Original article

Melanin in the dimorphic fungal pathogen *Paracoccidioides brasiliensis*: effects on phagocytosis, intracellular resistance and drug susceptibility

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Abstract

The fungal pathogen *Paracoccidioides brasiliensis* produces a melanin-like pigment in the presence of L-DOPA in vitro. We investigated whether melanization affected yeast uptake by alveolar and peritoneal macrophages, the intracellular resistance of fungal cells and their susceptibility to antifungal drugs. The interactions of melanized and nonmelanized *P. brasiliensis* with murine primary macrophages and J774.16 and MH-S macrophage-like cell lines were investigated. Melanized yeast cells were poorly phagocytosed by the cells even in the presence of complement. Melanization caused significant interference with the binding of cell wall components to lectin receptors on macrophages. Melanized cells were also more resistant than nonmelanized cells to the antifungal activity of murine macrophages. No difference in the susceptibilities of melanized and nonmelanized *P. brasiliensis* to antifungal drugs was observed using the minimum inhibitory concentration (MIC) method. However killing assays showed that melanization significantly reduced fungal susceptibility to amphotericin B and also protected against ketoconazole, fluconazole, itraconazole and sulfamethoxazole. The present results indicate that fungal melanin protects *P. brasiliensis* from phagocytosis and increases its resistance to antifungal drugs.

Keywords: Paracoccidioides brasiliensis; Melanin; Phagocytosis; Resistance

1. Introduction

Paracoccidioidomycosis (PCM) is a systemic granulomatous disease caused by *Paracoccidioides brasiliensis*, a thermally dimorphic fungus. It is widespread in Latin America, mainly affecting rural workers. Inhalation of conidia is the probable route of infection. According to McEwen et al. [1] approximately 10 million people may be infected with this fungus and up to 2% of them may develop disease. The incidence may increase in the setting of deforestation or soil disruption, and in immunocompromised individuals [2]. The acute and subacute form of PCM affects both sexes equally and primarily involves the reticuloendothelial/lymphatic system. The chronic form affects mainly adult males with pre-

dominant pulmonary and/or mucocutaneous involvement [3]. Activation of cellular responses is the primary effective mechanism for control of experimental and human PCM [4,5], and a correlation has been found between the severity of the disease and impaired delayed-type hypersensitivity (DTH) response [6].

Melanins are multifunctional polymers found in diverse species of all biological kingdoms [7]. In fungi, melanins are present in several fungal pathogens such as *Fonsecaea pedrosoi* [8], *Cryptococcus neoformans* [9,10], *Exophiala dermatitidis* [11], *Lacazia loboi* [12], *Histoplasma capsulatum* [13], *Sporothrix schenckii* [14], *Blastomyces dermatitides* [15], *Aspergillus fumigatus* [16] and *P. brasiliensis* [17]. In *C. neoformans* melanization of the encapsulated yeast is catalyzed by phenol oxidase (laccase) when cells are grown in the presence of exogenous dihydroxyphenolic compounds [9,18]. In vitro studies have shown that melanized *C. neofor-*

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mans cells are less susceptible than nonmelanized cells to UV-induced damage [19], macrophage-mediated phagocytosis [20], oxidative damage [21], and antimicrobial drugs [22]. Melanin synthesis is also associated with virulence for the human pathogenic fungi *E. dermatitidis*, *Aspergillus* spp., and *S. schenckii* (reviewed in [23]). Melanins have also been implicated in the virulence of several fungal plant pathogens [24].

Melanization of *C. neoformans* results from deposition of the polymer at the cell wall [25]. Treatment of melanized cells with enzymes, detergents, and hot acid results in the recovery of melanin "ghosts" that retain the size and shape of the original fungal cells [10]. The ability of *P. brasiliensis* to produce cell wall-associated melanin-like components in vivo and during infection has been reported [17]. Although conidial melanization occurs in the absence of phenolic substrate, yeast cells produce melanin in vitro only when L-DOPA is added to the culture medium. Furthermore, laccase-like activity has been shown in cell extracts from *P. brasiliensis* yeast [17].

Given the potential role of melanin in virulence of *P. brasiliensis*, we investigated the effect of melanin in yeast cells on their phagocytosis by alveolar and peritoneal macrophages, intracellular resistance, and in vitro susceptibility to antifungal drugs. The present data indicate that melanized yeast cells of *P. brasiliensis* show increased resistance to the antifungal activity of mouse macrophages and the drugs currently in use against this fungus.

2. Materials and methods

2.1. Animals

BALB/c mice were bred at the University of São Paulo animal facility under specific-pathogen-free conditions. Procedures involving animals and their care were conducted in conformity with the local Ethics Committee and international recommendations.

2.2. Fungal strain

Virulent *P. brasiliensis* 18 (Pb18) yeast cells were maintained by weekly passage in solid Sabouraud medium at 37 °C and were used after 7–10 days of growth. The fungal cells were washed in phosphate-buffered saline (PBS pH 7.2) and counted in a hemocytometer. The viability of fungal suspensions, determined by staining with Janus B (Merck, Darmstadt, Germany), was always higher than 90%. To avoid yeast clumps in the fungal suspensions these were vigorously shaken and let to sediment. Remaining clumps sedimented faster and were discarded. The top suspension contained mostly isolated yeasts with variable budding.

