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Short communication

Morphological aspects of *Paracoccidioides brasiliensis* in lymph nodes: implications for the prolonged latency of paracoccidioidomycosis?

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In order to determine if fungal morphology in tissues would furnish indications on the viability of *Paracoccidioides brasiliensis* yeast cells, lymph node biopsies from five patients with paracoccidioidomycosis, including one with residual circumscribed lesions, were examined. A program that allows transferring of microscopic images to the computer for further processing was used. In the four active cases, the infected lymph nodes had over 49% of healthy-looking yeast cells while in the case of the residual lesion, this figure was smaller (21%). The residual had a larger proportion of aberrant yeast cells, with predominance of shell-like, empty cells (33%) and crescent bodies (30%); balloon-like yeasts were also seen (16%). The last two types of cells were also seen in the active lesions, but in smaller proportions, $\leq 8\%$ and $\leq 9\%$, respectively. The number of multiple budding yeast cells, which clearly demonstrate fungal viability, ranged 24–33% in active cases but was only 5% in the residual lesion. Although the number of biopsies examined is small, the results tend to indicate that the morphology of *P. brasiliensis* yeast cells in walled-off tissues is abnormal and that the number of viable elements is small. There might be a connection between these findings and the long latency period illustrated by those patients with paracoccidioidomycosis that have been diagnosed in non-endemic areas. Additionally, if *P. brasiliensis* yeast cells were to be subjected to the micro-aerophilic environment present in walled-off lesions, they would probably require a long time to multiply. Under these circumstances, the mycosis would also need many years to manifest.

Keywords latency, *Paracoccidioides brasiliensis*, tissue morphology

Introduction

Paracoccidioidomycosis, a disease caused by the dimorphic fungus *Paracoccidioides brasiliensis*, is restricted to Latin American countries, where it afflicts an important number of the inhabitants in the region [1–4]. In Brazil, the center of the endemic, the disease causes approximately 200 deaths per year [5]. The asymptomatic primary infection may result in residual foci containing viable fungal cells; the latter can give rise to overt disease by endogenous reactivation, as shown by cases diagnosed outside of the geographic limits of the mycosis [6,7].

In 1985, Ajello & Polonelli [6] reviewed a series of 42 non-autochtonous cases diagnosed in Europe, the United States and Asia, and recorded a most puzzling aspect, namely, the long delay between the moment of infection

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in Latin America and the manifestation of the disease in non-endemic settings. Since then, 12 new cases have been added to this list for a total of 54 cases published to date [8–17]. All these records were reviewed for the present study and it was found that in 35 the length of the asymptomatic period had been given; the range was 5 months to 60 years, with an estimated mean of 14 years [6–8,11,13,14,17].

Additionally, a literature search conducted for the present purpose, revealed that in the recognized endemic areas, there were 11 cases of asymptomatic infections occurring in healthy individuals in whom biopsy specimens had revealed residual lesions that harbored *P. brasiliensis*. In these cases, the fungus was found accidentally or the infection was brought to the physician's attention because of the presence of secondary problems [18–27]. Additionally, in several of the cases, a qualitative description of the shape of *P. brasiliensis* yeast cells inside the residual lesion was also offered [19–21,25,27]. Although no latency period could be calculated for these cases, the histologically-proven lesions constitute residual foci and as such, they may reveal interesting aspects concerning the effects exerted on the fungus by the host's tissue response.

The above observations prompted the present microscopical study, aimed at determining if fungal shape and size within human nodular lesions could be taken as a measure of fungal activity.

Materials and methods

Five lymph node biopsies from patients with paracoccidioidomycosis were studied. Sections were prepared and stained with the methenamine silver (Gomori) technique [28]. Three of the biopsies had been taken from cervical and two from mesenteric lymph nodes. In four patients the disease was active (cases 1–4), while in the remaining one (case 5), the symptoms described below appeared 5 years after completion of an apparently successful course of treatment for paracoccidioidomycosis. This patient consulted because of ascites due to hypertrophy of the mesenteric lymph nodes and flow blockade [24].

