CHLAMYDOSPORE FORMATION BY Paracoccidioides brasiliensis MYCELIAL FORM

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SUMMARY

To investigate the role of some adverse environmental conditions in chlamydospore formation by the mycelial form of **P. brasiliensis**, we cultured four **P. brasiliensis** isolates (18, Bt4, 1183, Pb9) at 25°C within solid agar medium either rich or poor in nutrients. Isolates 18 and 1183 were also cultured under anaerobiosis in a nitrogen atmosphere. Isolate 18 produced great number of terminal and intercalary chlamydospore after 7-10 days of culture in a medium poor in nutrients (2% agar with 0.1% dextrose and polypepton).

The three other isolates also produced chlamydospores under the same conditions, but in lower numbers. Chlamydospore production by isolate 18 was abolished when the fungus was cultured in two agar media rich in nutrients (brain heart infusion and potato dextrose agar). Anaerobic incubation of isolate 18 under an atmosphere of N2 showed small mycelial outgrowth with numerous chlamydospores. At the electron microscopical level, the chlamydospores showed one or various nuclei and numerous mitochondria, indicating great potential for further development. Accordingly, chlamydospores produced multiple budding after only 24 h incubation at 35°C. The results demonstrate that under adverse environmental conditions **P. brasiliensis** mycelial form produces chlamydospores within a short period of time.

KEY WORDS: Chlamydospore; **Paracoccidioides brasiliensis**.

INTRODUCTION

Paracoccidioides brasiliensis, the causative agent of paracoccidioidomycosis, is a thermally dimorphic fungus producing a mycelial form at room temperature and yeast form at 37°C. In the mycelial phase, it may give rise to either few nonspecific sporulating structures or characteristic spores[19, 20].

The exact mechanism of infection is not known, because of uncertainty about the natural habitat of the fungus. Most authors believe that infection is acquired by inhalation, and that conidia may be the infectious propagules[8, 20]. Recently the substrate and cultural requirements were met to the production of various types of **P. brasiliensis** conidia. Using culture media poor in nutrients and incubation time ranging from 3 to 6 weeks, RESTREPO and co-workers described the development of arthroconidia, singlecelled conidia and aleuroconidia[3, 22, 23]. The same

authors were able to separate and quantitate the arthroconidia and successfully infect mice by intranasal instillation, producing active disease in healthy animals[13]. Despite the fact that chlamydospore formation has long been described at early stages in the mycelial growth of **P. brasiliensis**[4, 5, 7, 17, 18, 19, 24], and that chlamydospores seem to be the transitional form in the mycelial to yeast conversion[4, 16], little attention has been given to its optimal cultural conditions and potential infectivity. CARBONELL & RODRIGUEZ[4] gave indication that chlamydospore formation in **P. brasiliensis** mycelial form occurred under adverse condition, namely poor oxygenation; using slant cultures, the authors observed an outermost layer, outside the culture medium, consisting of slender hyphae without spores, and a middle layer, possibly under low oxygen atmosphere, with abundant intercalary and terminal chlamydospores.

While attempting to grow **P. brasiliensis** mycelia inside the agar of a culture medium poor in nutrients[16], we observed that within a short period of time, the mycelial growth produced great number of intercalary and terminal chlamydospores. We hypothesized that the resistant cells were being formed subsequent to starvation and low oxigen content. The present investigation was aimed at studying chlamydospore formation among different isolates of **P. brasiliensis** in media with different nutritional content and different levels of 02 atmosphere.

MATERIAL AND METHODS

1. Chlamydospore formation by P. brasiliensis **isolates** — Four isolates were examined. They were selected at random from a collection of stock cultures obtained worldwide, namely strain 18 (Department of Microbiology, São Paulo State School of Medicine, Brazil), strain Bt4 (Department of Pathology, Botucatu Medical School, São Paulo, Brazil), strain Pb9 (Instituto Venezolano de Investigaciones Cientificas, Caracas, Venezuela) and strain 1183 (Centers for Disease Control, Atlanta Georgia, USA). Fresh transfers were done to slants of brain heart infusion agar (Difco, Detroit, Michigan), supplemented with 1% dextrose, and incubated at 35°C for 5 to 7 days. the growth was harvested in sterile phosphate buffered saline (PBS; pH 7.4; 1/15 M) by carefully scraping the yeast mat with a pipet-