2.3. Growth of P. brasiliensis *yeast cells with or without* L-DOPA

Pb18 yeast were grown on a chemically defined liquid medium [26] supplemented with 1.0 mM L-DOPA during 15 days at 37 °C or in a defined liquid medium (15.0 mM glucose, 10.0 mM $MgSO_4$, 29.4 mM KH_2PO_4, 13.0 mM glycine, 3.0 μM vitamin B1 [pH 5.5] with or without 1.0 mM L-DOPA (Sigma Chemical Co., St Louis, MO) for 15 days at 37 °C in a rotary shaker at 200 rpm. All cultures were incubated in the dark to avoid photo polymerization of L-DOPA into melanin. Cells were collected by centrifugation at 2000 rpm for 30 min, washed with PBS, and stored at 4 °C until use. *C. neoformans* (fungal collection, ICB 163D), and *Candida albicans* (fungal collection, ICB 12A) were used as positive and negative controls, respectively.

2.4. Isolation and purification of yeast melanin particles

Melanin particles were isolated from melanized yeast by a modification of the methodology described previously [25]. Briefly, yeast cells were collected by centrifugation at 3000 rpm for 30 min. The pellet was autoclaved, washed with PBS, and suspended in 1.0 M sorbitol–0.1 M sodium citrate [pH 5.5]. Cell wall-lysing enzymes (from *Trichoderma harzianum* [Sigma, St Louis, MO]) were added at 10 mg/ml, and the suspension was incubated overnight at 30 °C to generate protoplasts. The protoplasts were collected by centrifugation, washed with PBS, and incubated in 4.0 M guanidine thiocyanate (denaturant) overnight at room temperature. Cell debris were collected by centrifugation, washed three times with PBS, and digested with proteinase K (1.0 mg/ml; Roche Laboratories; Indianapolis, IN) in reaction buffer (10.0 mM Tris, 1.0 mM $CaCl_2$, 0.5% sodium dodecyl sulfate [pH 7.8]), overnight at 37 °C. The resulting product was washed three times with PBS and then boiled in 6.0 M HCl for 1 h. The insoluble residue was collected by centrifugation, washed extensively with PBS, and dialyzed against distilled water for 10 days.

2.5. Transmission electron microscopy

P. brasiliensis melanized and nonmelanized cells were fixed overnight in Trump's EM reagent (1% glutaraldehyde, 4% paraformaldehyde in PBS), incubated in 1% osmium for 1 h, dehydrated, and embedded in araldite-Epon. Ultrathin sections were placed on nickel grids and examined using a JEOL (Tokyo, Japan) JEM 1010 electron microscope.

2.6. Scanning electron microscopy

P. brasiliensis melanized and nonmelanized cells were fixed in 2.5% glutaraldehyde for 1 h at r.t. Samples were then applied on a polylysine-coated coverslip and serially dehydrated in alcohol. The samples were fixed in a critical point drier (BAL-TEC CPD-030 – Electron Microscopy Sciences, USA), coated with gold–palladium (Balzers Union SCD-040 – Electron Microscopy Sciences, USA) and viewed using a JEOL (Tokyo, Japan) JEM 6100 electron microscope.

2.7. In vitro phagocytosis

Peritoneal and alveolar macrophages from BALB/c mice were harvested by lavaging the abdominal cavity or the lungs

and cultured in DMEM with 10% heat-inactivated FCS and 1% non-essential amino acids (Cultilab, Brazil). Other phago-cytosis experiments were carried out with the J774.16 mac-rophage-like cell line derived from a reticulum cell sarcoma [27] or with the MH-S line, originated from SV40 transfor-mation of an adherent cell-enriched population of alveolar macrophages [28]. The protocol for in vitro phagocytosis was as described in earlier studies [29,30] with minor modifica-tion. Cells were plated in 96-well tissue culture plates (TPP, Switzerland) at a density of 10^5 cells per well, were stimu-lated with 50 U/ml recombinant murine IFN-γ (PeproTech, Rock Hill, NJ) and incubated at 37 °C overnight. The medium in each well was then replaced with fresh medium containing 50 U/ml IFN-γ in the presence or absence of fresh mouse serum. Heat-inactivated hyperimmune mouse serum to the purified cell wall or melanin ghost of *P. brasiliensis* was added in some experiments. *P. brasiliensis* cells (melanized and non-melanized) were added at a ratio of 5:1 macrophages/fungi, following incubation at 37 °C for 6, 12 and 24 h. Cells were then washed several times with sterile PBS, fixed with cold absolute methanol, and stained with a 1/20 solution of Giemsa (Sigma). Though similar results were obtained with macroph-ages unstimulated by cytokines, higher phagocytic indices were obtained with IFN-γ pretreatment. Prior studies [29,30] have also used interferon-stimulated macrophages. Phagocy-tosed and surface bound noninternalized yeasts were counted by light microscopy at ×400 magnification. The phagocytic index (PI) is defined as PI = $P \times F$, where P is the percentage of macrophages with internalized yeast and F is the average number of yeast cells per macrophage. An estimate of the noninternalized bound yeast cells was made through a lateral binding index which used the same formula above but counted only the laterally adherent yeast cells onto the macrophage after the same time of phagocytosis and extensive washing. Phagocytosis was confirmed by transmission electron micros-copy that showed the internalization of yeast cells in mac-rophages. Experiments were carried out in triplicate and five to eight different fields were counted.

