EXPERIMENTAL MURINE CHROMOBLASTOMYCOSIS OBTAINED FROM
Fonsecaea pedrosoi ISOLATE CULTURED FOR A LONG PERIOD

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ABSTRACT: The present study aimed to describe *F. pedrosoi* propagules capable of causing chronic murine disease. Several changes in *F. pedrosoi* hyphae were identified in fungal cells cultured for a long period. Optical microscopy found many rounded cells with double-rigid melanin-rich walls. Terminal and intercalary chlamydoconidia were also frequently observed. Analyses of images from transmission electron microscopy (TEM) revealed several cells with walls composed of at least three layers and an outer layer enriched with melanin. Two groups of twenty BALB/c mice were subcutaneously infected in their footpads with *F. pedrosoi* cells at an inoculum concentration of approximately 1×10^4 cells/mL. In one group, long-term cultured *F. pedrosoi* cells were inoculated in one footpad, whereas in the other group, both footpads were infected. Active lesions were observed up to seven months post-infection, particularly in mice inoculated at two sites. After this period, animals were killed. Histological sections revealed characteristics bearing a strong resemblance to the human form of the disease such as tissue hyperplasia, granulomas with microabscesses and sclerotic cells. Based on this study, we identified fungal cells from old cultures capable of provoking chronic chromoblastomycosis under experimental conditions, especially when more than one site is infected.

KEY WORDS: *Fonsecaea pedrosoi*, experimental chromoblastomycosis, sclerotic cells.

CONFLICTS OF INTEREST: There is no conflict.

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INTRODUCTION

Chromoblastomycosis (CBM) is a chronic human disease caused by several dematiaceous fungal species, of which *Fonsecaea pedrosoi* and *Cladophialophora carrionii* are the most common throughout the world (1, 2). CBM therapy has produced limited success, especially in severe clinical forms (1, 3). Therefore, finding a suitable model for chronic experimental CBM is important for studying the immunological response and testing novel antifungal drugs. The presence of parasitic forms normally known as sclerotic (fumagoid or muriform) cells (also known as Medlar bodies) in tissues is a typical sign of the human form of the disease (2, 4). However, the fungal forms involved in establishing the infection and the mechanism of their adaptation to parasitism remain unknown.

Fungal cell modifications most often occur as resistance to chemical, biological and physical stress conditions. Fungal cells similar to the parasitic forms found in CBM can be induced *in vitro* under acidic conditions with low calcium concentration (5-7). Still, the infective potential of these forms requires further experimental study. The fungal cell alteration into sclerotic forms has been proposed as a virulence factor (6, 8). Sclerotic cells were detected in environmental samples of vegetables, suggesting the possible existence *in natura* of forms pre-adapted to parasitism (6, 8, 9). Recently, artificial inoculations of *C. carrionii* in cactus tissue revealed a morphological resemblance between sclerotic bodies and meristematic cells formed after vegetable transience (10).

Although hyphae and conidia are currently cited in the literature as potentially infectious in humans, several studies are have failed to reproduce a long-term stable chronic infection with these forms cultured in agar or broth for periods of 15 to 30 days (11-17). Fungal infections comprised of young culture forms at a single site generally produce acute inflammatory reactions mediated mainly by neutrophils, with animals healing within two months, independently of the infection site or inoculum concentration (11, 13, 14, 16-18).

Increased persistence of *F. pedrosoi* infection was found in animals inoculated at more than one site (16). A link between suppressor T cells and peripheral tolerance induced after *Leishmania major* inoculation at two sites has been described (19, 20). In our previous experiments, we observed that inoculation at two sites with 30-day-old forms is sufficient to cause this persistent infection. However, the infection sites in

most animals healed within 3 to 4 months post-inoculation and did not develop a stable chronic infection.

Therefore, the present study aims to describe the morphology of *F. pedrosoi* forms obtained from old cultures, and the ability of these fungal cells to provoke infection in laboratory mice inoculated at one or two sites.

MATERIALS AND METHODS

Fonsecaea pedrosoi Isolate

A *Fonsecaea pedrosoi* strain (EPM – 380/03) provided from a patient with CBM examined in 2003 in the Dermatology Outpatient Department, UNIFESP, was cultured on Sabouraud dextrose agar (SDA, Difco Laboratories, USA) supplemented with 80 mg/L of gentamycin (Schering-Plough, USA) at 28°C. The fungal strain employed in this study was preserved in sterile distilled water inside sealed penicillin tubes by the method of Castellani and maintained at room temperature. Aliquots containing fungal cells were collected using a sterilized insulin needle for cultivation.

