INFECTION AND IMMUNITY, Dec. 1995, p. 4944–4945 Vol. 63, No. 12
0019-9567/95/$04.00+0

Antioxidant Function of Melanin in Black Fungi

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Received 26 June 1995/Returned for modification 8 August 1995/Accepted 15 September 1995

1,8-Dihydroxynaphthalene melanin in *Wangiella dermatitidis* and *Alternaria alternata* was titrated in vivo with the oxidants permanganate, hypochlorite, and H_2O_2. Melanized strains neutralized more oxidant and withstood higher concentrations of permanganate and hypochlorite than albino strains did. H_2O_2 killing required 1,000-fold higher concentrations, and melanin did not protect *W. dermatitidis* against H_2O_2.

Leukocytes kill pathogenic microbes with a flux of secreted strong oxidants (1). Thus, any microbial product which neutralizes oxidants is likely to protect the pathogen and promote invasion. Melanin, a redox buffer composed of polymeric ortho-dihydroxylated aromatics (6, 13, 14, 17), is made by several pathogenic fungi, and melanin-deficient mutants have decreased virulence for mice (2, 11). Several types of evidence support the antioxidant role of melanin in *Cryptococcus neoformans* (7, 8, 15, 17a, 17b), which makes catecholamine melanin only when supplied with monomeric precursors (10). The melanotic pathogens which cause phaeohyphomycosis or chromoblastomycosis produce melanin constitutively from endogenous 1,8-dihydroxynaphthalene (DHN) (2, 3, 9, 16); the redox function of this type of melanin has not yet been examined. The availability of isogenic albino mutants of *Wangiella dermatitidis* (5) and *Alternaria alternata* (9) prompted us to study the role of DHN melanin in resistance to oxidants in those species.

W. dermatitidis 8656 (wild type) and *mel3* (an albino), gifts from Paul J. Szaniszlo, were cultured in flasks with 12-mm sidearms containing 300 ml of Czapek Dox broth at room temperature with agitation until the wild type contained 1.5 to 5 U of melanin. Cultures were exposed to fluorescent light according to the diurnal cycle. Melanization was estimated as optical density at 400 nm by comparison to the albino culture. Cultures with optical densities greater than 1.0 were diluted prior to reading; final wet weights of paired cultures were within 10% of each other. Melanized and albino cells were washed twice in phosphate-buffered saline, pH 7.5 (PBS), diluted equally to an optical density of 0.5 (albino culture against water at 700 nm) and quantitated in a counting chamber. One-milliliter aliquots were diluted to 6 ml with PBS containing various concentrations of oxidants (potassium permanganate, sodium hypochlorite [Clorox], or hydrogen peroxide) in 25-ml sidearm flasks. After 3 h, the absorbance of residual permanganate was determined at 520 nm, by using melanized or albino cell suspensions without permanganate used as optical blanks. Growth medium (5 ml) was added after 16 h, and growth was scored 2 days later. *A. alternata* 15A (wild type) and Alm^- (an albino), gifts from T. Tsuge, were grown to stationary phase in potato dextrose agar at room temperature until the wild type appeared quite black (1 week). Mycelial clumps were washed twice and resuspended in PBS in the original volume, and 10-ml samples were filtered and dried to constant weight. Aliquots (1 ml) were pipetted into flasks by using large pipettes without constrictions and were titrated with permanganate and with hypochlorite as described above. Because of imprecise pipetting of poorly dispersed *Alternaria* cultures, alternation of growing and nongrowing cultures was often seen in the vicinity of the minimal fungicidal concentration of oxidant. In such cases, an artificial endpoint was constructed by rearranging the results for growing and nongrowing strains into two homogeneous regions in the concentration curve. Statistical significance was estimated by using Student's *t* test.

In the case of *W. dermatitidis*, in vivo redox titrations with permanganate demonstrated a rightward shift of the concentration-absorption plot, indicating consumption of oxidant by biomelanin (Fig. 1A). The concentration axis intercepts were taken to represent the equivalence points of the redox titrations. The difference between the concentration axis intercepts for melanized and albino cells was taken to represent the quantity of permanganate consumed by melanin, as was done in the study of *C. neoformans* by Jacobson and Tinnell (8). The differences between concentration axis intercepts in various experiments were found to be roughly proportional to the amount of melanin present in the melanized cultures (Fig. 1B), indicating that melanin represents the major external antioxidant. The minimal fungicidal concentrations of permanganate were 0.131 mM for the melanized culture and 0.021 mM for the nonmelanized culture ($P < 0.01$) (Table 1); for hypochlorite, the minimal fungicidal concentrations were 0.039 and 0.006 mM, respectively ($P < 0.01$). In contrast, the melanized cultures were not more resistant to hydrogen peroxide than the albino cultures were (6.5 mM for both). All of these results closely resemble those obtained with *C. neoformans*. Among the reasons for the lack of protection from H_2O_2 the most important is that reaction of H_2O_2 with melanin requires alkaline conditions (18) (which ought not to be present at sites of infection). Thus, H_2O_2 appears to be a weak fungicide; killing with H_2O_2 under neutral conditions requires 1,000-fold-higher concentrations than does killing with hypochlorite, and the necessary quantities of H_2O_2 are far greater than the cellular redox capacity of fungal melanin (see below). According to this reasoning, one might expect melanin to protect against a chemically equivalent quantity of highly reactive oxidant but not against a much larger quantity of slowly reacting oxidant. Finally, the electrically neutral H_2O_2 molecule would be expected to cross the fungal cell membrane more freely than charged oxidants; the latter might be restricted to the (melanized) extracellular space. A quantitative estimation of the protective capacity of *Wangiella* melanin was made by dividing the difference in permanganate killing concentrations between melanized and albino cultures by the number of cells per aliquot. The average quantity obtained was 15.6 fmol of electron equivalents per cell, comparable to 21 fmol per cell obtained for *Cryptococcus* spp. (8) and relevant to reports that activated macrophages produce 2 to 32 fmol of oxidant per cell (4, 12).