te. The suspension was further homogenized by vortexing and then filtered through cotton gauze. The inoculum was adjusted to a concentration of 10^5 to 10^6 fungal cells/ml. Solid cultures in 90 x 20 mm petri dishes were then set up in duplicates: one ml of the yeast cell suspension was carefully mixed with 25 ml of 2% agar (immunodiffusion agar for immunoelectrophoresis, Oxoid, England), with 0.1% polypepton (Wako Pure Chemicals Ind., Osaka, Japan) and 0.1% anhydrous dextrose (Wako) (0.1% IDAgar), at 40-43°C. The cultures were prepared in duplicate for each strain. After solidification of the agar, the plates were incubated at 25°C for 5, 7 and 10 days. At day 10, the plates were incubated at 35°C for 5 days.

For direct examination of the cultures, a small sample of the medium was cut with a razor blade and placed on a microslide together with a drop of lacto-phenol cotton blue; a cover slip was then laid over it, carefully pressed, and the specimen was observed directly under a light microscope. For each dish, 2 slides were prepared. The numbers of chlamydospores present in 10 to 15 high power fields (HPF; 400X) of each slide selected at random were counted in a blind way. For histological examination, similar samples from each dish were formalin fixed, paraffin embedded, cut in 15 μm sections and stained with PAS, according to routine procedures.

2. Chlamydospore formation in poor and rich culture media — Isolate 18 and three different agar media were used, namely 0.1% IDAgar, brain heart infusion and potato dextrose agar (Difco). In all media, the agar concentration was 2%. One ml of yeast cell suspension was mixed with 25 ml of each melted medium (approx 40°C) and cultured in duplicates at 25°C for 10 days as described above. For the microscopical observation, lactophenol cotton blue preparations were made with a loopful of each plate; the numbers of chlamydospores were counted as described above.

3. Chlamydospore formation in anaerobic condition — The technique employed for culturing isolates 18 and 1183 of **P. brasiliensis** was the same described for the previous experiments. The culture medium used was 0.1% IDAgar. Cultures were kept at 25°C for 7 days under anaerobic conditions in a nitrogen atmosphere

produced as follows. A BBLGas Pak plastic jar (Becton, Dickinson Co., Cockeysville, USA) was placed inside a vinyl-isolated chamber for hazardous experiments, containing a sealed entrance and two vinyl-isolated windows for handling. A continuous flow of pure nitrogen (Nihon Sanso Co. Ltd, Tokyo, Japan) was then produced inside the chamber and the plastic jar. Afterwards the petri dishes containing the solid cultures were rapidly introduced inside the jar together with steel wool coated with copper for elimination of the residual oxygen. The jar was then sealed.

For coating the steel wool, approximately 50 g steel wool were dipped twice into 300 ml of a 1% cupric sulfate solution until decolouration of the solution. The copper-coated steel wool was squeezed to remove the excess of water and placed into the plastic jar. The copper in the surface of the wool reacts with the residual oxigen with the subsequent production of copper oxide. To ensure the presence of a atmosphere of N2 inside the jar, a balloon filled with the gas was connected to the jar through the lid and clamped when no further shrinkage of the balloon was observed.

For the microscopic examination, the procedures were the same as used in the previous experiments.

4. Electron microscopic morphology of chlamydospores — Isolate 18 was cultured as described above in 0.1% IDAgar at 25°C for 10 days. Afterwards, samples from the solid culture medium were fixed in 2.5% glutaraldehyde in Hank's solution (pH 7.2 - 7.4) at 4°C for 4 days, post-fixed in 1% osmium tetroxide in Hank's solution for 2 h, dehydrated in a graded series of ethanol and embedded in Epon-812 resin.

Ultrathin sections were doubly stained with 2% uranyl acetate in 60% ethanol, and lead citrate. They were examined with a Hitachi H-700H electron microscope at 100 kV.

RESULTS

We defined chlamydospores, as proposed by AINSWORTH[10], as thick-walled nondeciduous intercalary and terminal asexual spores formed by the rounding up of preexisting cells.