In the tissue sections, 100–150 *P. brasiliensis* yeast cells were counted and the differences in size and shape were recorded. The NIH Image 1.61/ppc computer program (Power Macintosh; National Technical Information Service, Springfield, VA, USA) was employed to transfer the images from the microscope to the computer and then to measure individual cells, as well as joining several of them in a single frame for detailed inspection [29].

Yeast morphology was assessed as follows:

1. Viable cells were considered to be intact yeasts with a complete cell wall that had an oval to round shape and presented an uniformly stained cytoplasm. Blastoconidia production, including multiple budding, was also recorded. Size was not taken into consideration as a measure of viability.
2. Non-viable cells were those that had broken cell walls, aberrant shapes (crescent, shell-like, distorted or balloon cells), that appeared empty and exhibited no budding.

Results

There were differences in yeast sizes among the specimens studied (Fig. 1); for instance, in case 1, most yeasts were large (>25 μm) while in case 2, most cells were smaller (<4–15 μm). In general, however, all specimens presented an array of variously-sized yeasts (4–50 μm).

As shown in Table 1, the morphology of the yeasts differed according to the activity of the lesion. In active lesions, yeasts exhibited a variety of shapes and forms (Figs 2a and 2b), with a large proportion (49–70%) of healthy-looking cells. In the residual lesion, on the contrary, there was a predominance (79%) of abnormal yeasts, classified as shell- (33%), crescent- (30%) and balloon-shaped (16%) (Fig. 3). Nonetheless, in this case there were also a certain number of well-formed, small-sized blastoconidia (21%) (Fig. 3).

The relative quantity of multiple budding yeast cells was also determined (Table 1). The proportion of multiple budding yeast cells was over 24% in the four patients with active lesions; in contrast, in the patient with residual pathology, this type of cell accounted for only 5% of all cells taken into consideration.

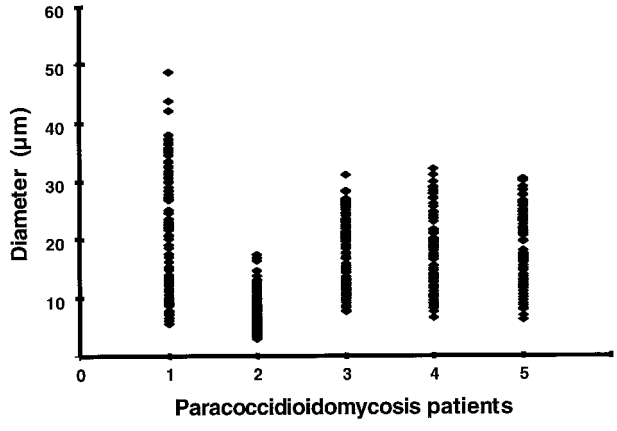

Fig. 1 Size distribution of *P. brasiliensis* blastoconidia in the lymph nodes (LN) of five patients with paracoccidioidomycosis.

Table 1 Morphological aspects of *P. brasiliensis* yeast cells in lymph nodes

		Yeast morphology (%)*				
		Normal†	Normal with multiple buds	Abnormal‡		
Case	Location of lymph node			1	2	3
1	Cervical	70	33	19	5	6
2	Cervical	60	33	26	7	7
3	Cervical	49	32	34	8	9
4	Mesenteric	52	24	36	7	5
5	Mesenteric	21	5	33	30	16

*, 100–150 yeasts counted/biopsy.
†, Normal: oval or round yeasts with intact cell walls, some with multiple buds and uniformly stained cytoplasm.
‡, Abnormal fungal cells: 1, empty or shell-like; 2, crescent shaped; 3, balloon shaped.

Discussion

Size variation in *P. brasiliensis* yeast cells is a common feature in tissues and clinical samples taken from patients with the mycosis [1–3,19–21,24,27,30]. The present quantitative observations made with a digital computer-based technique, agree with previous data and indicate that size alone does not reflect fungal viability.

On the other hand, as previously suggested by several workers, morphology of the yeast cells may be considered an indirect manifestation of fungal viability [1,19,21,27,30]. In KOH preparations or Gomori-stained tissues, viable *P. brasiliensis* cells tend to be seen undergoing multiplication and, often, producing multiple buds. The shape of the yeasts was regular, the cell-walls were intact and condensed material was present in the cytoplasm. In KOH preparations, the cytoplasm contained abundant lipid droplets [1,4,31].