2.8. Inhibition of phagocytosis by Saccharomyces cerevisiae mannan

The effect of mannan (Sigma) on phagocytosis was tested at 1, 10 and 100 µg/ml. Mannan was added before or together with melanized or nonmelanized yeast cells of *P. brasilien-sis*.

2.9. Laccase (EC 1.10.3.2) activity

P. brasiliensis 18, *C. neoformans* (positive control) and *C. albicans* (negative control) cells were washed three times with PBS (pH 7.2) and inoculated into an asparagine/glucose/salt (AGS) medium (6 mM asparagine, 1 M Na_2HPO_4 [pH 6.5], 0.4 mM $MgSO_4$, 0.5 M $CaCl_2$, 16 mM glucose) and either incubated at 30 °C (*C. albicans*, *C. neoformans*) or at 37 °C (*P. brasiliensis*) for 2 days with shaking. The cells were

harvested by centrifugation at $1000 \times g$ for 10 min and washed three times with AGS medium without glucose. The cells were suspended in 10 ml of the latter medium, incubated 2 days as described above for laccase production, harvested by centrifu-gation and then washed with PBS (pH 7.2). A cell suspension was adjusted to 10^8 cells per ml in 900 µl of 50 mM Na_2HPO_4 and mixed with 100 µl of 2,2′-azino-bis (3-ethylbenzo-thiazoline-6-sulfonate (ABTS, Roche, Indianapolis, IN). The suspension was incubated for 2 h in the same conditions described above. The cells were harvested by centrifugation at $1000 \times g$ for 10 min and the reaction was measured at 420 nm (DU 640, Beckman, USA).

2.10. Minimal inhibitory concentration (MIC) of antifungal drugs

MICs were determined by a modification of the method of Shadomy et al. [31]. Briefly, melanized and nonmelanized yeast cells were suspended in sterile saline and diluted to 10^5 cells per ml. A volume of 0.1 ml of melanized and nonmela-nized yeast suspension was completed to 1 ml with modified McVeigh–Morton medium [26] with the drugs to be tested. Final drug concentrations ranged from 4 to 0.0625 µg/ml for amphotericin B, 4–0.0005 µg/ml for ketoconazole, fluconazole and itraconazole and 600–9375 µg/ml for sulfamethox-azole. MICs were recorded after incubation at 37 °C for 7 days. For amphotericin B, the MIC was defined as the low-est concentration at which there was no fungal growth. The MICs for azoles and sulfametoxazole were defined as the low-est drug concentration that gave 90% growth inhibition com-paring with the growth of the drug-free control.

2.11. Antifungal killing assay

Melanized and nonmelanized yeast were suspended in ster-ile normal saline at a density of 10^5 cells per ml. Drugs were added at final concentrations in the same ranges as indicated above. After incubation at 37 °C for 7 days, aliquots were plated on brain heart infusion agar supplemented with 4% fetal calf serum (FCS) and 5% spent culture medium of *P. bra-siliensis* 192, to determine their viability, expressed as colony forming units (CFU). The rate of survival was compared to that of drug-free fungal cells used as a control.

2.12. Antifungal activity of macrophages

In this study the term "antifungal activity" describes the reduction in CFU that results from both fungicidal and fungi-static effects. The cell lines (J774.16 and MH-S) or primary macrophages from BALB/c mice (alveolar and peritoneal) were used in the phagocytosis assay described above. Mac-rophages were mixed with *P. brasiliensis* (melanized or non-melanized) in presence or absence of fresh mouse serum and incubated for 6, 12 or 24 h. After incubation the macroph-ages were washed with 0.15 M of methyl α-D-manno-pyranoside (Sigma) to remove most of the surface adherent

yeast cells. Cells were lyzed by adding 100 µl of sterile water for 30 min followed by repeated vigorous aspiration and ejection of well contents with a pipette for complete disruption of cells. Supernatant fluids and the lysate were pooled and plated on brain heart infusion agar supplemented with 4% FCS and 5% spent culture medium of *P. brasiliensis* 192 to determine cell viability. CFUs were counted after 6 days of incubation at 37 °C.

2.13. Statistical analysis

Pairwise comparison between groups was done by the *t*-test using Primer of Statistics (McGraw-Hill, New York, NY).

3. Results

3.1. Melanization of yeast cells of *P.* brasiliensis

After 15 days of culture in a defined liquid medium with L-DOPA, darkly pigmented yeast cells with multiple buds were observed (Fig. 1A). No pigmentation of *P. brasiliensis* was observed when yeasts were grown without L-DOPA (Fig. 1B). Positive and negative controls to this experiment were *C. neoformans* and *C. albicans*, respectively (data not shown). Pigmented yeast cells treated with proteolytic and glycolytic enzymes, denaturing agents, and hot concentrated acid yielded a dark residue that was collected by centrifugation. This residue contained melanin ghosts, as described previously by Gomez et al. [17] (data not shown). Nonmelanized yeast cells left no residue when submitted to the same treatments (data not shown). Scanning electron microscopy of melanized and nonmelanized yeast cells demonstrated that both had a smooth surface with no major differences between them (Fig. 2A, B). Transmission electron micrographs of yeast cells grown with L-DOPA showed a surface distribution of electron-dense granules, which were also detected in the cytoplasm (Fig. 3A) and were absent in yeast cells grown in the absence of L-DOPA (Fig. 3B).

