Effects of Melanin upon Susceptibility of Cryptococcus to Antifungals

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Abstract: Melanin is a recognized virulence factor in Cryptococcus neoformans; several pathogenetic mechanisms have been suggested. We studied melanin as an antifungal resistance factor. The growth of laccase-active strains of C. neoformans and C. albidos in L-DOPA resulted in the production of black pigment. The formal minimal inhibitory concentrations (MICs) of amphotericin B and fluconazole were not changed by melanization. However, when we examined those wells which contained inhibited cells, we found live cells only in wells containing melanized C. neoformans. In contrast, melanization did not protect C. albidos from killing by amphotericin B. In an amphotericin B time-kill study of C. neoformans, significantly more melanized cells than non-melanized survived for the first few hours. Fluorescence microscopy and flow cytometry analyses showed that fewer melanized cells were stained with the fluorescent dye MitoRed. Incubation of MitoRed (the model) or amphotericin B with melanin extracted from C. neoformans decreased the free concentrations of these substances. Fluconazole, in contrast, was not removed from solution by melanin. This suggests that neoformans cryptococcal melanin deposited amphotericin B in the cell wall, reducing its effective concentrations.

Key words: Cryptococcus neoformans, Cryptococcus albidos, Melanin, Susceptibility

Melanization effected by laccase is recognized as a virulence factor in the pathogen Cryptococcus neoformans (1–3, 6, 13, 14, 17, 20, 27, 28). Melanin is thought to protect C. neoformans from physical stress (18, 24) and from oxidants (8, 23). Melanin has been shown to bind proteins, and melanized cells are less susceptible to enzymatic degradation (19) and to killing by microbicial peptides (4). Melanin also can protect the pathogen against antifungal agents; C. neoformans cells cultured in the presence of L-DOPA are less sensitive to amphotericin B (25).

Clinical infections caused by Cryptococcus species other than C. neoformans have been reported (9, 11, 12, 15). We showed that strains of C. albidos, C. laurentii, and C. curvatus produced laccase, although the activity was lower than in C. neoformans (7). Here, we examined whether these species become black melanized cells when incubated with the laccase substrate and whether melanization causes drug resistance in these species. We used time-kill tests to compare the susceptibilities of melanized and non-melanized cells of C. neoformans and C. albidos to antifungal agents, and we investigated the role of melanin in resistance to antifungal agents.

Materials and Methods

Strains used. C. neoformans B-3501 was obtained from the National Institutes of Health (Bethesda, Md., U.S.A.), and C. albidos CBS142 was from the Centraalbureau voor Schimmecultures.

Preparation of melanized cells. Each strain was inoculated into 10 ml of 2% glucose and 2% yeast extract and incubated with agitation at 25 C for 24 hr. The culture (0.1 ml) was inoculated into 40 ml of asparagine salts medium (per liter: glucose, 3 g; asparagine, 1 g; MgSO4 \cdot 7H2O, 0.5 g; KH2PO4, 3 g; thiamine, 1 mg) and incubated for 24 hr with agitation. The cells were harvested by centrifugation at 1,000 \times g for 10 min at 4 C and washed.

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Abbreviations: L-DOPA, L-β-(3, 4-dihydroxyphenyl) alanine; MIC, minimal inhibitory concentration; PBS, phosphate buffered saline.
once with asparagine salts without glucose. The cells were resuspended in medium without glucose plus L-DOPA at a final concentration of 1 mM. The cells were incubated at 25 °C in the dark for at least one week, until black cells were obtained. As controls, tubes without L-DOPA or without cells were incubated under the same conditions.

**Antifungal agents.** Stock solutions of amphotericin B (Wako, Osaka, Japan) in DMSO and fluconazole (ICN Biomedical Inc., Aurora, Ohio, U.S.A.) were diluted using RPMI1640 (Sigma) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS). The minimal inhibitory concentrations (MICs) of amphotericin B and fluconazole were determined for melanized and non-melanized cells using the broth microdilution techniques described by the National Committee for Clinical Laboratory Standards (16).