The present exercise, although carried out with a small number of biopsies due to the infrequent elucidation of a patient with a well-contained lesion, corroborates the above findings and further suggests that during active infection, lesions contain an elevated number (49–70%) of healthy-looking yeasts, 24–33% of which present multiple budding. In old, walled-off lesions, such as seen in case 5, almost all cells (79%) are broken, have irregular contours and appear empty. Additionally, in this case, multiple budding was markedly reduced (5%). The changes observed in this study correspond to those noted by Angulo-Ortega [19,20], who attributed morphological variations in yeast cells to the environment predominating within the lesions. According to him, when the tissues are fibrotic, calcified or cretified, as observed in residual lesions, multiple budding is sparse and the yeast cells are smaller than usual [19,20].

Variations in the morphology of *Histoplasma capsulatum* yeast cells in tissues have also been recorded in the past. In old lesions, histoplasmomas, and caseous and calcified nodules, the yeast cells may adopt aberrant morphologies. According to Pine [32], giant cells with little internal structure, a presentation suggestive of de-

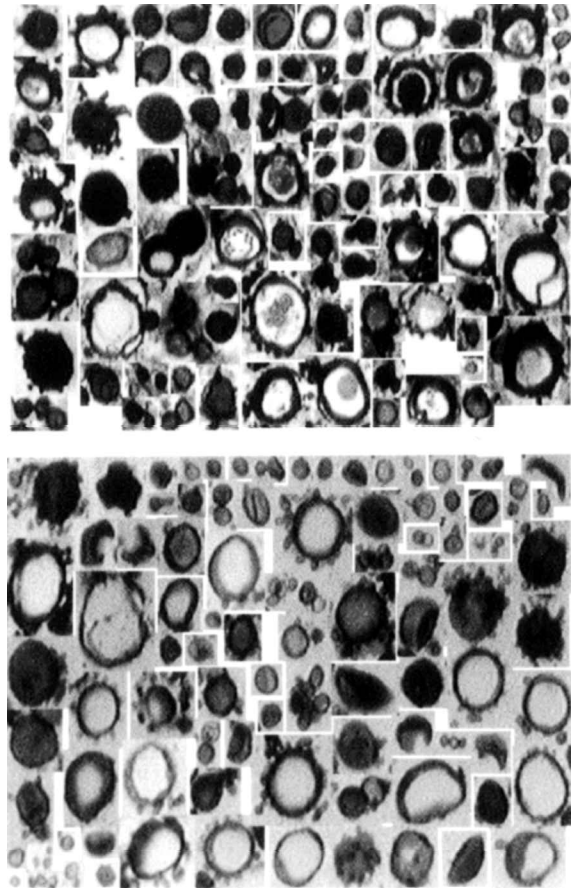

Fig. 2 Computer (NIH Image 1.61/ppc program) analysis of >100 *P. brasiliensis* yeasts as observed in patients with active paracoccidioidomycosis. (a, b) Cervical lymph nodes from patients with active disease.

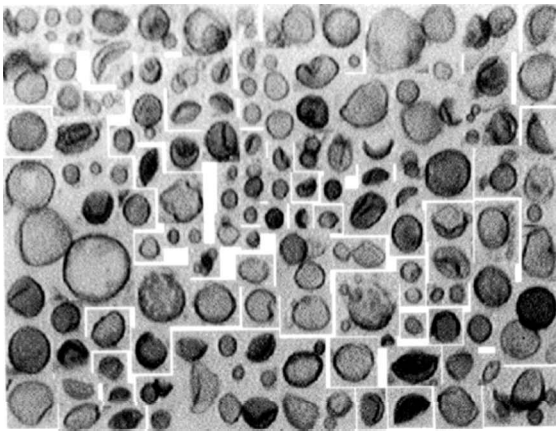

Fig. 3 Computer (NIH Image 1.61/ppc program) analysis of 150 *P. brasiliensis* yeasts as observed in the mesenteric lymph nodes of a patient with residual lesions. A few photos depict healthy-looking fungal cells. Note aberrant forms and sparse multiple budding.

generation, can be seen extracellularly in necrotic tissues. Sweany *et al.* [33] described unusual forms that were 2–3 times the width of the yeast bodies and additionally, observed some elongated dumbbell-shaped forms. Schwarz [34] also found giant forms and considered them to be moribund, non-reproductive cells. Rippon [28] commented that *H. capsulatum* yeasts up to 20 μm in diameter can be visualized in old necrotic lesions along with small normal looking cells. These data indicate that in residual lesions, morphological changes may also appear in other dimorphic Onygenalean fungi.