The numbers of chlamydospores produced by the four **P. brasiliensis** isolates, expressed by the median of spores present in 60 HPF (15 HPF in each cotton blue preparation: 2 microslides per plate, 2 plates per isolate) at day 10, are shown in Fig. 1. Isolate 18 produced a great number of both intercalary (Fig. 2A, B, C) and terminal (Fig. 2A, B, D, E, F) chlamydospores after 10 days of culture at 25°C. As the spores were counted at randomly selected microscopical fields, the number of spores per HPF shown by isolate 18 varied from 9 to 49. However, the process of sporulation was homogeneous throughout the fungal colonies inside the solid culture medium (Fig. 3a). The chlamydospores ranged in diameter from 5 to 20 μm and presented a thick cell wall (Fig. 2). In the cytoplasm, there were intensely blue-stained areas (Fig. 2). At day 10, when the plates containing numerous spores
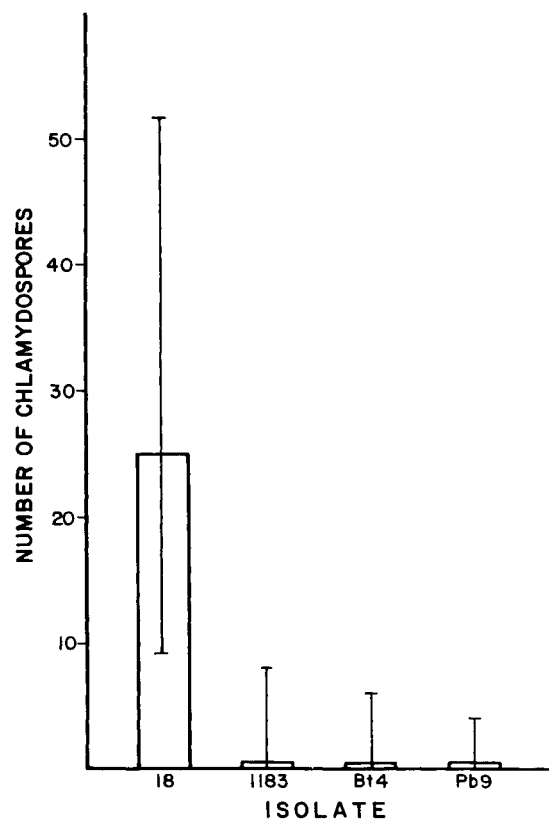

Fig. 1 — Chlamydospore formation by four **P. brasiliensis** isolates cultured in 2% agar with 0.1% polypepton and dextrose for 10 days at 25°C. Columns are the medians of the numbers of spores in 60 high power fields counted in 4 microslides (2 slides per plate; 2 plates per isolate). Bars represent the range between the highest and lowest numbers.

were incubated at 35°C, chlamydospores already showed multiple budding after 24 hours (Fig. 2 G. H, I). Chlamydospore formation by isolates 1183, Bt4 and Pb9 was significantly less frequent than by isolate 18 (Student's t test; p < 0.001).
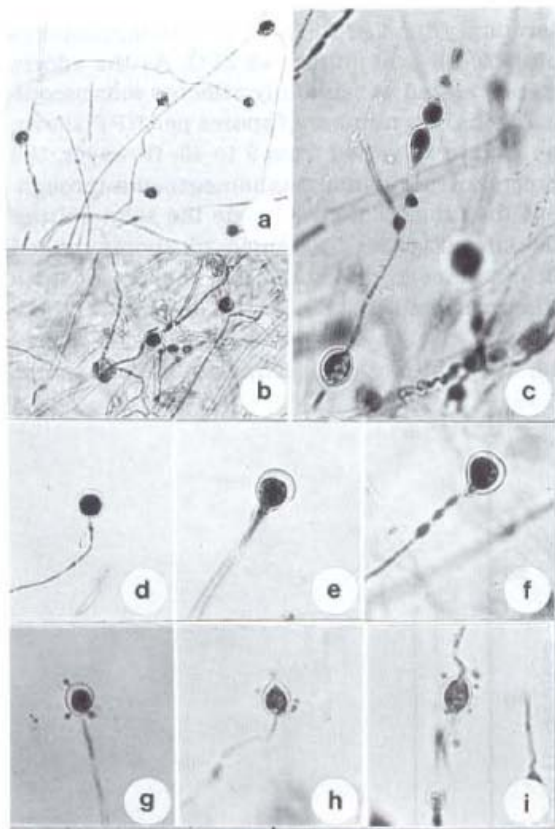

Fig. 2 — Terminal (A, B, D, E, F) and intercalary (A, B, C) chlamydospores. Note the thick cell wall and deeply stained matrix inside. Irregular swellings (immature chlamydospores) are less than 5 μm in diameter and show normal cell wall (A, B, C). Chlamydospores with multiple budding after 1 day at 35°C (G, H, I) (cotton blue preparations; A, C = 200x, B — I = 400x).