**Time-kill tests.** Time-kill studies were based on the method described by Klepser et al. (10). The adjusted cell suspension in RPMI (2×10^3 or 2×10^5 cells/ml) was added to the same volume of a solution of RPMI containing antifungal agent or medium alone. The solutions were incubated at 27 °C. After 1, 2, 5, 24, or 48 hr, each solution was diluted in sterile physiological saline solution (PSS) and plated on Sabouraud dextrose agar to determine viable colony counts.

**Staining with fluorescent dye.** The dye MitoRed, which is used to stain mitochondria, was purchased from Dojindo Laboratories and dissolved in dimethylsulfoxide (DMSO). A suspension of 2×10^5 melanized or non-melanized cells per ml in PBS was incubated with 200 nmol MitoRed at 37 °C for 1 hr. After centrifugation, the cells were resuspended in 10% formalinized PBS and kept in a refrigerator overnight. After washing the cells with PBS, the cells were observed by fluorescence microscopy and analyzed using a flow cytometer FACScan (Becton Dickinson, San Jose, Calif., U.S.A.). A total of 10,000 cells were analyzed.

**Preparation of melanin from C. neoformans.** Melanin was extracted from melanized C. neoformans using the method described by Wang et al. (26). Briefly, melanized C. neoformans cells were suspended in 1.0 M sorbitol in 0.1 M sodium citrate (pH 5.0). Novozyme234 (10 mg/ml) was added to the cell suspension. The suspension was incubated at 30 °C for 1 hr. After centrifugation, 4 M guanidinium isothiocyanate was added and incubated at room temperature for 30 min. The cell debris was suspended in 6 M HCl at 100 °C for 30 min. The black pellet was dialyzed against H_2O and particulate melanin was obtained. The concentration is expressed as particles per ml.

**Results**

**Melanization of Non-neoformans Cells**

Each strain was incubated with L-DOPA in asparagine salts medium without glucose. C. neoformans became black within 5 days. C. albidus also became black; however, the color change took longer. The medium containing L-DOPA without cells remained clear for ten days. Figure 1 shows melanized C. albidus.

**Susceptibility Tests**

The antifungal susceptibilities of melanized and non-melanized cells were compared. The MICs for amphotericin B to non-melanized and melanized C. neoformans and C. albidus were all 0.25 µg/ml. The MICs for fluconazole to non-melanized and melanized cells of C. neoformans were 2 µg/ml. The strain of C. albidus was known to be azole-resistant (21), therefore MICs for fluconazole for C. albidus are not shown. These results showed that the MICs for melanized cells were equivalent to the MICs to non-melanized cells. After determining MICs, the contents of the wells were spread on Sabouraud agar to determine viable colony counts.

In those wells which contained inhibited cells, only melanized cells were alive even at the concentration of 16 µg/ml amphotericin B. Non-melanized cells were all dead. The live colonies from Sabouraud agar were found to have reverted to sensitivity.

**Time-Kill Test**

In C. neoformans, after 2 and 5 hr significantly more melanized cells than white non-melanized cells were alive at the MIC for amphotericin B, as shown in Fig. 2. Curiously, the viability using 10^6 cells/ml (A) was less than with 10^5 cells/ml (B), although there were more live cells in the experiments using 10^6 cells/ml. Similar but weaker results were obtained for fluconazole (Fig. 3) at concentrations of 1× and 4× the MIC; after 1 and 2 hr, the viability of melanized cells was slightly higher.
than that of non-melanized cells.

**Staining Cells with Fluorescent Dye**

To model the effect of the melanization of *C. neoformans* and *C. albidos* on susceptibility, cells were stained with MitoRed dye, accumulation in mitochondria. Under a fluorescent microscope, melanized cells were observed to be less well stained than non-melanized cells (Fig. 4). These results were confirmed by flow cytometry (Fig. 5, A–D). Next, equal amounts of black and white cells were mixed and stained. The peak of fluorescence intensity of the mixture of white and black cells was midway between those for the separate cells. However, the level of intensity was reduced below that expected in the case of a simple mixture (E). These results could not be explained by a change in the permeability of the dye due to melanin deposited in the cell wall, rather, they suggest absorption of dye by melanin. To examine whether there was direct binding of melanin and dye, the dye (200 nmol) was pre-incubated with melanin (10^7 particles/ml) from *C. neoformans* at 37 C for 1 hr. After centrifugation, the color of the dye in the supernatant was greatly diminished. Moreover, the intensity was decreased remarkably when dye pre-incubated with melanin was used to stain
Fig. 4. Light (A and B) and fluorescence (C and D) microscopy of non-melanized (A and C) and melanized (B and D) cells following staining with MitoRed.

