Melanin, Melanin “Ghosts,” and Melanin Composition in *Cryptococcus neoformans*

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Melanin synthesis is associated with virulence for the pathogenic fungus *Cryptococcus neoformans*. Exposure of nonmelanized *C. neoformans* 24067 cells to 4 M guanidinium isothiocyanate followed by 6 M HCl at 100°C resulted in complete solubilization of cellular structures. However, exposure of melanized *C. neoformans* 24067 to the same conditions produced a suspension of black particles. Analysis of black particles with transmission and scanning electron microscopy revealed cell “ghosts” with electron-dense walls. Electron spin resonance (ESR) spectroscopy of suspensions of black particles revealed a signal indicative of a stable free radical population. The magnitude of the ESR signal was increased by illumination, Zn^{2+} ions, and basic pH. Boiling the black particles produced only a small reduction in the magnitude of the ESR signal. These ESR changes are characteristic of melanin. Exposure of melanized black cells to 0.1 or 0.5% NaOCl bleached the cells white and abolished the ESR signal, consistent with melanin disruption. ESR spectroscopy was used to study the rate of stable free radical formation, taken as an index of melanization, of *C. neoformans* 24067 cells growing in medium with 1.0 mM L-dopa at 30 and 37°C over the course of 14 days. Melanization was slower at 37°C, but by day 14, cells grown at 30 and 37°C had comparable levels of melanin. ESR spectroscopy of seven melanized *C. neoformans* strains revealed that some strains differed by up to eightfold in melanin content. The ESR spectra of *C. neoformans* 24067 grown in minimal medium containing either 1.0 mM catechol, dopamine, or L-dopa revealed large differences in signal amplitude. The relative melanin contents were 0.05, 0.45, and 1.0 for *C. neoformans* grown in media with catechol, dopamine, and L-dopa, respectively. Quantitative analysis revealed that melanin comprised 15.4% of the dry mass of the cell after 10 days of growth in medium containing 1.0 mM L-dopa. The results suggest (i) a useful protocol for isolating *C. neoformans* melanin, (ii) a role for melanin in maintaining cell wall integrity, (iii) disruption of melanin by NaOCl, (iv) strain differences in melanin content after growth in L-dopa, and (v) quantitative and/or qualitative differences in the melanin produced after growth in various substrates.

Cryptococcus neoformans is an opportunistic fungal pathogen that causes life-threatening infections in 6 to 8% of patients with AIDS (3). *C. neoformans* has a phenoloxidase enzyme (laccase) which can catalyze the synthesis of a black pigment from a variety of catechol precursors (15, 23). The black pigment was demonstrated by electron spin resonance (ESR) spectroscopy to be a type of melanin (18). Melanin synthesis is associated with virulence in *C. neoformans* (9, 10, 16, 18). Melanized cells are more resistant to killing by chemically generated oxygen and nitrogen-derived oxidants (7, 8, 18), UV light (19), amphotericin B (20), macrophages (18), and microglia (1). Melanization also reduces antibody-mediated phagocytosis of yeast cells by murine macrophages (18). These observations have led to the suggestion that melanin enhances virulence by protecting the *C. neoformans* cells against host antimicrobial defense mechanisms (7, 8, 15, 18).

Despite the potential importance of the phenoloxidase system and melanin for virulence in *C. neoformans*, little is known about the composition of melanin or the melanin content of yeast cells. Melanin is deposited in the cell wall of *C. neoformans* and is believed to be a polymer (18, 22). In the course of experiments to produce RNA, we serendipitously discovered that melanized cells were resistant to solubilization by the

detergent guanidinium isothiocyanate. We followed this observation by studying the black particulate material that remained after treatment of melanized cells with guanidinium isothiocyanate and HCl and established that it is a melanin. ESR spectroscopy was used to study several aspects of melanization of *C. neoformans*.

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MATERIALS AND METHODS

Chemicals. Glucose, MgSO_4 , KH_2PO_4 , HCl, guanidinium isothiocyanate, and NaOCl were purchased from Fisher Chemical Inc. (Fair Lawn, N.J.). Vitamin B₁, L-dopa, catechol (1,2-benzenediol), dopamine (3-hydroxytyramine), sorbitol, and sodium citrate were purchased from Sigma Chemical Co. (Cleveland, Ohio). Novo-zyme 234 was purchased from BioPacific Inc. (Emoryville, Calif.).

