Growth of Cryptococcus neoformans in Presence of L-Dopa Decreases Its Susceptibility to Amphotericin B

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Cryptococcus neoformans grown with l-dopa was melanized and was less susceptible to amphotericin B. The results suggest that in vivo and in vitro susceptibilities to amphotericin B may differ.

Cryptococcus neoformans is an opportunistic fungal pathogen which causes meningitis in approximately 6 to 8% of AIDS patients (4, 21). Amphotericin B (AmB) remains the mainstay of therapy for C. neoformans meningitis (3). However, AmB often fails to eradicate the infection, and relapses are frequent in patients with AIDS (3, 21). AmB is a polyene antibiotic which is believed to mediate antifungal effects by binding to cell membrane sterols and damaging the cell membrane (2). The fungicidal activity of AmB may also be the result of cell damage by reactive oxygen species (2).

Melanin synthesis has been associated with virulence in C. neoformans (10, 15). Melanin synthesis is catalyzed by a phenoloxidase (laccase) enzyme from catechol precursors such as l-dopa (14, 20). The addition of l-dopa to fungal media results in the rapid melanization of C. neoformans cells. Melanin pigment is deposited in the fungal cell wall (17). Melanization of C. neoformans cells in human brains has been described previously (9, 16). Although melanin pigments are ubiquitous in nature, their physiologic functions are poorly understood (6). Melanins can protect cells from free radical and UV light damage (6, 18). Melanins are also capable of binding drugs and chemicals (6). Structurally, melanins are polyanionic molecules which contain stable populations of organic free radicals (5). Given that AmB is believed to mediate fungicidal action by disrupting the cell membrane and that melanins have been proposed to have multiple roles including free radical-scavenging functions, we hypothesized that the melanization of C. neoformans cells could affect the organism’s susceptibility to AmB. We tested this hypothesis by determining the survivals of melanized and nonmelanized C. neoformans cells after exposure to AmB.

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C. neoformans 24607 (serotype D) was obtained from the American Type Culture Collection (Rockville, Md.) and was grown in defined minimal medium (15 mM glucose [EM Science, Gibbstown, N.J.], 10 mM MgSO4 [J. T. Baker Inc., Phillipsburg, N.J.], 29.4 mM KH2PO4 [J. T. Baker], 13 mM glycine [United States Biochemical Corp., Cleveland, Ohio], 3.0 μM vitamin B1 [Sigma Chemical Co., St. Louis, Mo. [pH 5.5]) with or without 1.0 mM L-dopa (Sigma). L-Dopa was chosen as the phenoloxidase substrate because it is a human neurotransmitter precursor. The L-dopa concentration of 1.0 mM was chosen because it results in rapid melanization and has been used in previous studies (7, 14, 18, 19). For AmB susceptibility experiments cells were washed two times with distilled water, suspended in distilled water, and exposed to various concentrations of AmB (Boehringer Mannheim Inc., GMBH, Mannheim, Germany). AmB suspensions in distilled water were made fresh before each experiment. The susceptibilities of cells grown with or without the l-dopa were studied by removing aliquots from the culture for testing at various time points of growth. After 1 h of exposure to AmB, C. neoformans cells were plated on Sabouraud dextrose agar plates (Difco Laboratories) to determine their viabilities, as measured by the number of colonies. Percent survival was obtained by comparing the number of colonies relative to the

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number of cells not exposed to AmB. Student's t test was done with Primer of Statistics version 3.0 (McGraw-Hill Inc., New York, N.Y.). All data are expressed as averages ± standard deviations.

Figure 1 shows that the survival of 10-day-old melanized cells is significantly greater than that of 10-day-old nonmelanized cells after exposure to AmB at concentrations of 0.1 and 0.2 μg/ml, respectively. At an AmB concentration of 0.3 μg/ml most cells were killed, and there were no survival differences between melanized and nonmelanized cells. To determine the relationship between melanization and susceptibility to AmB, cells were grown in the presence and absence of l-dopa for various time intervals, exposed to AmB (0.2 μg/ml), and plated to determine percent survival (Fig. 2). Decreased susceptibility of C. neoformans to AmB for cells grown with l-dopa was evident after 1 day of growth, at which time little or no melanization was apparent by visual inspection. Day-to-day variation in susceptibility to AmB was noted in the time course, which may reflect subtle metabolic changes or experimental variation (Fig. 2). However, at all time points cells grown with l-dopa were less susceptible to AmB than cells grown without l-dopa.

The mechanism by which cells grown in the presence of l-dopa are less susceptible to the fungicidal effects of AmB is unknown. Our experiments do not allow a distinction between decreased AmB susceptibility resulting from growth in l-dopa versus cell melanization. l-Dopa is not utilized as a nitrogen or carbon source by C. neoformans (15, 19) but is oxidized by the phenoloxidase enzyme into short-lived unstable intermediates which polymerize to form melanin (13, 20). The effect of the growth of l-dopa on membrane sterol composition, if any, is unknown. Although l-dopa oxidation products could conceivably react with AmB, this is unlikely given that l-dopa oxidation probably occurs intracellularly (phenoloxidase is found in either the cytoplasm [7] or the inner cell membrane [14]), whereas the AmB-sterol interaction is likely to occur at the outer cell membrane. A more likely explanation is that the decreased susceptibility of cells grown with l-dopa is a result of melanin synthesis. Melanin is deposited in the cell wall, where it is in a position to interact with extracellular substances. Melanin is a complex polymer which can bind to multiple types of drugs and chemicals such as organic amines and polycyclic hydrocarbons (11). This suggests that melanin could protect the organism against the effects of AmB by binding the drug or reducing the permeability of the cell wall to AmB. Alternatively, melanin could protect the organism against oxidant fluxes resulting from disrupted cell membranes by AmB. In this regard melanin has been shown to be a free radical scavenger and antioxidant (8, 12). Regardless of the mechanism by which growth in the presence of l-dopa decreases AmB susceptibility, the results may have clinical implications. C. neoformans in human infections are melanized in brain tissues (9, 16), possibly as a result of the presence of neurotransmitter precursors in the central nervous system. Melanized C. neoformans cells may be less susceptible to AmB in vivo, and this could contribute to the difficulty in eradicating C. neoformans with antifungal therapy. The AmB concentrations used in our experiments (0.1 to 0.3 μg/ml) are in the range of plateau levels in serum (0.2 to 0.5 μg/ml) after the administration of conventional intravenous doses (1). Furthermore, the results raise the possibility that in vitro and in vivo AmB susceptibilities are different, suggesting a potential confounding variable for the correlation of in vitro susceptibility with clinical outcome.

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REFERENCES


