTENEGRO, M. R. - Experimental paracoccidioidomycosis in Syrian hamster: morphology, ultrastructure and correlation of lesion with presence of specific antigen and serum levels of antibodies. **Mycopathologia** (Den Haag), **67**: 131-141, 1979.

12. LARRONDO, J. V. & CALVO, M. A. - Ultrastructure of concentric membrane system in *Arthrinium aureum*. **Mycopathologia** (Den Haag), **117**: 153-155, 1992.

13. McEWEN, J. G.; BRUMMER, E.; STEVENS, D. A. & RESTREPO, A. - Effect of murine polymorphonuclear leukocytes on the yeast form of *Paracoccidioides brasiliensis*. **Amer. J. trop. Med. Hyg., 36**: 603-608, 1987.

14. MINGUETTI, G.; HOFMEISTER, R. M.; FAVARO, M. & FREITAS, O. T. - Ultraestrutura do *Paracoccidioides brasiliensis* - na fase leveduriforme. **Rev. Inst. Med. trop. S. Paulo, 25**: 161-167, 1983.

15. SAN-BLAS, G. - The cell wall of fungal human pathogens: its possible role in host-parasite relationship. **Mycopathologia** (Den Haag), **79**: 159-184, 1982.

16. SESSO, A. & BARUZZI, R. G. - Interaction between macrophage and parasite cells in lobomycosis. The thickened cell wall of *Paracoccidioides loboi* exhibits apertures to the extracellular milieu. **J. Submicrosc. Cytol. Path., 20**: 537-548, 1988.

17. URIBE, F.; ZULUAGA, A. I.; LEON, W. & RESTREPO, A. - Histopathology of cutaneous and mucosa lesions in human paracoccidioidomycosis. **Rev. Inst. Med. trop. S. Paulo, 29**: 90-96, 1987.

Recebido para publicação em 11/08/1993.
Aceito para publicação em 27/09/1993.