***C. neoformans*.** Strains were maintained in Sabouraud dextrose agar (Difco Laboratories, Detroit, Mich.). Strain 24067 was obtained from the American Type Culture Collection (Rockville, Md.). Strains SB4A and SB6A were obtained from Eric Spitzer (Stony Brook, N.Y.). Strain NIH371 was obtained from J. E. Bennett (Bethesda, Md.). Strains J9A, J11A, and J22A were isolated from Bronx, N.Y. Melanized and nonmelanized *C. neoformans* were obtained by growing *C. neoformans* in defined minimal medium (15 mM glucose, 10 mM MgSO_4 , 29.4 mM KH_2PO_4 , 13 mM glycine, 3.0 μM vitamin B₁) with or without 1 mM L-dopa, respectively, as described previously (21).

Effect of guanidinium isothiocyanate and HCl. Melanized and nonmelanized *C. neoformans* 24067 cells were collected from a day 10 culture, washed once with 1.0 M sorbitol in 0.1 M sodium citrate (pH 5.0), and resuspended in 5 ml of the same solution. Novo-zyme 234 was added into the cell suspension at a concen-

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tration of 10 mg/ml, and the suspension was incubated for 1 h at 30°C to generate protoplasts. The protoplasts from melanized and nonmelanized cells were collected by centrifugation and suspended in 4 M guanidinium isothiocyanate for 30 min at room temperature. Cell debris was collected by centrifugation and suspended in 6 M HCl for 30 min at 100°C. After guanidinium isothiocyanate and HCl treatment, nonmelanized cells dissolved completely. However, for melanized cells, a black pellet remained. The black pellet contained particulate material that was dialyzed against distilled water for 10 days and in some experiments was lyophilized. The carbon and nitrogen quantitative analysis was performed by Quantitative Technologies Inc. (Whitehouse, N.J.), using 2400 Perkin-Elmer CHN elemental analyzer. Briefly, combustion converted the sample elements to gases such as CO₂, H₂O, and N₂; the product gases were separated under steady-state conditions and measured as a function of thermal conductivity.

ESR spectroscopy. ESR spectra were obtained as described previously (18). Briefly, black particles were weighed in an analytical balance and suspended in distilled water at 6 mg/ml. ESR spectra were obtained with a model ER 200D EPR/ENDOR spectrometer with ESP 300 upgrade (Bruker Instruments, Inc., Billerica, Mass.) operating with the following parameters: modulation frequency, 100 KHz; modulation amplitude, 1.4 G; center field, 3,310 G; sweep width, 100 G; microwave frequency, 9.29 GHz; microwave power, 15.9 mW; and temperature, 77 K. The ESR spectra of black particles were studied in alkaline pH, acid pH, 0.1 M Zn²⁺ ions, and after visible light illumination from a 300-W slide projector bulb.

TEM and SEM. Transmission and scanning electron microscopy (TEM and SEM) images were obtained as described previously (13, 18). Briefly, for TEM (18), black particles were fixed in 2% glutaraldehyde for 2 h, then incubated overnight in 4% formaldehyde–1% glutaraldehyde–0.1% phosphate-buffered saline (PBS), and subjected to 1.5 h of post fixation in 2% osmium. Dehydration was accomplished by serial incubation in graded ethanol and two final incubations in 100% ethanol. Black particles were embedded in Spurr's resin. TEM pictures were then obtained with a Siemens (Berlin, Germany) model 102 electron microscope. For SEM, the black particles were fixed overnight in a 4% glutaraldehyde solution in PBS, transferred on polylysine-coated coverslips, dehydrated by incubation in graded ethanol, mounted with gold-palladium, and viewed in a JEOL (Tokyo, Japan) JAM-6400 electron microscope (13).

Carbohydrate determination. Carbohydrate content in the black particulate suspension and in synthetic melanin (Sigma) was determined by the phenol-sulfuric method (4). Carbohydrate content was calculated relative to a standard solution of glucose.

Bleaching with NaOCl. Ten-day-old melanized *C. neoformans* 24067 cells were exposed to 0.01% (1.0 mM), 0.1% (10 mM), or 0.5% (50 mM) NaOCl for 2 h. Pellets were washed three times with distilled water, suspended in distilled water (pH 7.0), and analyzed by ESR spectroscopy.

Melanization with different catechol substrates or at different temperatures. *C. neoformans* 24067 cells were grown in minimal medium with either 1.0 mM catechol, dopamine, or L-dopa for 10 days at 30°C. A suspension of 2.7×10^8 melanized cells per ml was analyzed by ESR spectroscopy. For the study of melanization of *C. neoformans* at 30 and 37°C, *C. neoformans* 24067 cells were grown in minimal medium with 1.0 mM L-dopa. At different time points, cells growing at 30 or 37°C were collected, washed, and analyzed by ESR spectroscopy.

