JOURNAL OF BACTERIOLOGY, Nov. 1993, p. 7102–7104
0021-9193/93/217102-03$02.00/0

Vol. 175, No. 21

Antioxidant Function of Fungal Melanin

ERIC S. JACOBSON[1,2]* AND SHERI B. TINNELL[3]

Research Service, Box 151, McGuire Veterans Affairs Medical Center, Richmond, Virginia 23249,[1] and Departments of Medicine[2] and Microbiology and Immunology,[3] Virginia Commonwealth University, Richmond, Virginia 23298*

Received 11 March 1993/Accepted 9 August 1993

Polyphenols have been implicated in the virulence and oxidant resistance of *Cryptococcus neoformans*. Although monomeric polyphenols did not protect against the prooxidant, plumbagin, polymeric dopamine-melanin conferred resistance both to hypochlorite and to permanganate. The physiologic antioxidant capacity conferred by melanin was found to be 21.3×10^{-15} mole-equivalents per cell, a value which approximates oxidant production by stimulated macrophages.

Cryptococcus neoformans is one of several melanotic fungi which cause infections of the central nervous system (3). Melanin, although required for virulence (3, 12, 16), plays an unknown role in invasive disease (19).

Antimicrobial oxidants are part of the inflammatory response (1, 13, 18), yet the similarity of several leukocytic oxidants to potentially toxic products of normal aerobic metabolism has suggested that microbes might deploy metabolic antioxidants as a defense against phagocytes. Moreover, the demonstration of albinism in oxygen-sensitive cryptococcal mutants implicated melanization in oxidant resistance (9). Since biochemical investigation of these mutants revealed reductions both in polyphenol uptake and in phenol oxidase activity (9) and since polyphenols are reductants, it followed that either intracellular monomeric catechol pools (2) or finished polymeric melanin might protect against oxidants. In order to distinguish between these two possibilities, the effects of polyphenols upon resistance to oxidants was studied under conditions that either promoted or did not promote melanization.

Strain B-3502 (wild type) was obtained from K. J. Kwon-Chung, strain 567 (Mel^-) was obtained from I. Polacheck, and strain 569 (Mel^-), mutated at *oxy2* (9), was obtained from H. S. Emery. All strains were maintained on brain heart infusion agar. For physiological experiments, a loopful of the culture was inoculated into 10 ml of 2% glucose–2% yeast extract broth, which was then agitated at 37°C until stationary phase was reached. This culture (0.3 ml) was inoculated into 145 ml of asparagine broth (pH approximately 4.5) consisting of (per liter) asparagine (1 g), magnesium sulfate heptahydrate (0.5 g), potassium dihydrogen phosphate (3 g), glucose (3 g), and thiamine (1 mg) contained in a 300-ml flask with a 12-mm side arm. The medium was supplemented with dopamine hydrochloride or other polyphenols as indicated, generally at 1 mM. Growth was monitored in 12-mm side arms at 700 nm; melanization was monitored at 400 nm as the incremental optical density (OD) with reference to a comparable culture without dopamine. Cultures with melanization beyond an OD of 1 were diluted 10-fold and measured against a similarly diluted reference culture. Wet weight of the culture was determined prior to each experiment. Cryptococcal melanin was isolated from melanized cultures by exhaustive solvent extraction and acid digestion (15). Catechols, tannins, and plumbagin were supplied by the Sigma Chemical Co., and sodium hypochlorite was supplied by the Chlorox Co.

Susceptibility to plumbagin was determined in exponential-phase (nonmelanized) cultures that were adjusted to an OD of 0.05 in 10 ml of broth and agitated at 37°C with or without polyphenol. Various concentrations of plumbagin were added, and overnight growth was scored. Susceptibility to extracellular oxidants was determined in cultures grown to stationary phase in dopamine (1 mM), washed twice with saline, suspended in phosphate-buffered saline (pH 7.5) at an OD of 0.5, and counted in a hemocytometer. The suspension (1 ml), along with various amounts of oxidant and phosphate-buffered saline (to 6 ml), was agitated at 37°C for 3 h. When permanganate was used as the oxidant, residual permanganate was determined at 520 nm with reference to an identical suspension without permanganate. Asparagine broth (5 ml) was added, and growth was monitored for 24 h, after which each culture was scored as viable or not viable. Statistical confidence was estimated with Student's *t* test. In reconstruction experiments, 0.6 mg of purified melanin was added to each 6-ml incubation mixture immediately prior to the addition of oxidant.