3.2. Laccase activity

To confirm that *P. brasiliensis* had laccase activity, *P. brasiliensis*, *C. albicans* (ICB 12A) (negative control) and *C.*
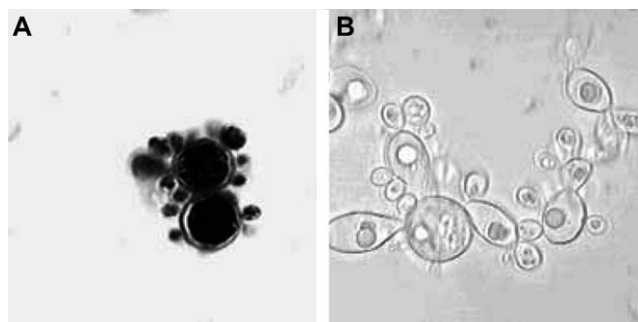

Fig. 1. Light microscopy of *P. brasiliensis* yeast cells grown in liquid medium with (A) and without (B) L-DOPA. Original magnification, 400×.

neoformans (ICB 163D) (positive control) were grown in AGS medium. The specific substrate ABTS was added and after 2 h we observed increased absorbance at 420 nm in *P. brasiliensis* and *C. neoformans* but not in *C. albicans* (Fig. 4). A control with no cells was added and the absorbance was similar to that obtained with *C. albicans* (data not shown).

3.3. In vitro phagocytosis

We investigated the effect of melanization of *P. brasiliensis* on phagocytosis in vitro using both primary macrophages
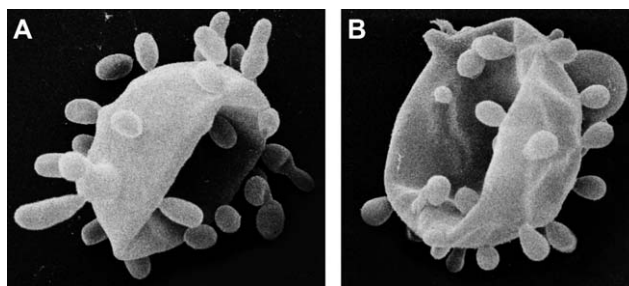

Fig. 2. Scanning electron micrographs of *P. brasiliensis* yeast cells grown with (A) and without (B) L-DOPA. No difference was observed in the morphology of both groups of yeast cells.
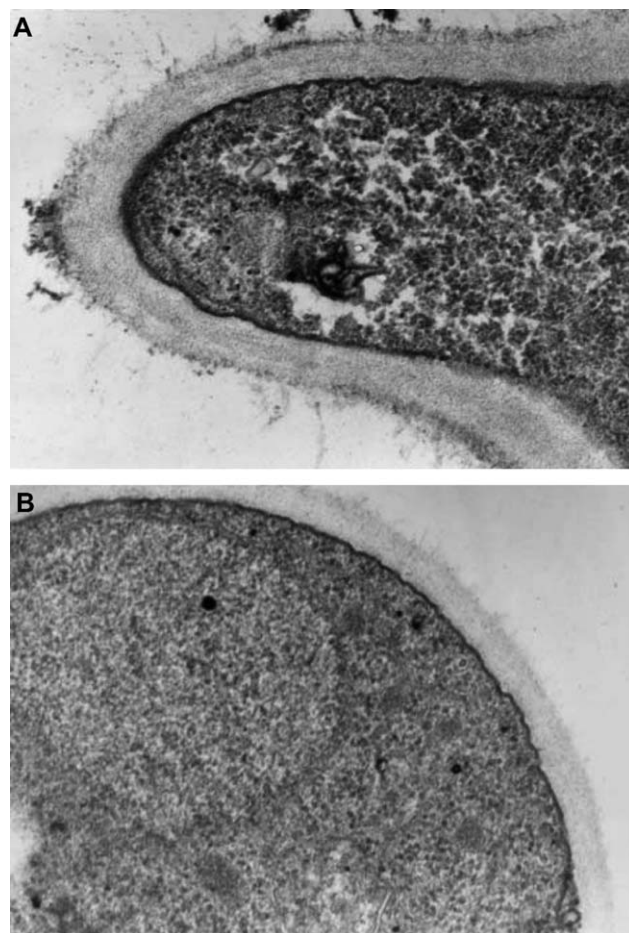

Fig. 3. Transmission electron micrographs of *P. brasiliensis* yeast cells grown with (A) and without (B) L-DOPA. Cells grown with L-DOPA show electron-dense particles at the cell surface and cytoplasm, unlike the untreated cells.
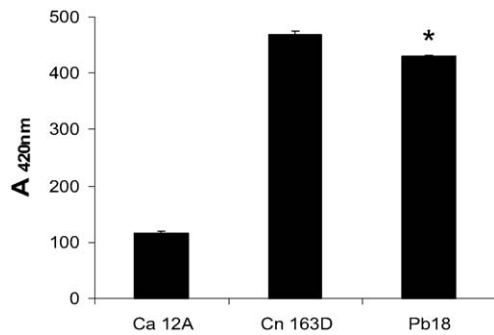
Fig. 4. Laccase activities in strains of *C. albicans* (Ca, negative control), *C. neoformans* (Cn, positive control) and *P. brasiliensis* yeast cells. Bars are averages of three measurements with standard deviations.
*Significant difference ($P < 0.05$) relative to the negative Ca control.