Melanization in *C. neoformans*. *C. neoformans* 24067, SB4A, SB6A, NIH371, J9A, J11A, or J22A strains were grown in minimal medium with 1.0 mM L-dopa for 10 days. Melanized cells were collected, and equal numbers of cells (2.7×10^8 /ml) were analyzed by ESR spectroscopy.

RESULTS

Effect of guanidinium isothiocyanate and HCl on *C. neoformans*. Exposure of nonmelanized *C. neoformans* to 4 M guanidinium isothiocyanate followed by hot 6 M HCl resulted in complete solubilization of cellular components. However, exposure of melanized *C. neoformans* cells to guanidinium isothiocyanate and HCl yielded a black particulate material. Light microscopy of suspensions of the black particulate material at a magnification of $\times 400$ revealed structures resembling yeast cells (not shown). Scanning electron microscopy revealed that the black particulate material was composed of hollowed cell remains (Fig. 1). TEM revealed that the cell remains had electron-dense walls without internal structures (Fig. 2). On the basis of the microscopic appearance of the black particulate material, the fact that it originated from melanized cells, and the fact that it consisted of black electron-dense structure resistant to acid hydrolysis, we surmised that these structures represented melanin "ghosts" of melanized cells. Phenol-sulfuric acid reaction indicated that both the black particulate

FIG. 1. Scanning electron micrograph of black particulate material remaining after treatment of *C. neoformans* with guanidinium isothiocyanate and hydrochloric acid (magnification, $\times 15,000$).

material and synthetic melanin contained 6 to 7% carbohydrate by weight. Elemental carbon and nitrogen analysis of black particulate material revealed a C/N ratio of $12.5 (30.0\% \pm 0.5\% \text{ C}/2.4\% \pm 0.06\% \text{ N}; n = 3; \text{ values are averages } \pm \text{ standard deviations})$; the C/N ratio of Sigma melanin was 7.5 ($48.2\% \text{ C}/6.4\% \text{ N}$), which is in excellent agreement with published values (2).

ESR spectroscopic studies. Demonstration that a compound is melanin is difficult by chemical means because melanins are insoluble and poorly characterized (5, 22). However, melanins bear stable free radicals and therefore have a characteristic ESR spectrum which can be used to identify and define them (5, 17). ESR spectroscopic studies of isolated black particulate material revealed a stable free radical signal like that described for melanin (5, 17) (Fig. 3). Illumination of the black particulate material for 15 min with white light from a 300-W projector lamp increased the ESR signal by 74.7%. Suspension of the black particulate material in a solution of 0.1 M ZnCl₂ increased the ESR signal by 436%. Suspension of the black particulate material in water at basic pH adjusted by 1 N NaOH resulted in a higher magnitude of ESR free radical signal than suspension in water at pH 7.0. Boiling the black particulate material (pH 7.0) or suspending it in water at acidic pH adjusted by 1 N HCl had little effect on the ESR signal of melanin in water at pH 7.0. These ESR spectrum changes indicate that the black particulate material is or contains melanin.

Hypochlorite reaction with *C. neoformans*. Hypochlorite is produced by the neutrophil and macrophage myeloperoxidase and is an important antimicrobial compound of host effector cells (12). Melanized *C. neoformans* cells are less susceptible to killing by hypochlorite, and the effect has been attributed to shielding of the fungal cell by melanin (8). To investigate the interaction of melanin and hypochlorite, we used ESR spectroscopy. Incubation of 8-day-melanized *C. neoformans* cells in 0.1 or 0.5% NaOCl solution at room temperature resulted in rapid bleaching within 10 min, with the cells turning from black to white. ESR spectroscopic studies of NaOCl-treated melanized cells revealed disappearance of the distinctive melanin spectra (Fig. 4). Incubation of melanized cells in a 0.01% NaOCl solution had no effect on the ESR spectra (data not shown).