Culture

The *F. pedrosoi* strain was cultured in 1 L cylindrical glass bottles (Kimax® GL-45, Daigger Lab Equipment and Supplies, USA) containing 300 mL of Sabouraud dextrose broth (SDB, Difco Laboratories, USA) with gentamycin, pH 5.7, at 28°C in a incubator (TE-420®, Tecnal, Brazil) and shaken at 100 rpm for six months.

F. pedrosoi Cells

Cultures of *F. pedrosoi* were vortexed and filtered through sterile gauze to retain macroscopic fungal balls and mycelial aggregates. Filtrates were drawn up into a 50 mL glass needle and ejected into a beaker several times to disrupt small mycelia clusters and to obtain solitary cells and small fragments of hyphae. Cells were then recovered by centrifuging homogenates at 3000 *g* for ten minutes and subsequently observed by optical microscopy. Images were captured using a Q-Color5® digital camera fitted to an Olympus BX50® microscope (Olympus Optical Co. Ltd., Japan). Images were processed using Image Pro Express® software (version 6.3, Media Cybernetics, USA). Samples from old cultures were cultivated on blood agar and stored at 28°C for bacterial contamination tests.

Transmission Electron Microscopy (TEM)

For TEM, *F. pedrosoi* cells cultured for six months were fixed for two hours at room temperature with 3% glutaraldehyde and 4% formaldehyde in 0.1 M cacodylate buffer, pH 7.2. Fixed cells were washed in cacodylate buffer three times for 30 minutes (each wash) and once overnight, and then fixed for two hours with 2% osmium tetroxide in 0.1 M cacodylate buffer. Cells were subsequently washed with 0.1 M cacodylate buffer three times for 15 minutes, then gradually dehydrated in a series of ethanol solutions and embedded in Eppon resin. Ultrathin sections were mounted on Formvar grids, stained with 4% uranyl acetate and lead citrate, and observed under a transmission electron microscope (JEOL 1200 EX II®, USA) at 80 kV (21). Note: materials cited for TEM were purchased from Electron Microscopy Sciences Company (USA).

Inocula

Inoculum viability was assessed using LIVE/DEAD® Cell Vitality Assay kit (L34951, Invitrogen, USA) in fluorescent microscopy. Suspensions containing fungal forms were adjusted to a concentration of 1×10^4 cells/mL in a Neubauer chamber. The minimal concentration of the fungal inoculum for animal infection was standardized by a pilot study. Short hyphal fragments had approximately six to ten septa. The separation among septa was employed to distinguish individual hyphal cells that were then counted. The inocula population contained a cellular mix of approximately 23% solitary chlamydoconidia cells, 18% roundish cells, 26% conidia, and 33% hyphal fragments. The suspension had a viability of approximately more than 95% of total cells.

Infection

Two groups of 20 BALB/c male mice weighing approximately 23 g each (6 to 8 weeks old) were used throughout the study. These pathogen-free animals were purchased from CEDEME/UNIFESP (São Paulo, Brazil). The protocol employed herein was approved by the UNIFESP Ethics Committee (project number 0808/05). Ten minutes prior to infection, animals were intraperitoneally anesthetized with 0.4 µL Anasedan® and 0.35 mL/kg Dopalen® (both from Vetbrands, Brazil). Approximately 50 µL of the *F. pedrosoi* cell suspension was inoculated subcutaneously into the footpads (about 500 cells/footpad). Swelling was monitored every 30 days up to a

maximum of seven months using a Mitutoyo digital caliper (USA). Footpad volume was calculated based on height and width measurements and the mathematical formula for a cylinder volume. Results are expressed as mean ± SE. Based on normality data as defined by the D'Agostino and Pearson omnibus test, significant differences were determined using an ANOVA test (Prism software). Seven months after infection, mice were killed and their footpads were removed in accordance with guidelines on the use and care of laboratory animals (22-24). A sample of infected tissue was analyzed and cultivated on SDA. Fresh tissue samples were clarified with 20% KOH and examined directly by optical microscopy.