(lung and peritoneal) and cell lines J774.16 and MH-S. Non-melanized yeast cells were 3.5-fold more internalized than melanized cells by J774.16 cells (Fig. 5A) as well as by primary macrophages (data not shown). The MH-S cell line was much less efficient in the phagocytosis of both nonmelanized and melanized yeast. Similar results were obtained with primary lung macrophages (data not shown). The addition of complement in the system increased phagocytosis by both J774.16 and MH-S cells (Fig. 5A). An estimate of lateral binding of noninternalized yeasts was also made. Melanized yeast cells showed higher lateral binding indices to both J774.16 and MH-S cells (Fig. 5B). Similar results were obtained with primary macrophages (data not shown). The internalization of nonmelanized and melanized cells was confirmed by electron microscopy (data not shown). Phagocytosis was observed regardless of whether the macrophages were activated with IFN-γ or not but the indices were significantly higher with cytokine stimulated cells (data not shown).

The interference of melanin on the phagocytosis mediated by antibodies was also investigated. Addition of inactivated a polyclonal mouse antiserum to the cell wall of *P. brasiliensis* increased the phagocytosis indices with nonmelanized but not with melanized yeast cells (Fig. 6A). Similar results were obtained with MH-S cells (data not shown). When inactivated polyclonal antibodies generated against the melanin ghost were added, the phagocytosis index with melanized yeast cells increased in both J774.16 and MH-S (Fig. 6B).

3.4. Inhibition of in vitro phagocytosis with mannan

To determine if melanin interfered with the recognition of carbohydrate epitopes via mannose (MR) and/or other lectin-like receptors expressed on macrophages, yeast mannan was used as an inhibitor of cell interaction. Although a reduction in phagocytosis of nonmelanized cells occurred, adherence and uptake of melanized yeast cells was almost abrogated in both macrophage lines, J774.16 (Fig. 7) and MH-S (not shown). The present data suggest that melanin interfered directly with the interaction between carbohydrate residues on the cell wall and their receptors on macrophages.
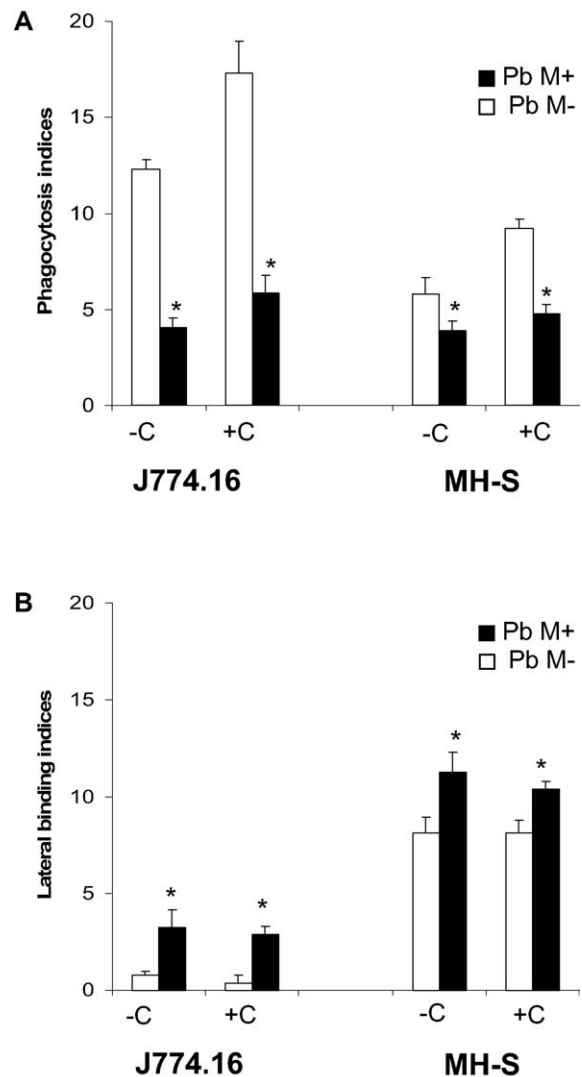

Fig. 5. Phagocytosis and laterally adherent noninternalized yeast cells after 24 h with (+C) or without complement (–C); (M+) melanized and (M–) non-melanized *P. brasiliensis* yeast cells. **A**, Phagocytosis indices. **B**, Lateral binding indices. The values are averages of three measurements with standard deviations.
*Significant difference ($P < 0.05$) comparing M+ and M– yeast cells.