Fig. 5. Analyses of MitoRed uptake of *C. neoformans* by flow cytometry. (A) Non-melanized cells and PBS, (B) melanized cells and PBS, (C) non-melanized cells and MitoRed, (D) melanized cells and MitoRed, (E) mixture of non-melanized and MitoRed and melanized cells, (F) MitoRed was pre-incubated with extracted melanin before staining of non-melanized cells.
non-melanized cells (F).

Treatment of Drugs with Extracted Melanin

Next, amphotericin B (256 µg/ml, 0.3 ml) or fluconazole (256 µg/ml, 0.3 ml) was pre-incubated with melanin (1 or 2×10³ particles/0.3 ml) at 37°C for 3 hr; the melanin was subsequently removed by centrifugation and the supernatant was serially diluted and tested for inhibition of C. neoformans. The nominal MICs for pre-incubated amphotericin B were found to be increased compared to those by untreated amphotericin B (Fig. 6). To confirm the melanin effect, further experiments with different doses of melanin were done. As shown in Fig. 7, MICs were increased in a dose-dependent manner. This suggests that cryptococcal melanin binds amphotericin B and blocks its movement across the plasma membrane. Rather, pre-incubation of fluconazole with melanin did not increase the nominal MICs. We infer that C. neoformans is not protected because melanin does not absorb this drug.

Discussion

We investigated the role of melanin in resistance to antifungal agents. Although MICs appeared not to be affected by melanin, melanized C. neoformans cells were more resistant to killing by amphotericin B than non-melanized cells. We examined whether melanin constituted a permeability barrier and blocked the uptake of amphotericin B. However, when we pre-incubated amphotericin B with melanin, the antifungal appeared to be removed from solution. We compared this result to absorption of the mitochondrial dye, MitoRed, by melanin. In each case, the results suggested that both substances bound to melanin, decreasing the concentrations of the free substance in solution. In contrast, melanin did not absorb fluconazole detectively and did not protect against fluconazole nearly as well.

Very recently van Duin et al. reported the effects of melanization of C. neoformans and Histoplasma capsulatum on the susceptibility to antifungal agents (22). They found no difference in MICs between melanized and non-melanized cells and showed that in a 2 hr killing assay melanization reduced the susceptibility to amphotericin B and caspofungin. They also reported that the binding of amphotericin B and caspofungin to melanin reduced the antifungal activities. Our results are in complete agreement.

In conclusion, the growth of laccase-active Cryptococcus strains in L-DOPA resulted in the production of black pigment. Pigmented C. neoformans was much more resistant to killing by amphotericin B and slightly so to killing by fluconazole. The melanin deposited in the cell wall appears to capture drugs, preventing them from reaching their active sites. However, the affinity of melanin and drugs could be modified by factors not yet known.

It seems strange that melanin protects C. neoformans from killing but does not prevent inhibition of growth. The explanation may come partly from the way in which Cryptococcus makes melanin and in the way the experiments were performed. Exogenous substrate is required for melanization in this genus, but the accepted medium for determining the MIC does not provide melanin substrate. Thus, melanized cells used to test antifungals were first exposed to very high concentrations of catecholamines (1 mM) but were not able to continue to make melanin when they were exposed to the drugs.
Van Duin et al. (22) have suggested that over the time course of such an MIC determination, the cells outgrow their melanin layer and lose protection. In contrast, during the short time course of a killing experiment, the melanin layer remains intact. In the clinical situation, melanin substrate is believed to be continuously present (13). However, in infections, the amount of melanin per cell is believed not to be as large as it can be in vitro (8), probably because mammals contain relatively small amounts of free catecholamines in their tissues. If the protection afforded by melanin depends upon the amount of melanin on the fungal cells, infecting fungi may not contain sufficient melanin to be protected. Thus, the significance of our results to therapy of cryptococcosis is unclear. However, it is perfectly consistent with our results that chromomycetes, richer in melanin than Cryptococcus, respond much better to triazole antifungals than to amphotericin B (5).

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References