In the endemic areas for paracoccidioidiomycosis, rare reports document asymptomatic patients in whose tissues *P. brasiliensis* was found accidentally. To obtain comprehensive information on this subject, a literature survey was undertaken. The hypothesis was that in these cases, the lesions should have corresponded to residual foci bearing the potential for future endogenous re-activation. Eleven of these cases were found. The tissue responses are described in Table 2. It was found that the infected foci were all surrounded by fibrous capsules, had calcium deposits or corresponded to well-circumscribed pneumonic or granulomatous lesions [18–26]. According to Franco & Montenegro [27,30], the tissue barrier thus created by the host immune defenses hinders blood access and, consequently, limits oxygen supply. This circumstance probably exerts pressure on fungal morphology resulting in the abnormalities mentioned above, as described by Angulo-Ortega [19,20] and Severo *et al.* [25]. Furthermore, the capacity to reproduce could also be impaired, as *P. brasiliensis* is an aerobic microorganism [4,31]. Nonetheless, some yeasts undoubtedly remain viable. This was demonstrated directly in one case with residual, walled-off lesions: the fungus was isolated successfully only under microaerophilic conditions. Repeated attempts at recovering the fungus from the pathological specimen in either liquid or solid media incubated both aerobically and anaerobically, failed [24].

With regard to the oxygen requirements of *P. brasiliensis*, experiments conducted by Restrepo *et al.* [35] and by Sano *et al.* [36] indicated that under reduced oxygen tensions, a proportion of the yeasts managed to retain their viability. With prolonged incubation, however, a decrease was noticed. Additionally, total oxygen deprivation was not well-tolerated. *In vivo* animal experiments performed concurrently by Sano *et al.* [36] showed that isolates resistant to oxygen stress were more virulent. It appears, then, that the fungus becomes tolerant of low oxygen tensions and adapts to them. Such an adaptation would be critical in survival, which would probably rely on the ability to enter a state of dormancy. Whether or not the evidence for oxygen stress tolerance as a virulence factor can be extrapolated from animal models to human patients remains to be explored.

Prolonged latency has been demonstrated in other infectious diseases. This phenomenon has been most extensively studied in tuberculosis [37]. As is the case in

Table 2 *Paracoccidioides brasiliensis* in residual lesions observed in Latin American residents

Case	Lesions: location, quantity	Histopathological description	Reference
1	Lungs, solitary	Solid, circumscribed nodule	[21]
2	Lungs, multiple	Encapsulated, partially calcified caseous nodules	[19,20]
3	Lungs, multiple	Fibrous nodules, calcified	[19,20]
4	Lungs, solitary	Fibrous nodules, calcified	[19,20]
5	Adrenal solitary	Calcified, caseous, encapsulated nodule	[19,20]
6	Lungs, solitary	Necrotic nodule with fibrous capsule	[23]
7	Abdominal, multiple	Fibrous nodules partially calcified	[24]
8	Lungs, solitary	Necrotic, encapsulated nodule with fibrous capsule	[22]
9	Lungs, multiple	Granulomatous nodules with caseification	[25]
10	Abdominal, multiple	Calcified fibrous necrotic nodules	[26]
11	Lungs, solitary	Granulomatous, caseous necrotic nodule	[18]

paracoccidioidomycosis, latent mycobacterial infections can reactivate after many years in connection with immunosuppressive conditions, such as acquired immune deficiency syndrome (AIDS). During latency, *Mycobacterium tuberculosis* loses its acid fast properties; furthermore, it becomes oxygen-intolerant and is no longer susceptible to otherwise effective antibacterial agents. The reasons for these changes are being explored and several important research avenues are being pursued, some of them focused on the newly discovered genetic traits connected to *M. tuberculosis* dormancy (*sigF* and *acr* genes) [37]. *M. tuberculosis* is a bacterial agent but the resemblance of its dormancy to that *P. brasiliensis* is strong.