3.5. Antifungal activity of macrophages

The relative efficacies of peritoneal (J774.16) and alveolar (MH-S) macrophage cell lines to kill nonmelanized and melanized *P. brasiliensis* yeast were assessed by measuring the CFUs 6, 12 and 24 h after incubation with IFN-γ-activated macrophages. The efficacy of J774.16 cells to kill nonmelanized cells of *P. brasiliensis* was greatest at 24 h of incubation. No significant differences were detected with melanized *P. brasiliensis* (Fig. 8). Similar results were obtained with MH-S cells. Addition of complement increased the efficiency of J774.16 macrophages to eliminate nonmelanized and melanized yeast cells (Fig. 8) with virtually no effect on MH-S cells (not shown).
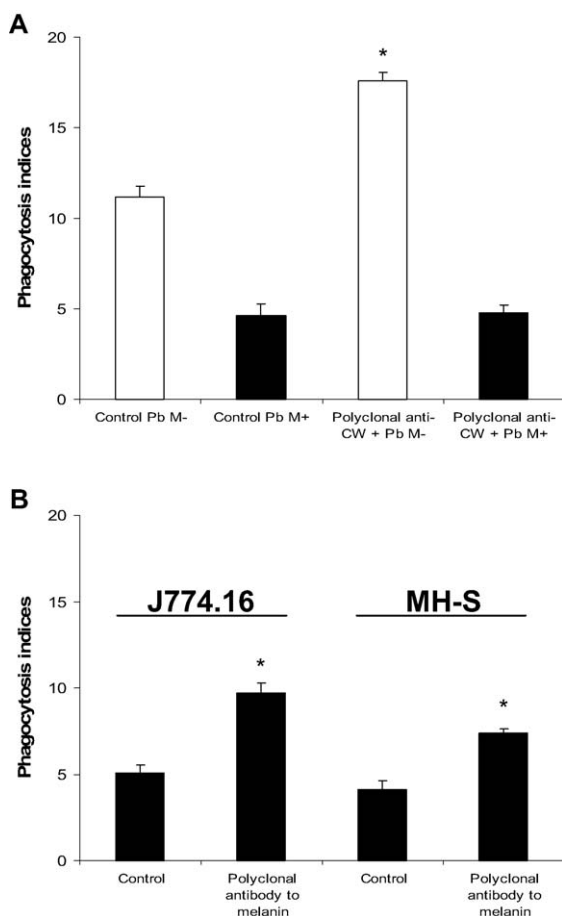
Fig. 6. Phagocytosis after 24 h of melanized (M+) and nonmelanized (M–) *P. brasiliensis* yeast cells opsonized with polyclonal antibodies against the cell wall (**A**) or the melanin ghost (**B**). In **A**, yeast cells were incubated with anti-CW antiserum and phagocytosed by J774.16 cells. Controls show the phagocytosis without antibodies. Values are averages of three measurements with standard deviations.
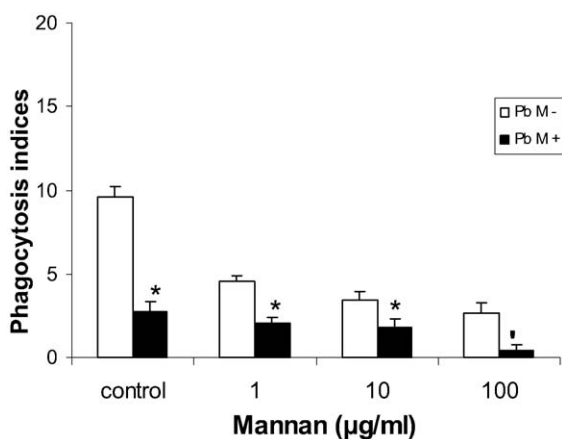*Significant differences ($P < 0.05$) comparing with controls.

Fig. 7. Inhibition of phagocytosis of melanized (M+) and nonmelanized (M–) *P. brasiliensis* yeast cells with different concentrations of yeast mannan. Values are averages of three measurements with standard deviations using J774.16 cells. Similar but less pronounced results were obtained with MH-S cells.
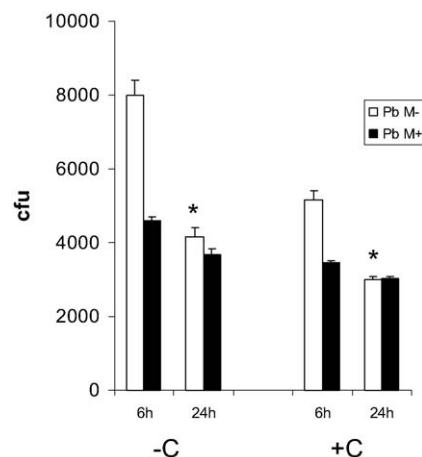*Significant difference ($P < 0.05$) relative to nonmelanized cells.

Fig. 8. Antifungal activity of macrophages after 6 and 24 h of incubation of nonmelanized (M–) or melanized (M+) yeast cells with J774.16 cells previously treated with 50 U/ml of IFN-γ. The effect of complement (C) was also verified. Similar but less pronounced results were obtained with MH-S cells. Values are averages of three measurements with standard deviations. *Significant difference ($P < 0.05$) relative to the 6 h bar.