Sporulation by isolate 18 was already observed at day 7, although it was significantly less frequent than at day 10, as demonstrated by the median counts of 4 and 25 spores in 60 HPF, respectively (Student's t test; p < 0.001). No chlamydospore formation was detected at day 5.

In addition to typical chlamydospores, there were numerous segmentary swellings with diameter less than 5 μm and thin cell wall (Fig. 2 A, B, C); these structures were interpreted as immature chlamydospores.

The histological examination of the solid culture medium of both mature and immature chlamydospores showed similar findings to those described above (Fig. 3).
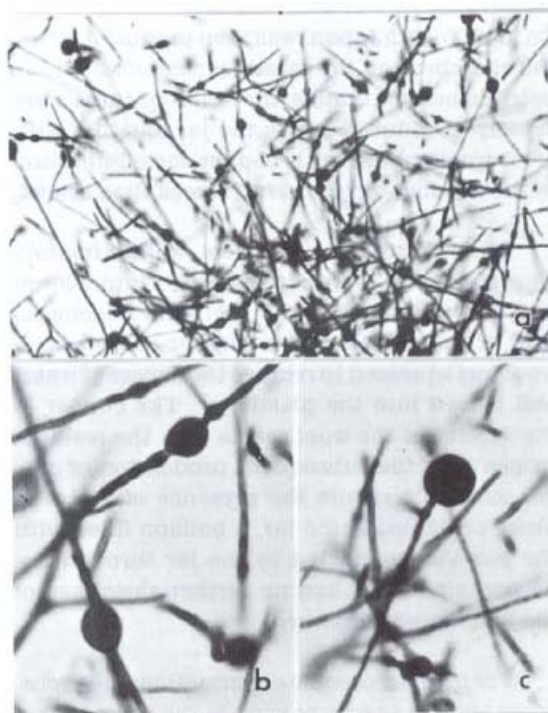

Fig. 3 — Sporulation by **P. brasiliensis** isolate 18, cultured in 2% agar with 0.1% polypepton and dextrose for 10 days at 25°C. Note terminal (C) and intercalary (B) chlamydospores with thick cell wall and deeply stained material inside (PAS; 15 μm sections of paraffin embedded solid culture medium; A = 200x, B, C = 400x).

Sporulation by isolate 18 showed a positive correlation with the poor nutritional content of the culture medium. The medium poor in nutrients (0.1% IDAgar) yielded a greater number of spores (median of spores per HPF = 22; lowest count = 14; highest count = 35). The two rich media (BHI and PDA) did not induce chlamydospore formation.