Histopathology

Tissue specimens were left in 10% formalin for over 12 hours. Bone tissue from the paws was decalcified with 5% ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich, USA) (17). The specimens were embedded in paraffin, and 3 to 5 µm serial sections from the blocks were stained with hematoxylin-eosin (Sigma-Aldrich, USA) and periodic acid-Schiff (PAS, Merck Chemicals, Germany).

RESULTS

Light Microscopy and TEM

Different morphological forms were found through optical microscopy. There were spherical or ovoid-shaped cells and hyphae with intercalary or terminal chlamydoconidia shapes. In general, these forms were 5 µm to 20 µm in diameter, sometimes with planate divisions and a large nucleus (Figure 1). A significant number of cells were more pigmented than others (Figure 2). By TEM, we verified that cell walls of these forms were composed of a thick multilayer containing at least three layers (Figure 3). The outer layer had a dark, electron-dense appearance, and was probably comprised of melanin (Figure 3D). Bacterial contamination was not detected in samples taken from old cultures.
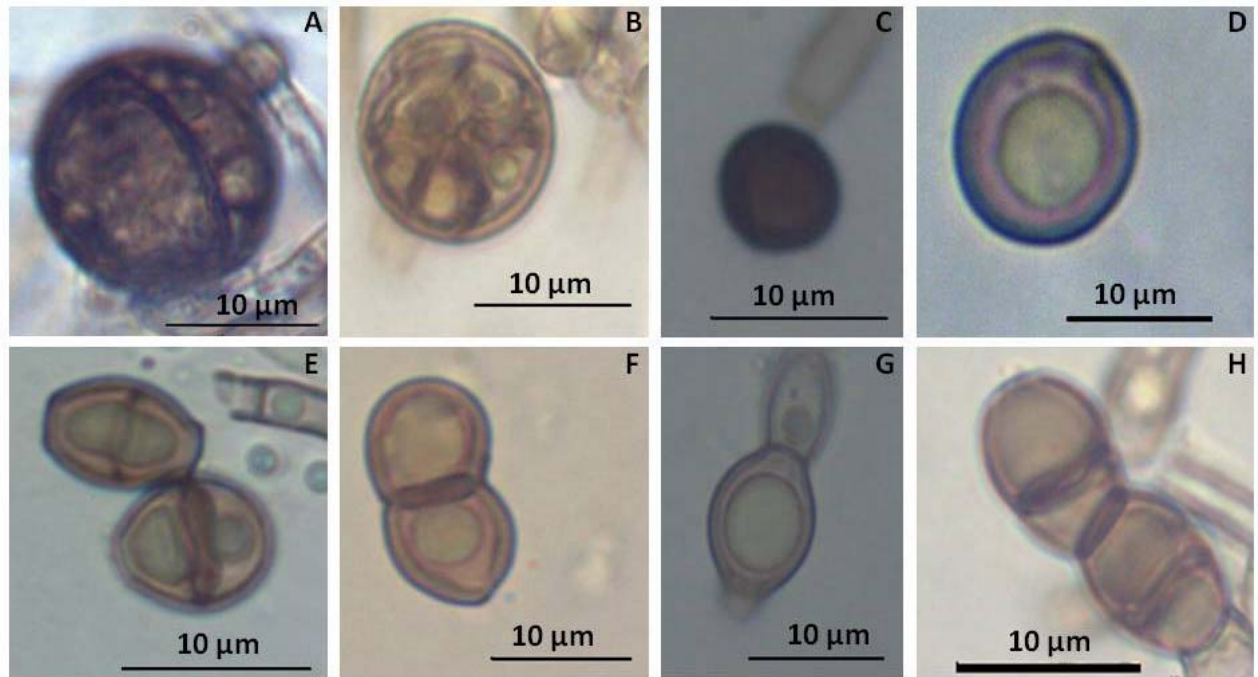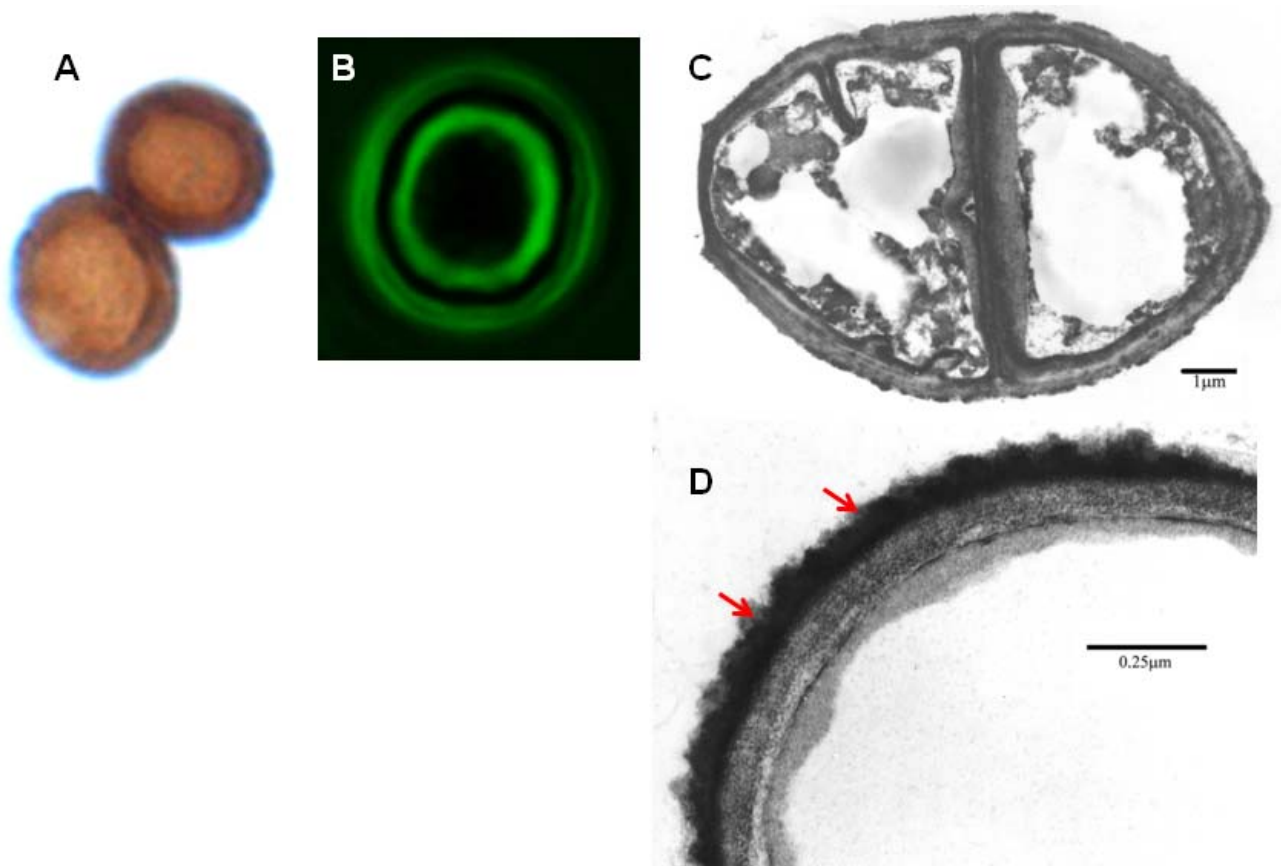
Figure 1. Highly melanized fungal forms with diameters ranging from 5 to 20 μm from pure cultures of *F. pedrosoi* cultivated for six months (1000x). (**A** and **B**) Multicellular and (**C** and **D**) unicellular chlamydoconidia; (**E**-**H**) roundish cells.