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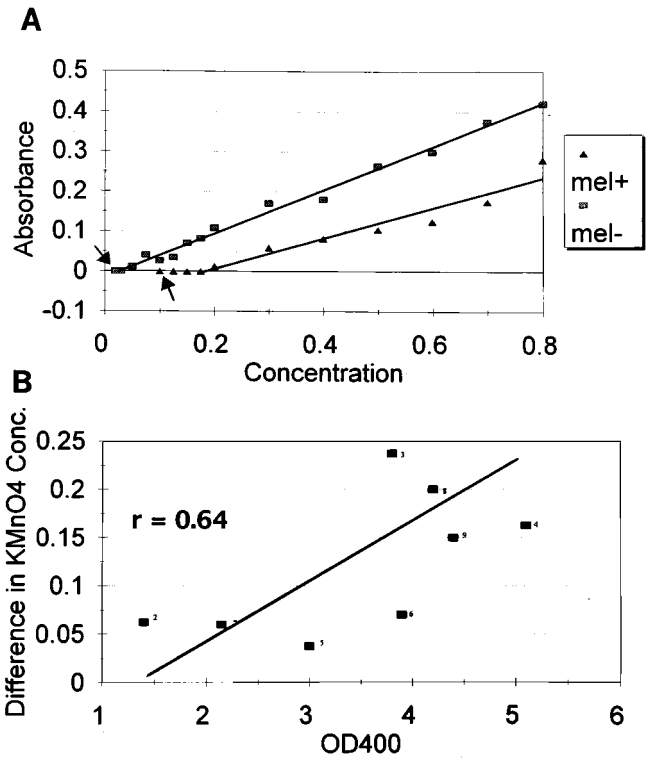

FIG. 1. (A) In vivo redox titrations of melanized and albino strains of *W. dermatitidis* with $KMnO_4$. A_{520} of MnO_4^- was measured with reference to cell suspensions without $KMnO_4$. Arrows indicate minimal fungicidal concentrations (the concentration was lower for the albino). The optical density of melanin was 4.4. (B) Dependence of MnO_4^- consumption upon cultural melanin. Differences in $KMnO_4$ concentration (conc.) axis intercepts in various experiments were plotted against optical densities at 400 nm (OD400) of melanin of the respective wild-type cultures.

It was not possible to monitor the growth of *A. alternata* as optical density, because of the unevenly dispersed cultures. However, 10-ml aliquots of wild-type and albino cultures contained almost equal dry weights of fungus (45 and 50 mg, respectively). Therefore, washed, undiluted suspensions were studied for sensitivities to oxidants, and sensitivities were expressed as minimal fungicidal ratios in micromoles per milligram of fungus (dry wt) (Table 1). The differences between the ratios were significant for both permanganate and hypochlorite ($P < 0.05$ and $P \sim 0.05$, respectively). H_2O_2 was not studied in *A. alternata*, since it was not protective in either *C. neoformans* (8) or *W. dermatitidis.*

TABLE 1. Minimal fungicidal oxidant concentrations for melanized and nonmelanized fungi

Oxidant	Melanin	MFC for *W. dermatitidis* (mM)[a]	*P*	MFC for *A. alternata* (μmol/mg)[a]	*P*
MnO^-_4	+	0.131 ± 0.081	<0.01	7.4 ± 1.0	<0.05
	−	0.021 ± 0.013		3.1 ± 0.4	
ClO^-	+	0.039 ± 0.000	<0.01	4.0 ± 0.8	0.05
	−	0.006 ± 0.005		2.7 ± 0.6	
H_2O_2	+	6.5 ± 4.9	NS[b]		
	−	6.5 ± 4.9			

[a] MFC, minimal fungicidal concentration. Values are means ± standard deviations.
[b] NS, not significant.

In both species, DHN melanin was associated with resistance to permanganate and hypochlorite. Thus, DHN melanin in *W. dermatitidis* and *A. alternata* appeared to confer cellular redox properties which were quantitatively (for *W. dermatitidis*) and qualitatively (for *A. alternata*) similar to those conferred by dopamine melanin upon *C. neoformans*. Since the magnitude of the protection was comparable to the amount of oxidant generated by macrophages, the redox buffering capacity of melanin is likely to contribute to pathogenesis of infections caused by melanotic fungi.

This work was supported by the Department of Veterans Affairs (E.S.J.) and by Public Health Service grant R15 AI29628 (H.S.E.).

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Editor: D. H. Howard