Anaerobic incubation of isolate 18 and 1183 under an atmosphere of N2 resulted in a limited outgrowth as observed grossly by the small size of the colonies in the solid medium. Cotton blue preparations of the cultures of both isolates revealed hyphal growth with chlamydospore formation (Fig. 4).
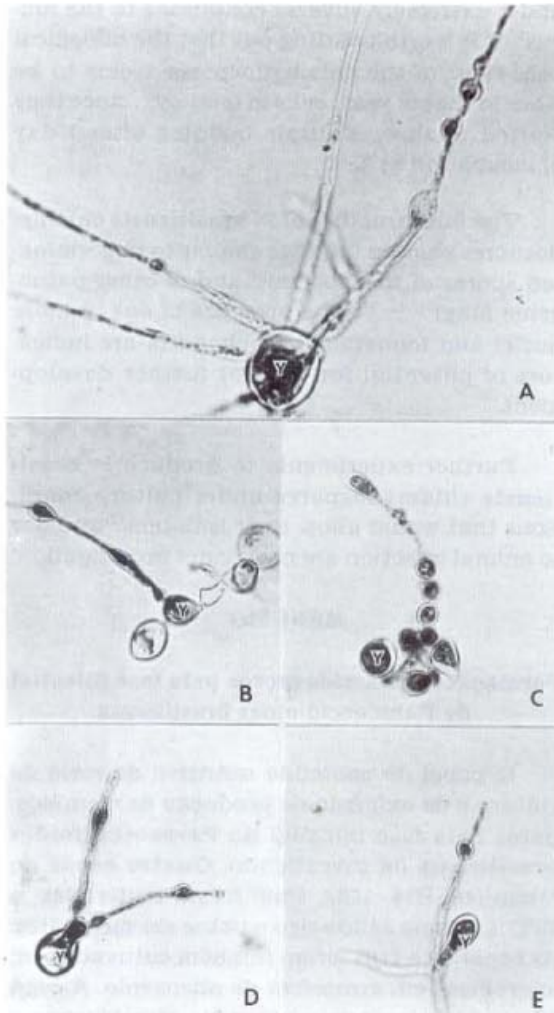
Fig. 4 — Anaerobic incubation of P. **brasiliensis** under an atmosphere of nitrogen. Yeast cells (Y) from the inoculum show hyphal growth with intercalary (A, B, D, E), and terminal chlamydospores (C, D) (cotton blue preparations; A = 200x, B, C, D, E = 400x).

reticulum, vesicles and mitochondria. The cell wall was characteristically thick (≃ 260 nm) and consisted of an electron-translucent inner layer and an electron-dense granular outer layer continuous with the outer layer of the suspensor cell wall.
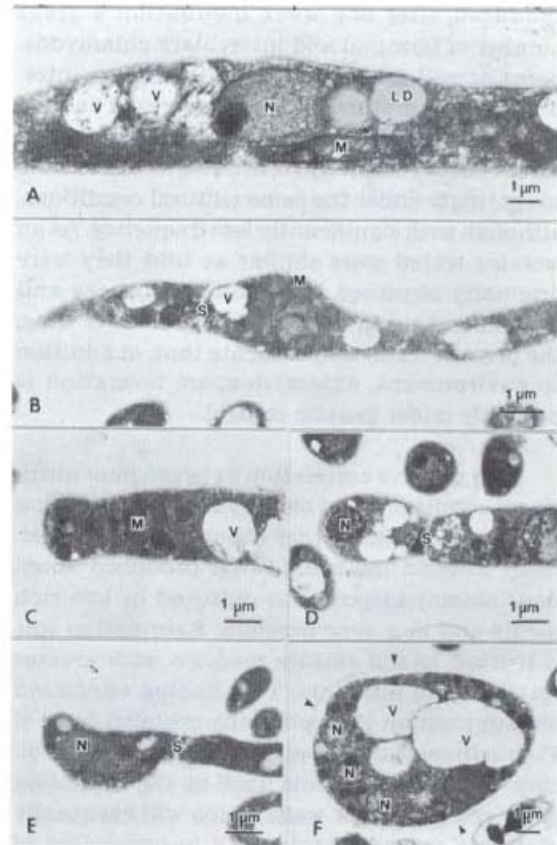

Fig. 5 — Chlamydospore formation by isolate 18 of **P. brasiliensis** cultured at 25°C for 10 days. Irregular intercalary (B) and terminal (C, D, E) swellings (immature forms), and mature chlamydospore (F). Note septum (S), one or more nuclei (N), mitochondria (M), vacuoles (V), lipid droplets (LD) and thick cell wall of the mature form (arrowheads) (E).

The transformation process from hyphae to terminal chlamydospores of isolate 18 cultured in 0.1% IDAgar for 10 days was studied by electron microscopy. At the early stage intercalary and terminal cellular components of the hyphal form began to round up (Fig. 5). These irregular swellings or immature chlamydospores contained one or more nuclei, and showed all the organelles present in the hyphae counterpart; the cell wall had also the same morphological appearance and thickness. The fully developed spores were round structures with one or several nuclei, numerous vacuoles, lipid droplets, endoplasmic

DISCUSSION

It is well known that several fungi under adverse conditions, such as starvation, exposure to membrane-altering or enzyme-activating agents, develop units of hyphal differentiation produced by isotropic growth[9, 25]. These resistant cells are called chlamydospores and they need dissolution of the supporting hyphae before dispersal is possible[6, 9]. The release can be accomplished by mechanical fracture of a non-dif-

ferentiated cell wall or by natural breaking down of the supporting hyphae through bacterial action, fungal lysis and/or weathering[9, 25].