* Many cells have an enlarged nucleus (light green).

**Figure 2**. Highly melanized hyphal cells from *F. pedrosoi* cultured for six months (400x).

Figure 3. *F. pedrosoi* chlamydoconidia cells from 6-month cultures. (**A**) Light microscope image (1000x); (**B**) autofluorescent wall layers (1000x); (**C**) TEM of a roundish cell with longitudinal and transverse septa; (**D**) TEM of a wall from terminal chlamydoconidia, showing four layers with an external electrondense layer.

**Infection with Cells from Old Cultures**

Mouse footpads were monitored over the course of seven months, while animals maintained a stable and chronic infection process. At 4 to 5 days post-infection, animals developed a small inflammatory response on their footpad. By the end of the first month, we noted footpad swelling, particularly in mice inoculated at two sites. The swelling lasted for two months, and it acquired a firm consistency. After five months, several animals that were inoculated in two footpads developed foot hyperkeratosis. All animals infected showed progressive inflammatory process of footpads in the early post-infection period (Figure 4). However, animals inoculated in two sites exhibited more swelling, chronicity and stability of the infectious process. Eight mice inoculated at one site healed after five months. Infection metastasis to other organs did not occur. In general, a small number of sclerotic bodies (3 to 4

cells/optical field) were observed in lesions during direct examination (Figure 5) and in anatomopathological sections (Figure 6). Histopathological findings revealed tissue hyperplasia, granulomas and proliferation of pseudoepitheliomatous cells. Additionally, observed lesions presented microabscesses surrounded by foamy macrophages with polymorphonuclear leukocyte (PMN) infiltration, and encapsulated by fibroblasts. Rare eosinophils and lymphocytes were found. Neutrophils were around and inside microabscesses. Granulomas of mice inoculated at one site presented more PMN infiltration than those of animals inoculated at two sites, suggesting that the migration and action of these cells at the infection site may be important for the infection control. Viable fungal cells were recovered from 18 animals (90%) injected at two sites and from seven animals (35%) infected at one site.
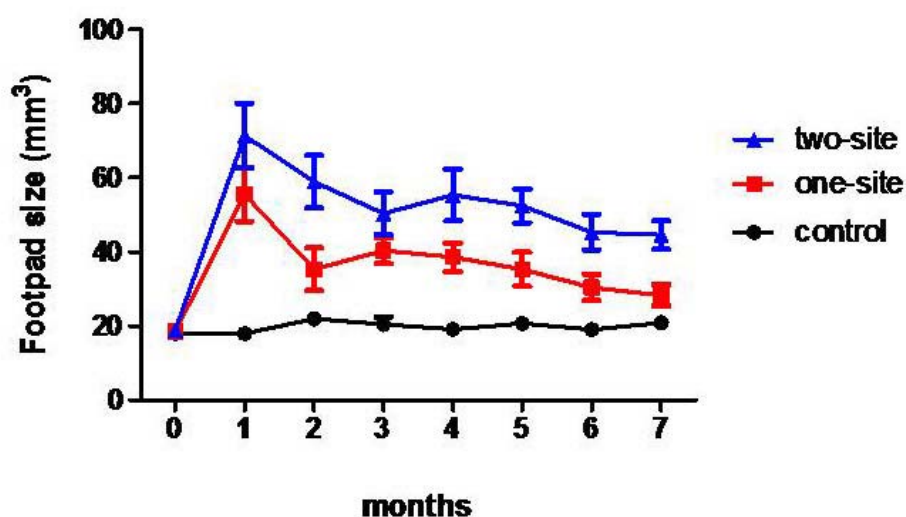