A potentially lethal intracellular superoxide flux was induced in cultures grown in polyphenol for 2 or 24 h by subverting aerobic metabolism by addition of plumbagin (7) (dihydroxyphenylalanine inhibited growth moderately at 1 mM and totally at 10 mM, even without plumbagin). Cationic (dopamine hydrochloride), anionic (caffeic acid), and zwitterionic (L-dihydroxyphenylalanine) catechol species and neutral tannins [(+)-catechin or hematoxylin] were tested at 1 mM, caffeic acid, (+)-catechin, hematoxylin, dihydroxyphenylalanine, and dopamine were tested at 1 and 10 mM, and dopamine was tested at 1, 10, and 30 mM. Growth was inhibited at plumbagin concentrations between 25 and 50 μM, and no polyphenol protected against growth inhibition in these rapidly growing, nonmelanized cultures. In contrast, melanin protected cultures against oxidants. When the wild-type strain was cultured in broth containing 1 mM dopamine hydrochloride, the culture entered the stationary phase of growth after 1 day, whereas melanization occurred only during the stationary phase, increasing steadily for one week (Table 1). Wet weights of cultures with added dopamine equalled wet weights of cultures without added dopamine. In comparison to cultures grown without dopamine, resistance to hypochlorite was demonstrable after 3 days and peaked at fivefold to sixfold after 4 days, corresponding closely to melanization. In contrast, albino mutants did not produce melanin and did not acquire resistance from prolonged growth in dopamine medium.

* Corresponding author.

TABLE 1. Melanization and hypochlorite resistance in *C. neoformans*

Strain (phenotype)	Cultural age (days)	Melanization (OD)	Minimal fungicidal hypochlorite concn (μM) of culture grown in:		Resistance factor	*P*
			Asparagine broth	Asparagine broth + dopamine		
B-3502 (Mel^+)	1	0.0	12	12	1.0	NS[a]
B-3502 (Mel^+)	2	0.7	30	39	1.3	NS
B-3502 (Mel^+)	3	1.3	32	79	2.5	<0.01
B-3502 (Mel^+)	4	1.6	21	118	5.6	<0.01
B-3502 (Mel^+)	6	2.2	25	118	4.7	<0.05
567 (Mel^-)	4	0.1	39	39	1.0	NS
569 (Mel^-)	4	0.2	32	39	1.2	NS

[a] NS, difference not significant.

The protective capacity of melanin was quantitated. After incubation of washed, melanized yeast (relative OD, 1.9) in permanganate, residual permanganate concentrations were compared with concentrations of pure potassium permanganate in buffer (Fig. 1A). The melanized culture exhibited a threefold-increased resistance to permanganate (minimal fungicidal concentration, 0.200 mM versus 0.063 mM; $P < 0.01$). The concentration-absorbance curve of the nonmelanized culture could almost be superimposed on the standard curve of permanganate in buffer, indicating that the nonmelanized culture contained little exposed reductant. In contrast, melanized cultures neutralized substantial quantities of permanganate, as reflected by downward and rightward displacements of their plots. It follows, then, that most extracellular reducing power resides in melanin. The minimal fungicidal concentration for a culture was located just to the right of the *x* intercept for that culture and corresponded to the smallest concentration not able to be neutralized in 3 h. The physiologic neutralizing capacity of melanin appears to be represented by the distance between plots for the nonmelanized and melanized cultures. By reference to the standard curve, the half-reaction for the reduction of permanganate ion to manganese dioxide, $MnO_4^- + 4H^+ + 3e^- = MnO_2 + 2H_2O$, and the number of cells in the suspension, the physiologic neutralizing capacity of melanin in the wild-type strain was calculated as $21.3 \times 10^{-15} \pm 6.3 \times 10^{-15}$ mole-equivalents per cell (four trials).