3.6. MIC of antifungal drugs

Table 1 shows the susceptibilities of melanized and non-melanized cells of *P. brasiliensis* for amphotericin B, fluconazole, itraconazole, sulfametoxazole and ketoconazole. MICs were within the range of values previously reported for these fungi [32–36]. No significant differences were observed between MICs of antifungal drugs in melanized and nonmelanized cells.

In vitro drug susceptibility measured in laboratory conditions not always correlates with clinical efficacy against subacute or chronic mycotic infections. Consistent with such limitation, the MICs for the available antifungal agents may not predict their successful use in *P. brasiliensis* infections. In fact, results with the NCCLS method showed a correlation with the clinical outcome only for *C. albicans* infections [37–39], a fungus that does not melanize its cell wall.

3.7. Killing assay

Melanized cells were significantly less susceptible to all drugs tested. The differences were statistically significant with all concentrations tested of amphotericin B, ketoconazole, fluconazole and itraconazole (Fig. 9). Melanized yeast cells were

Table 1
MICs for *P. brasiliensis* with melanized and nonmelanized yeast cells

Drug	MIC (µg/ml)		
	Present results		Previous reference*
	With L-DOPA	Without L-DOPA	
Amphotericin B	0.5	0.5	0.06–0.243 [34]
Ketoconazole	0.008	0.008	0.001–0.007 [32,35]
Fluconazole	0.5	0.5	0.125–64 [32,35]
Itraconazole	0.025	0.025	0.0005–0.031 [32,35]
Sulfamethoxazole	300	300	300–600 [36]

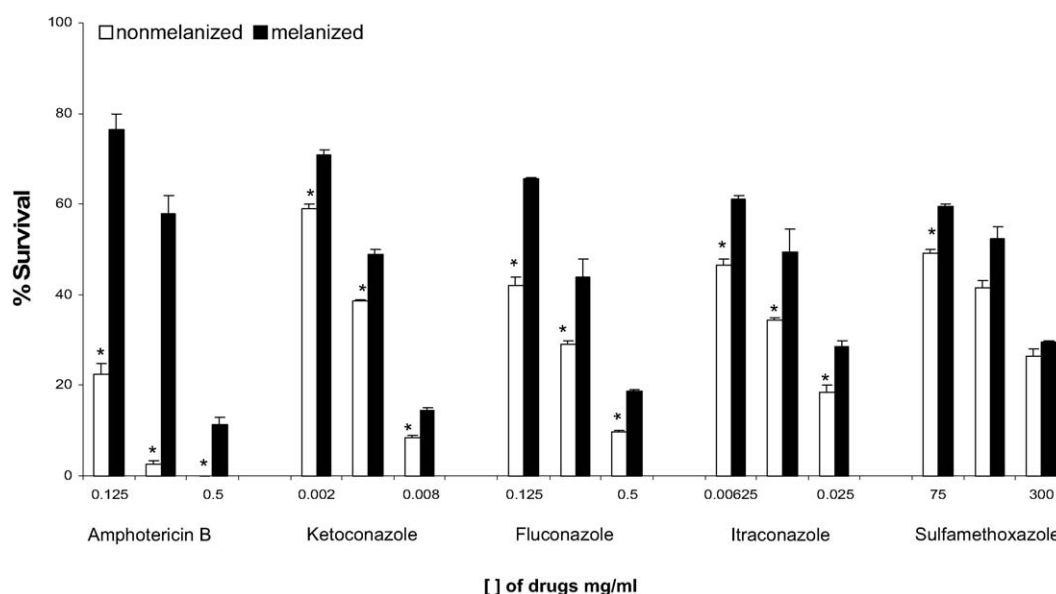* Growth without L-DOPA of different strains of *P. brasiliensis*.

Fig. 9. Antifungal killing assay. The survival rates of melanized and nonmelanized yeast cells after exposure to various concentrations of amphotericin B, ketoconazole, fluconazole, itraconazole and sulfamethoxazole are shown. Bars are averages of three measurements with standard deviations. *Significant differences ($P < 0.05$) relative to melanized cells.

less susceptible to sulfametoxazole, but a significant difference was observed only at 75 μg/ml.

4. Discussion

Melanin has been reported to be a virulence factor in several fungal pathogens. Some of them accumulate melanin in the cell wall constitutively [8,12], whereas others need a substrate to produce the pigment [13,15,40]. Early studies using Fontana–Masson stain suggested that *P. brasiliensis* did not produce melanin [12]. However, this stain is non-specific for melanin (reviewed in [9]) [41]. More recently, Gomez et al. [17] showed that growth of *P. brasiliensis* yeast in a defined medium with L-DOPA resulted in melanization of the cells. Furthermore, they isolated black particles similar in size and shape to *P. brasiliensis* yeast cells from infected murine tissues that were also reactive with a specific anti-melanin Mab [17]. The present results confirm the production of a melanin-like pigment in *P. brasiliensis* yeast growing in the presence of L-DOPA (Fig. 1). A laccase-like activity was also demonstrated (Fig. 4), using a different method from that described previously [17]. Both features strongly suggest the synthesis of melanin in yeast cells.