Figure 4. Kinetics of lesions from BALB/c mice that were subcutaneously infected with aged inocula of *F. pedrosoi* at one (squares, red) and two footpads (triangles, blue). The control group was treated with 50 µL of PBS (circles, black). Footpads of mice were measured every 30 days for seven months after inoculation. Animals infected at two sites had strong swelling and a more chronic infectious process. Data are shown with mean and SE values, ANOVA test, $p < 0.05$.
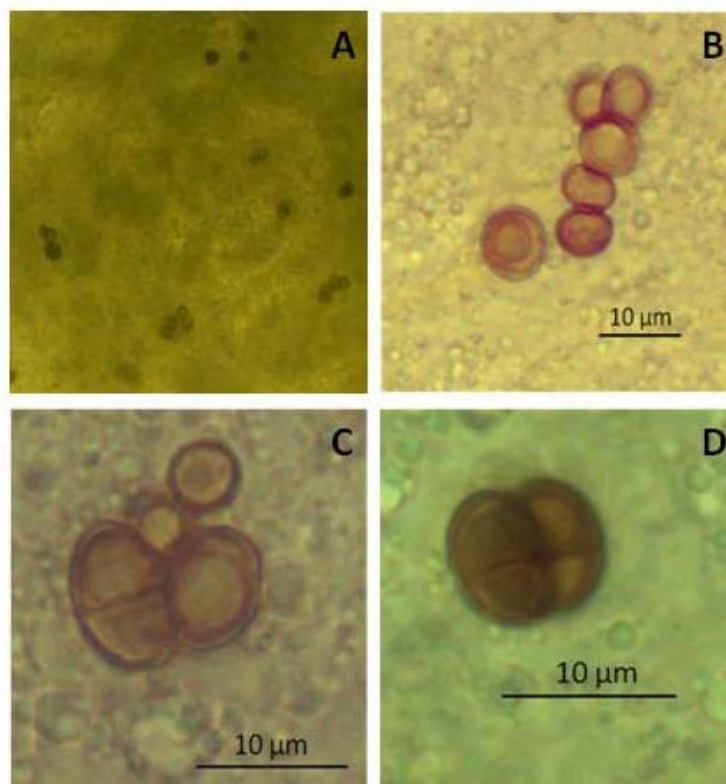
Figure 5. Sclerotic cells from footpad tissue samples obtained seven months after infection observed by direct examination; (**A**) 100x, (**B**) 400x, (**C** and **D**) 1000x.
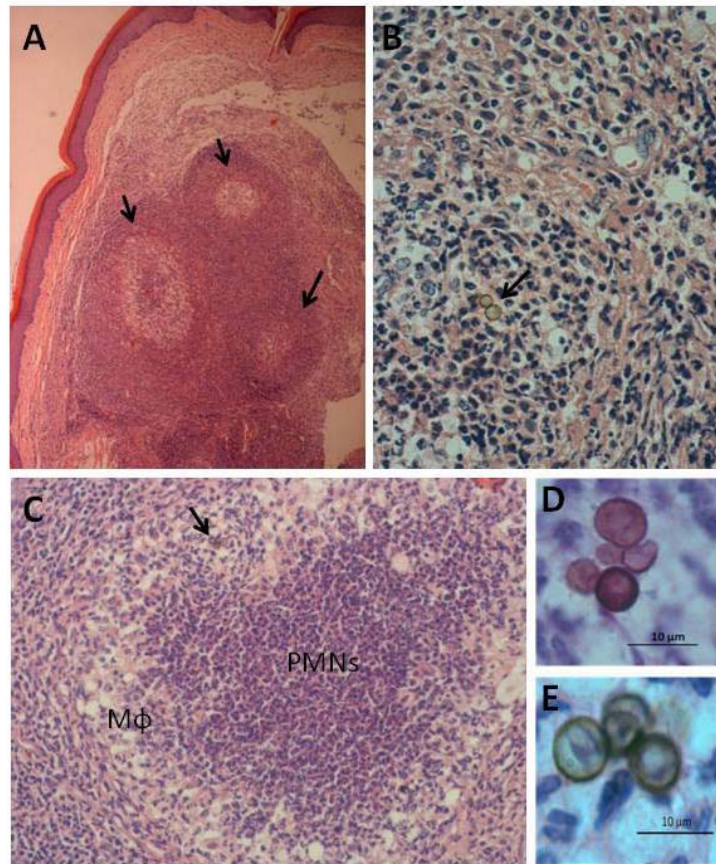
Figure 6. Photomicrography of histopathological sections from footpad tissue of animals with chronic experimental CBM infection at seven months after infection; tissue was stained with PAS. (**A**, arrows) Granulomatous reaction containing microabscesses with neutrophilic infiltration, foamy macrophages, low numbers of lymphocytes and eosinophils, and (**B**) recovery by fibroblasts. Few sclerotic cells of *F. pedrosoi* were found in granulomas of mice inoculated at (**C**) one site or (**B**) two sites. (**C**) PMN rich cellular infiltration surround by macrophages (Mφ) of animal tissue infected at one site. (**D** and **E**) Sclerotic cells with diameters ranging from 5 to 10 µm.

(**A**) 40x, (**B**) 200x, (**C**) 100x, (**D** and **E**) 1000x.

*Panel **E** is a magnified view of **C** (arrow).

Mφ: macrophages, PMN: polymorphonuclear leukocytes.

DISCUSSION

Since the discovery of CBM, a number of studies involving experimental inoculation with CBM agents have been carried out in an attempt to develop an animal model of the disease (11-13, 15, 16, 25). However, a suitable model for chronic experimental CBM has yet to be established (4, 26). *Fonsecaea pedrosoi* inoculated in various peritoneal sites caused persistent infection (18, 27). Suppressed host immune response in the presence of chromoblastomycotic agents may be related to the induction of a specific population of T cells (4). Inoculation of *Leishmania major* at two sites on BALB/c mice, for example, stimulated the migration of regulatory T cells to one of the infection sites, resulting in greater infection chronicity than that observed in animals inoculated at a single site (20). In this study, we verified that *F. pedrosoi* cells cultured for a long period of time could cause chronic infection in BALB/c mice, especially if the inoculation occurred at more than one site. We also verified that low concentrations of aged inoculum are capable of producing a stable experimental infection, in contrast to other studies that employed a high number of *F. pedrosoi* cells to obtain acute infections (13, 15, 16).