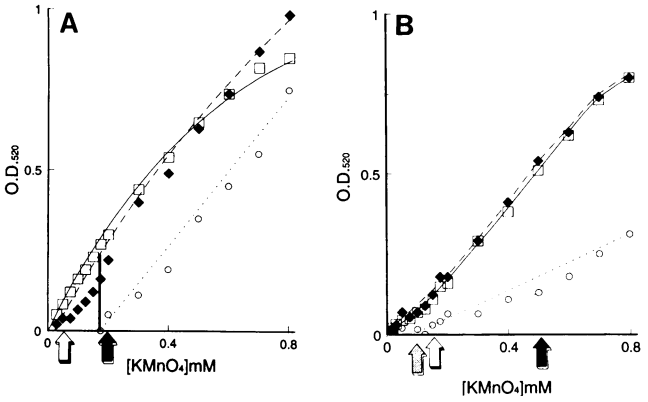

FIG. 1. (A) Titration of the wild-type strain with permanganate. ——, absorbance of permanganate in buffer; – – –, ODs of nonmelanized yeast incubated in permanganate for 3 h; . . . . , OD of melanized yeast treated in a similar manner. White and black arrows indicate minimal fungicidal concentrations for nonmelanized and melanized cells, respectively. The vertical plot represents the physiologic antioxidant capacity of melanin. (B) Titration of strain 567 (Mel^-) with permanganate. ◆, ODs of suspensions of yeast grown in unsupplemented broth and incubated in permanganate for 3 h; □, ODs of suspensions of yeast grown in broth with 1 mM dopamine and incubated in permanganate; ○, ODs of suspensions of yeast grown in unsupplemented broth but incubated with melanin (0.1 mg/ml) and permanganate. The white arrow indicates the minimal fungicidal concentration for cells grown without dopamine; the cross-hatched arrow indicates that concentration for cells grown with dopamine. The black arrow represents that concentration for cells grown without dopamine but incubated with melanin.

Exogenous melanin was used for a reconstruction experiment in an albino. In this strain, the permanganate titration plot for the dopamine culture coincided with that for the culture without dopamine (Fig. 1B), indicating that albino cells could not make an extracellular reductant. The mutant also lacked protection against the oxidant (only one dilution difference in the minimum fungicidal concentrations; not statistically significant), but exogenous melanin neutralized permanganate and protected the cells. In quantitative terms, 0.6 mg of purified melanin provided approximately twice as much protection as the melanin in a culture with an incremental optical density of 1.9.

The nonionic oxidant, hydrogen peroxide, was tested against melanized and nonmelanized washed cultures of the wild-type strain. The average minimal fungicidal concentration for each condition was 7.7 ± 4.0 mM (three trials), or approximately 100 times that for hypochlorite or permanganate. No protection was afforded by melanin.

These experiments demonstrate a protective antioxidant function for melanin, which contains quinone-like and hydroquinone-like residues and has been observed to consume superoxide and other oxidants (5, 6, 17). Circumstantial evidence for an antioxidant role in pathogenesis is provided by the recognition that hypochlorite is a major oxidative product of inflammatory cells (18) and that invading *C. neoformans* surrounds itself with melanin (11), which, at 4.4 feq per cell, would be sufficient to neutralize a large proportion of the cellular oxidant produced by stimulated human macrophages (2 to 32 fmol per cell [4, 14]). Thus, the yeast might inactivate and survive all but the largest fluxes of macrophage oxidants; disease states in which macrophage activation fails are those that are often complicated by cryptococcosis.

It was notable that melanin afforded no protection from hydrogen peroxide, which is one of the most often measured oxidative products of macrophages. Since extracellular (11) melanin protected against the negatively charged oxidants, hypochlorite and permanganate, but not against the uncharged hydrogen peroxide, it is likely that hydrogen peroxide molecules freely cross the cell membrane to attack intracellular

targets, whereas electrically charged oxidants are excluded from the cytoplasm and react primarily with melanin. Moreover, hydrogen peroxide reacts only very slowly with melanin (10). Further perspective is provided by the finding that hypochlorite is 100 times more fungicidal than hydrogen peroxide (minimum fungicidal concentration, 80 μM versus 8 mM). These considerations suggest that hypochlorite may be the more important leukocytic oxidant in cryptococcosis. Indeed, the ratio of hydrogen peroxide to hypochlorite in the macrophage's secreted oxidants in inflammatory tissues has yet to be defined. Certainly, cells of the monocyte-macrophage lineage do contain various amounts of myeloperoxidase, the enzyme which converts hydrogen peroxide to hypochlorite in the presence of chloride (14). In summary, melanin appears to protect the pathogen against the more effective leukocyte oxidant.

An alternative hypothesis, in which catecholamines are viewed as animal tannins, was tested. Since an antioxidant function for plant polyphenols (tannins) has been proposed (2, 8), the possibility of a protective antioxidant function for intracellular, monomeric catechols in *Cryptococcus* spp. was examined; that predicted effect was not found.

This work was supported by the United States Department of Veterans Affairs.