Melanization of *C. neoformans* as a function of time and temperature. ESR spectroscopy was used to study the rate of melanization of *C. neoformans* at 30 and 37°C over the course

FIG. 2. Transmission electron micrograph of black particulate material remaining after treatment of *C. neoformans* with guanidinium isothiocyanate and hydrochloric acid (magnification, $\times 45,000$).

of 14 days. Melanization in medium containing 1 mM L-dopa increased in an approximately linear rate over the course of the 14-day growth period (Fig. 5). With the exception of the day 10 measurement, melanin levels were comparable for cells grown at 30 and 37°C. A 10-day-old culture of *C. neoformans* grown at 37°C was washed and divided in half; one portion was lyophilized to obtain dry cell weight, and the other portion was treated with guanidinium isothiocyanate and HCl to make melanin particulate material. From the weight of the lyophilized melanin particulate material divided by the weight of lyophilized cells, we estimate that melanin constitutes 15.4% of the dry weight of the cell (153 mg of melanin was obtained from 994.7 mg of lyophilized cells which had been grown in a 500 ml of minimal medium containing 1.0 mM L-dopa for 10 days).

FIG. 3. ESR spectrum of black particulate material remaining after treatment with guanidinium isothiocyanate and hydrochloric acid. ESR spectra of two independent preparation were similar.

Melanization of different strains of *C. neoformans*. The intensity of the ESR signal is directly proportional to the concentration of unpaired electrons in melanin and increases with the content of melanin. We have therefore taken the ESR signal to estimate the relative content of melanin in a cell suspension. Melanin content of *C. neoformans* was expressed in systemic double integration of the signal (Table 1). Among the seven strains of *C. neoformans* studied, J9A and NIH371 had the highest and the lowest double integration values, respectively.

Melanization of *C. neoformans* on catechol, dopamine, and L-dopa. Dopamine is a neurotransmitter in the human brain which can be used as substrate for *C. neoformans* melanization (14). *C. neoformans* grown in medium containing 1.0 mM dopamine resulted in melanized cells which were visually indis-

FIG. 4. Effect of NaOCl on melanized *C. neoformans*. Spectrum A was obtained from melanized cells in distilled water; spectrum B was obtained from melanized cells treated with 0.1% NaOCl and then washed and suspended in distilled water; spectrum C was obtained from melanized cells treated with 0.5% NaOCl and then washed and suspended in distilled water. This experiment was done twice with the same results.

FIG. 5. Melanization of *C. neoformans* cells at 30 and 37°C. Melanin intensity of *C. neoformans* cells was expressed as systemic double integration per cell (10^{-11}). This experiment was done twice with similar results.

tinguishable from cells grown with L-dopa as the phenoloxidase substrate. *C. neoformans* growing in medium with 1.0 mM catechol became only slightly melanized, which indicated that catechol was not a good substrate for melanization of *C. neoformans*. Double integrals of melanin ESR signal from cells grown in media with catechol, dopamine, and L-dopa revealed relative melanin contents of 0.05, 0.45, and 1.0, respectively.

DISCUSSION

During attempts to obtain mRNA for Northern (RNA) blot analysis from melanized and nonmelanized cells, we noted that treatment with 4 M guanidinium isothiocyanate dissolved most of the nonmelanized cells but did not dissolve melanized cells. Since melanin is very insoluble and resistant to detergents, we hypothesized that melanized cells were resistant to solubiliza-

tion because of the melanin in their cell walls. Since melanins are also resistant to acid hydrolysis (22), we digested the guanidinium isothiocyanate-treated melanized and nonmelanized cells with hot HCl. The hot HCl treatment resulted in complete solubilization for the yeast cell debris from nonmelanized cells but not the black particulate material that remained from melanized cells. Electron microscopy of the black particulate material revealed structures of approximately the same dimensions as yeast cells but consisting of empty shells of an electron-dense substance. ESR spectroscopy of this material revealed a spectrum like that of *C. neoformans* melanin (18). The changes in the magnitude of the ESR signal of the black particulate material with light, pH, Zn^{2+} , and boiling are those expected for melanin (5). Given the absence of internal structures, the treatment of detergent and acid, and the prolonged dialysis, we conclude that the black particulate material contains melanin (and may in fact be all melanin). This protocol appears to be useful for the production of milligram quantities of *C. neoformans* melanin for structural studies. The observation that melanized cells were resistant to solubilization suggests that melanin may function by contributing to cell wall integrity.

The C/N ratio of the black particulate material was 12.5. This C/N ratio is different from those of synthetic melanin or melanin from human sources (2), indicating a different structure for fungal melanin. Phenol-sulfuric acid reaction is a spectrophotometric technique used to measure carbohydrate content whereby the presence of carbohydrate is deduced by the formation of furfural derivatives (4). The polysaccharide content measured by this technique was 6 to 7% and may reflect the presence of attached polysaccharide or chemical moieties in the melanin which react with phenol and H_2SO_4 to produce products with furfural-like absorbance spectra. The fact that commercially available synthetic melanin (which presumably has no carbohydrate) gave a virtually identical polysaccharide composition by weight when assayed by the phenol-sulfuric acid method suggests the latter possibility. Hence, we cannot infer with confidence that polysaccharide is present in our melanin preparation by this colorimetric technique. Nevertheless, the similarity in the absorbance of phenol-sulfuric acid product between our preparation and synthetic melanin is consistent with and supports the identification of the black particulate material as *C. neoformans* melanin.