A variety of cellular forms of the *F. pedrosoi* strain were observed in the present study (Figure 1). The nutritional starvation in old cultures may be a reason for the abnormal fungal morphology observed *in vitro*. Many other dematiaceous fungi such as *Alternaria alternata*, *Cladosporium cladosporiodes*, *Scytalidium lignicola*, *Phialophora verrucosa*, *P. parasitica*, *P. richardsiae*, *C. carrionii*, *C. boppii* and *Rhinocladiella aquaspersa* as well as several clinical strains of *F. pedrosoi* (n = 8) showed high melanized forms, including chlamydoconidia and sclerotic-like cells, after culturing for longer than six months. Prominent melanization of certain fungal forms was observed in TEM as electron-dense pigments, particularly in the outer layer of the cell wall. The microbial ability to synthesize melanins can be related to virulence and pathogenicity (28).

Many pathogenic fungi have melanized forms or secrete melanin in order to increase their resistance to environmental hazards and to enable them to evade host defense mechanisms (29). However, in addition to wall coverage and melanin secretion, infective forms of CBM agents may possess additional virulence factors harmful to hosts. The transformation of dematiaceous cells into resistant forms similar to sclerotic bodies should be considered an important virulence factor. For this, meristematic forms (resistant thick-walled globose cells) found in vegetables may be

possible precursor cells of CBM-parasitic forms. In plant hosts they can be regarded as an extremotolerant survival phase (6, 10).

Production of sclerotic cells *in vitro* results from morphological changes in hyphae and conidia of *F. pedrosoi* and *Cladophialophora* spp. chemically induced by growth under stress conditions (5, 6, 7, 30). Hence, the fungal changes in hostile environments could be considered a step for pre-adaptation of CBM agents to human/animal parasitism. Interestingly, several fungal forms of *F. pedrosoi*, including terminal chlamydoconidia-shaped cells, have been observed in samples collected from thorns of *Mimosa pudica*, reported to be a probable natural source of CBM agents (31). A significant number of roundish chlamydoconidia-shaped melanin-enriched cells were observed in inocula utilized to cause animal infections in the present study. Such forms may be precursors of sclerotic cells subsequently observed at seven months after the infection. Both melanized cells and sclerotic cells had similar morphology and diameters of approximately 5 to 10 μm.

Effective acute cellular response in animals against *F. pedrosoi* cells from young cultures involves intense neutrophil infiltration (11-13, 17). In this study, however, sclerotic bodies were resistant to neutrophil killing mechanisms and macrophage phagocytosis. Sections taken from infected tissues in animals inoculated at a single site showed a higher number of neutrophils than those taken from animals inoculated at multiple sites, while several mice from the unifocal infection group healed after five months. Histopathological sections of tissue from human disease revealed hyperkeratotic pseudoepytheliomatous hyperplasia with microabscesses rich in polymorphonuclear neutrophils (4, 32).

According to our histological results, we detected tissue hyperplasia and granulomatous reactions with microabscesses containing central neutrophil infiltrates surrounded by foamy macrophages, sclerotic bodies inside giant cells, rare lymphocytes and eosinophils, and an external layer encapsulated by fibroblasts. We also noted additional pathological characteristics that further indicated the similarity in the disease process between our infected mice and human CBM patients, such as fibrosis, acanthosis and hyperkeratosis.

In the current investigation, experimental CBM disease with chronic outcomes was demonstrated to be associated with fungal morphology and host responses. Old cultures may represent an alternative means to obtain chlamydoconidia-shaped and well-melanized roundish cells capable of provoking a stable chronic infection

resistant to the host response, particularly if these forms are inoculated into mice at more than one site. In the future, such *F. pedrosoi* forms may be a suitable method for investigating infections *in vivo*, although the time required can be rather long, at least ten months.

ACKNOWLEDGEMENTS

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