It is apparent that much remains to be done before we begin to understand the basis for the remarkable survival capacities of *P. brasiliensis* within residual lesions. We should also address the host conditions that promote the awakening of the dormant microorganism, especially in apparently immunocompetent hosts.

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References

1 Brummer E, Castañeda E, Restrepo A. Paracoccidioidomycosis: An update. *Clin Microbiol Rev* 1993; **6:** 89–117.
2 Londero AT, Ramos CD. Paracoccidioidomicose: Estudo clínico-micológico de 260 casos observados no interior do Estado do Rio Grande do Sul. *J Pneumol (Brazil)* 1990; **16:** 129–132.
3 Franco MF, Mendes RP, Moscardi-Bacchi M, Rezkallah-Iwasso M, Montenegro MR. Paracoccidioidomycosis. *Clin Trop Med Commun Dis* 1989; **4:** 185–220.
4 Lacaz CS, Porto E, Martins JEC. Paracoccidioidomicose. In: *Micologia Medica*, 8th edn. São Paulo, Brazil: Sarvier Editora, 1991: 248–261.
5 Coutinho Z, Silva D, Lázera M, Petri V, Sabrozo PC, Wanke B. Mortalidade por paracoccidioidomicose. Brasil 1980–1995. *II Congreso brasileiro de Micologia*, Rio de Janeiro, Brasil, April 1998. Rio de Janeiro: Sociedade Brasileira de Micologia, 1998: Abstract A57.
6 Ajello L, Polonelli L. Imported paracoccidioidomycosis: A public health problem in non-endemic areas. *Eur J Epidemiol* 1985; **1:** 160–165.
7 Greer DL, Restrepo A. La epidemiología de la paracoccidioidomicosis. *Bol Ofic Sanit Panamer* 1977; **82:** 428–445.
8 Chikamori T, Saka S, Nagano H, *et al.* Paracoccidioidomycosis in Japan. Report of a case. *Rev Inst Med Trop São Paulo* 1984; **26:** 267–271.
9 Sugar A, Restrepo A, Stevens DA. Paracoccidioidomycosis in an immunocompromised host. *Am Rev Resp Dis* 1984; **129:** 340–342.
10 Neveling F. Parakokzidioidose-Infektion bei Abentuerurlaub in Amazonasgebiet. *Prax Klin Pneumol* 1985; **42:** 722–725.
11 Washburn RG, Bennett JE. Paracoccidioidomycosis case report: cure with amphotericin B and triple sulfa. *J Med Vet Mycol* 1986; **24:** 235–237.
12 Koehler C, Klotz M, Daus H, Schwarze G, Dette S. Vizcerale Paracoccidiodiomycosis bei einem Goldgräber aus Brasilien. *Mycoses* 1988; **31:** 395–403.
13 Coelho KIR, Sano A. Paracoccidioidomycosis in Japan. *Annual Report of the Research Center for Pathogenic Fungi and Microbial Toxicoses*. Chiba, Japan: Chiba University, 1992: 55–59.
14 Manns BJ, Baylis BW, Urbanski SJ, Gibb AP, Rabin HR. Paracoccidioidomycosis: case report and review. *Clin Infec Dis* 1996; **23:** 1026–1032.
15 Pereira Jr M, Pereira M, García-Garcìa A, Toribro J. Immunologic features of a case of paracoccidioidomycosis treated with fluconazole. *Acta Derm Venereol (Stockh)* 1996; **76:** 84–85.
16 Koya G. An autopsy case of mixed infection of *Leishmania donovani* and *Blastomyces brasiliensis*: emphasis in the histopathogenesis of reticuloendotheliosis. *Acta Pathol Jpn* 1964; **14:** 223–229.
17 Joseph EA, Mare A, Irving WR. Oral South American blastomycosis in the United States of America. *Oral Surg Oral Med Oral Pathol* 1966; **6:** 732–737.
18 Alves JWS, Michel GT, Londero AT. Paracoccidioidoma: case record and review. *Mycopathologia* 1997; **137:** 83–85.
19 Angulo-Ortega A. Calcifications in paracoccidioidomycosis: are they the morphological manifestations of subclinical infections? In: *Paracoccidioidomycosis. First Panam Symp.* Washington: PAHO Sci Public. N° 254, 1972: 129–133.
20 Angulo-Ortega A. Lesiones numulares de origen inflamatorio. Paracoccidioidomas. *Tórax*, *Bol Postgr Neumol Clin Integr (Venezuela)* 1975; **11:** 25–34.
21 Brass K. Observaciones sobre la antomía patológica, patogenia y evolución de la paracoccidioidomicosis. *Mycopathologia* 1969; **37:** 119–138.
22 Melo IS, Londero AT. Spontaneously resolving pulmonary lesions in paracoccidioidomycosis. *Mycopathologia* 1983; **82:** 57–59.
23 Restrepo A, Robledo M, Giraldo R, *et al.* The gamut of paracoccidioidomycosis. *Am J Med* 1976; **61:** 33–42.
24 Restrepo A, de Bedout C, Cano LE, *et al.* Recovery of Paracoccidioides brasiliensis from a calcified lymph node by microaerophilic incubation in liquid media. *Sabouraudia* 1981; **19:** 295–300.
25 Severo LC, Porto NS, Camargo JJ, Geyer GR. Multiple paraccocidioidomas, simulating Wegener's granulomatosis. *Mycopathologia* 1985; **91:** 117–119.
26 Silva AL, Giacomin RT, Silva BD. Paracoccidioidomicose ganglionar abdominal calcificada. *Rev Soc Brasil Med Trop* 1991; **24:** 253–255.
27 Franco MF, Montenegro MRG. Anatomia Patológica. In: Del Negro G, Lacaz CS, Fiorillo AM, eds. *Paracoccidioidomicose (Blastomycosis Sulamericana)*. São Paulo, Brasil: Sarvier-EDUSP, 1982: 97–117.
28 Rippon JW. Histoplasmosis. In: *Medical Mycology. The pathogenic Fungi and the Pathogenic Actinomycetes*, 3rd edn. Philadelphia: Saunders, 1988: 381–423.
29 Seebacher T, Bade EG. Quick and easy molecular weight determination with Macintosh computers and public domain image analysis software. *Electrophoresis* 1996; **17:** 1573–1574.