The scanning electron micrographs did not show any difference between melanized and nonmelanized yeast cells (Fig. 2), but when these cells were analyzed by transmission EM electron-dense granules were detected around the cell surface and in the cytoplasm (Fig. 3). Similar results were observed in melanized *H. capsulatum* [13], but scanning electron micrographs showed tufts at the surface of melanized *H. capsulatum* yeast cells in contrast with the smooth surface of nonmelanized cells.

The mechanism of phagocytosis of unopsonized *P. brasiliensis* is not completely understood. Macrophages have several receptors that recognize carbohydrate structures, such as β-glucan receptors, CR3 and MR [42]. The mannose/fucose receptor has been implicated in the binding and phagocytosis of bacteria, fungi, and protozoa [42]. MR expressed on macrophages can bind mannan and activate the host immune system via a nonself-recognition mechanism [43]. We have observed that peritoneal macrophages rather than alveolar macrophages take up *P. brasiliensis* more efficiently even after opsonization with complement (Fig. 5). In contrast, alveolar macrophages have significantly higher lateral binding indices of noninternalized yeast cells than peritoneal macrophages (Fig. 5B). The poor efficacy of alveolar macrophages in comparison with peritoneal macrophages to phagocytose *P. brasiliensis* yeast cells may influence the initial phase of infection depending on the number of conidia, or even yeast cells inhaled by the host. When the assays were conducted with melanized yeast cells, the phagocytosis indices were significantly reduced in all conditions and with macrophages from different sources (alveolar or peritoneal). When phagocytosis was conducted with *P. brasiliensis* opsonized with anti-cell wall polyclonal antibodies, we observed that melanin interfered with the binding of these antibodies since no increase in the phagocytosis was detected; significant increase of phagocytosis was only observed when polyclonal mono-specific antibodies against melanin ghost were added (Fig. 6). Similarly, the phagocytosis of melanized antibody-opsonized *C. neoformans* was less efficient than that of opsonized non-melanized cells [20]. A dose dependent reduction in yeast cell uptake occurred with the addition of mannan as a binding competitor in the phagocytosis assay (Fig. 7). The most significant reduction occurred with nonmelanized cells using peritoneal macrophages while only high concentrations of mannan significantly reduced phagocytosis of melanized cells. Using MH-S cells inhibition of phagocytosis was more pro-

nounced with high levels of mannan. Several other studies have shown that monosaccharides [44], mannan [45], and other MR ligands [42] compete with the microbes for binding to macrophages. Most of these studies are difficult to interpret due to the complexity of the system. It is clear from the present studies that melanin can interfere directly with the binding of fungi to phagocytic cells of different sources. Melanization has also been shown to diminish the efficacy of phagocytosis in the *C. neoformans* system but that system is very different since a polysaccharide capsule separates the cell wall-associated melanin from cellular receptors [20].

In certain fungal species, melanized cells have been shown to be less susceptible to killing by oxygen- and nitrogen-derived free radicals [21,46]. Laccase of *C. neoformans*, as a virulent factor, can protect fungi against the cytotoxicity of alveolar macrophages [47]. Melanized *P. brasiliensis* were more resistant than nonmelanized yeast cells to the fungicidal and fungistatic effects of J774.16 and MH-S cells. The mechanism by which macrophages reduce *P. brasiliensis* CFU in the presence or absence of complement seems to involve fungal cell damage by oxygen- and nitrogen-derived free radicals [42,48] as well as fungicidal proteins [49]. Melanized cells were more resistant to complement-mediated phagocytosis than nonmelanized cells (Fig. 8). The mechanisms by which melanin enhances the resistance of *P. brasiliensis* to J774.16 and MH-S cells may in fact be a combination of reduced phagocytosis and increased resistance to oxygen- and nitrogen-derived antimicrobial products generated in these cells after IFN-γ activation [48].

Melanization did not affect the cytotoxicity of amphotericin B, ketoconazole, fluconazole, itraconazole, sulfametoxazole as measured by standard broth macrodilution procedures for assessing the susceptibilities of yeast cells to antifungal drugs. Similar results have previously been observed with melanized and nonmelanized *C. neoformans* and *H. capsulatum* [22]. However, an increased resistance of melanized cells to antifungal drugs was revealed using a killing assay. We show that melanization of *P. brasiliensis* protected yeast cells against all drugs tested in at least one dilution. The killing assay with melanized *C. neoformans* and *H. capsulatum* showed less susceptibility to amphotericin B and caspofungin, but melanization did not affect cell resistance when fluconazole or itraconazole was used [22]. No significant killing of fungal cells was obtained with the potentially fungicidal drug sulfamethoxazole. In the case of amphotericin B, incubation with synthetic or *C. neoformans* melanin reduced the fungicidal activities of both amphotericin B and caspofungin [22]. Hence, the findings with *P. brasiliensis* parallel results with *C. neoformans* [50] but we extend the findings to sulfonimide-type drugs.

In conclusion, *P. brasiliensis* production of melanin appears to contribute to virulence by reducing the uptake of yeast cells by peritoneal and alveolar macrophages, thereby enhancing the resistance of fungal cells to attack by immune effector cells. Melanized yeasts of *P. brasiliensis* are also less susceptible to potent antifungal drugs, particularly amphot-

ericin B. Hence, melanization may significantly impact our ability to treat patients with PCM, and melanin is therefore a potential target for therapeutic intervention.

Acknowledgments

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