Isolate 18 of **P. brasiliensis**, when grown at 25°C inside a solid agar medium with low nutritional content and poor availability of oxygen, produced after one week incubation a great number of terminal and intercalary chlamydospores, as well as irregular hyphal swellings, interpreted as immature chlamydospores. The phenomenon was not restricted to one isolate, since three other **P. brasiliensis** isolates were also able to sporulate under the same cultural conditions, although with significantly less frequency. As all isolates tested were similar as that they were originally obtained from clinical sources and have been kept in vitro for long period of time, the present result may indicate that, in addition to environment, chlamydospore formation is probably under genetic control.

The positive correlation between poor nutritional conditions and chlamydospore formation by isolate 18 was further checked in an experiment wherein the isolate that produced abundant chlamydospore was cultured in two rich media and in a poor medium. Sporulation was restricted to the culture medium with greater restriction of nutrients. This finding reinforced the supposition that when the mycelial form of **P. brasiliensis** meets adverse nutritional condition, the fungus defends itself by the formation of spores with thick walls which will eventually stay in a dormant state until improvement of the environmental conditions.

Based on our pilot experiments, we hypothesize that chlamydospore formation in **P. brasiliensis** might be multifactorial depending on nutrition and on the oxygen atmosphere. Accordingly, we carried out a further experiment to analyze the phenomenon under anaerobic condition.

Like other dimorphic pathogenic fungi[1, 14], **P. brasiliensis** showed growth under anaerobiosis although in a small scale. The growing hyphae of both isolates tested rapidly presented rounding up of some cells, with the formation of chlamydospores. This finding represents further evidence for the role of these structures as asexual spores primarily involved in perenation

under extremely adverse conditions to the fungus[9]. It is worth pointing out that the biological behaviour of the chlamydospores seems to be close to that of yeast cells in latency[21], since they started to show multiple budding after 1 day of incubation at 35°C.

The fine structure of **P. brasiliensis** chlamydospores showed findings similar to ungerminated spores of this species[24] and of other pathogenic fungi[11, 15, 26]. The presence of one or more nuclei and numerous mitochondria are indicators of potential for prompt further development.

Further experiments to produce **P. brasiliensis** chlamydospores under culture conditions that would allow their isolation[12] and use to animal infection are now under investigation.

RESUMO

Formação de clamidósporos pela fase micelial do Paracoccidioides brasiliensis

O papel do conteúdo nutritivo do meio de cultura e de oxigênio na produção de clamidósporos pela fase micelial do **Paracoccidioides brasiliensis** foi investigado. Quatro cepas do fungo (18, Bt4, 1183, Pb9) foram cultivadas, a 25°C, em meio sólido rico e pobre em nutrientes. As cepas 18 e 1183 foram também cultivadas em anerobiose em atmosfera de nitrogênio. A cepa 18 produziu grande número de clamidósporos terminais e intercalares após 7-10 dias de cultura em meio sólido pobre em nutrientes (agar 2%, com dextrose e polipeptona 0,1%). As outras três cepas produziram número significativamente menor de esporos. A cepa 18 não produziu clamidósporos quando cultivada em dois meios ricos em nutrientes (infusão de cérebro e coração, e agar dextrose de batata). A incubação anaeróbica da cepa 18 em atmosfera de nitrogênio apresentou pequeno crescimento micelial com a presença de numerosos clamidósporos. À nivel ultraestrutural, os clamidósporos apresentaram um ou mais núcleos e numerosas mitocôndrias, indicativos de potencial para posterior desenvolvimento. Assim, os esporos produziram gemulação múltipla 1 dia após incubação a 35°C. Os resultados demonstraram que, sob condições ambientais adversas, a fase micelial do **P. brasiliensis** produz clamidósporos em curto período

de tempo. É possível que o fungo encontre condições semelhantes no solo, produzindo os esporos, que poderiam desempenhar papel na propagação da paracoccidioidomicose.

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