30 Montenegro MR, Franco M. Pathology. In: Franco M, Lacaz CS, Restrepo A, Del Negro G, eds. *Paracoccidioidomycosis*. Boca Raton, FL: CRC Press, 1994: 131–150.
31 Lacaz CS. *Paracoccidioides brasiliensis*: morphology, evolutionary cycle, maintenance during saprophytic life, biology, virulence, taxonomy. In: Franco MF, Lacaz CS, Restrepo A, Del Negro G, eds. *Paracoccidioidomycosis*. Boca Raton FL: CRC Press, 1994: 13–25.
32 Pine L. Morphological and physiological characteristics of *Histoplasma capsulatum*. In: Sweany HC, ed. *Histoplasmosis*. Springfield IL: CC Thomas, 1960: 40–75.
33 Sweany HC, Gorelick D, Coller FC, Jones JL. Pathology and some diagnostic features of histoplasmosis in patients entering a Missouri hospital. The "B" group. *Dis Chest* 1962; **42:** 128–150.
34 Schwarz J. Histoplasmosis. In: Baker RD, ed. *The Pathologic Anatomy of the Mycoses. Human Infections with Fungi, Actinomycetes and Algae*. New York: Springer Verlag, 1971: 67–130.
35 Restrepo A, Jimenez BE, de Bedout C. Survival of *Paracoccidioides brasiliensis* under microaerophilic conditions. *Sabouraudia* 1981; **19:** 301–305.
36 Sano A, Miyaji M, Nishimura K, Franco MF. Studies on the relationship between pathogenicity of *Paracoccidioides brasiliensis* in mice and its growth rate under different oxygen atmospheres. *Mycopathologia* 1991; **114:** 93–101.
37 Parrish NM, James DD, Bishal WR. Mechanisms of latency in *Mycobacterium tuberculosis*. *Trends Microbiol* 1998; **6:** 107–112.