REFERENCES

1. **Babior, B. M., R. S. Kipnes, and J. T. Cornutte.** 1973. Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. J. Clin. Invest. **52:**741–744.
2. **Cohen, G., and R. E. Heikkila.** 1977. *In vivo* scavenging of superoxide radicals by catecholamines, p. 351–365. *In* A. M. Michelson, J. M. McCord, and I. Fridovich (ed.), Superoxide and superoxide dismutases. Academic Press, Inc., New York.
3. **Dixon, D. M., A. Polak, and P. J. Szaniszlo.** 1987. Pathogenicity and virulence of wild-type and melanin-deficient *Wangiella dermatitidis*. J. Med. Vet. Mycol. **25:**97–106.
4. **Fels, A. O. S., C. F. Nathan, and Z. A. Cohn.** 1987. Hydrogen peroxide release by alveolar macrophages from sarcoid patients and by alveolar macrophages from normals after exposure to recombinant interferons alpha A, beta and gamma and 1,25-dihydroxyvitamin D_3. J. Clin. Invest. **80:**381–386.
5. **Gan, E. V., H. F. Haberman, and I. A. Menon.** 1976. Electron transfer properties of melanin. Arch. Biochem. Biophys. **173:**666–672.
6. **Geremia, E., C. Corsano, R. Bonomo, R. Giardinelli, P. Pappalardo, A. Vanella, and G. Sichel.** 1984. Eumelanins as free radical trap and superoxide dismutase activities in amphibia. Comp. Biochem. Physiol. **79B:**67–69.
7. **Hassan, H. M., and I. Fridovich.** 1979. Paraquat and *Escherichia coli*. Mechanism of production of extracellular superoxide radical. Arch. Biochem. Biophys. **196:**385–395.
8. **Hatano, T., R. Edamatsu, M. Hiramatsu, A. Mori, Y. Fujita, T. Yasuhara, T. Yoshida, and T. Okuda.** 1989. Effects of the interaction of tannins with co-existing substances. VI. Effects of tannins and related polyphenols on superoxide anion radical, and on 1,1-diphenyl-2-picrylhydrazyl radical. Chem. Pharm. Bull. **37:** 2016–2021.
9. **Jacobson, E. S., and H. S. Emery.** 1991. Catecholamine uptake, melanization and oxygen toxicity in *Cryptococcus neoformans*. J. Bacteriol. **173:**401–403.
10. **Korytowski, W., and T. Sarna.** 1990. Bleaching of melanin pigments: role of copper ions and hydrogen peroxide in autooxidation and photooxidation of synthetic DOPA-melanin. J. Biol. Chem. **265:**12410–12416.
11. **Kwon-Chung, K. J., W. B. Hill, and J. E. Bennett.** 1981. New, special stain for histopathological diagnosis of cryptococcosis. J. Clin. Microbiol. **13:**383–387.
12. **Kwon-Chung, K. J., and I. Polacheck.** 1982. Melanin-lacking mutants of *Cryptococcus neoformans* and their virulence for mice. J. Bacteriol. **150:**1414–1421.
13. **Murray, H. W., and Z. A. Cohn.** 1980. Macrophage oxygen-dependent antimicrobial activity. III. Enhanced oxidative metabolism as an expression of macrophage activation. J. Exp. Med. **152:**1596–1609.
14. **Nakagawara, A., C. F. Nathan, and Z. A. Cohn.** 1981. Hydrogen peroxide metabolism in human monocytes during differentiation in vitro. J. Clin. Invest. **68:**1243–1252.
15. **Nicolaus, R. A., M. Piatelli, and E. Fattorusso.** 1964. On some natural melanins. Tetrahedron **20:**1163–1172.
16. **Rhodes, J. C., I. Polacheck, and K. J. Kwon-Chung.** 1982. Phenoloxidase activity and virulence in isogenic strains of *Cryptococcus neoformans*. Infect. Immun. **36:**1175–1184.
17. **Sealy, R. C., C. C. Felix, J. S. Hyde, and H. M. Swartz.** 1980. Structure and reactivity of melanins: influence of free radicals and metal ions. Free Rad. Biol. **4:**209–259.
18. **Test, S. T., and S. J. Weiss.** 1986. The generation and utilization of chlorinated oxidants by human neutrophils. Adv. Free Rad. Biol. Med. **2:**91–116.
19. **Wheeler, M. H., and A. A. Bell.** 1988. Melanins and their importance in pathogenic fungi. Curr. Top. Med. Mycol. **2:**338–387.