Hypochlorite (bleach) is routinely used as a disinfectant because of its microbicidal properties (12) and is an important antimicrobial product of the myeloperoxidase system of neutrophils and macrophages (12). Jacobson and Tinnell have demonstrated that melanin can protect *C. neoformans* against the fungicidal effects of hypochlorite, presumably by reacting with the hypochlorite or hypochlorite-derived oxidants (8). In this study, incubation of melanized *C. neoformans* cells with 0.1 or 0.5% NaOCl (roughly 100-fold the concentration of hypochlorite used by Jacobson and Tinnell [8]) resulted in a bleaching effect whereby the cells turned from black to white. ESR spectroscopy of NaOCl-treated melanized cells revealed absence of the distinctive free radical spectra of melanin. Since melanin is defined, in part, by an ESR spectrum which results from a stable free radical population (5, 17), we interpret this finding as indicative of oxidative disruption of melanin structure by reaction with hypochlorite. Hence, melanin can protect against a low concentration of hypochlorite (8) but is disrupted by a high concentration of this oxidant. Hypochlorite produced by the myeloperoxidase system may exert an important antimicrobial action against pathogens capable of synthesizing melanin in vivo.

Analysis of melanin content per *C. neoformans* cell as a function of time and temperature revealed a progressive in-

TABLE 1. Melanin contents of different strains of *C. neoformans*

Strain	Serotype	Origin	Relative melanin content ^a
24067	D	ATCC	1.0
SB4A	AD	Stony Brook, N.Y., clinical isolate	1.9
SB6A	A	Stony Brook, N.Y., clinical isolate	0.7
NIH371	D	Thailand, environmental isolate	0.4
J9	D	Bronx, N.Y., clinical isolate	3.3
J22A	D	Bronx, N.Y., clinical isolate	1.3

^a The relative melanin concentration is calculated from the double integral. The double integral, $DI_k(a)$, is defined by the following formula:

$$DI_k(a) = \sum_{j=n_k}^a \sum_{i=n_k}^j [f(i) - m(i - n_k) - b]$$

The double integral is normalized to instrumental parameters and carried out over the range of points specified by n_k , a , and j . Baseline correction is accomplished by the adjustable terms $m(i - n_k)$ and b . b is a constant compensating for spectrum offset from zero. Four independent measurements of the same sample revealed that the standard deviation was 2.5% of the mean, indicating high reproducibility of double integrals in melanin ESR free radical signals. Melanin content was calculated relative to that of strain 24067.

crease in melanin content during a 14-day growth period. Melanin content increased linearly with time at both 30 and at 37°C. The melanin contents of *C. neoformans* cells grown at 30 and 37°C were comparable despite the fact that the phenoloxidase enzyme is more active at 30 than at 37°C (6). Quantitative analysis of melanin content done by extracting melanin by the guanidinium isothiocyanate and hot HCl method described here revealed that at 10 days, melanin comprised at least 15.7% of the dry weight of the cells. ESR spectroscopy was used to measure differences in melanin content among *C. neoformans* strains grown in 1 mM L-dopa for 10 days. At that time, all cultures appeared black and melanin content could not be estimated visually. Double integration of ESR signals revealed eightfold differences in melanin content between some strains (i.e., between J9A and NIH371). The variation in melanin content suggests that the contribution of melanin to the virulence potential of *C. neoformans* may vary among strains.

Since *C. neoformans* has a predilection for the dopaminergic areas of the human brain (11), we studied the ESR spectra of melanized cells produced by growth in minimal medium containing 1.0 mM dopamine. Dopamine was used because it is a human neurotransmitter. The ESR spectrum of melanized cells grown with dopamine was indistinguishable from that obtained by growing cells with L-dopa, but the melanin content of *C. neoformans* cells grown with L-dopa was 2.2 times greater than that of cells grown with dopamine. *C. neoformans* became only slightly melanized when grown in medium with catechol as the phenoloxidase enzyme substrate. This result demonstrates quantitative and/or qualitative differences in the melanin produced after growth in various substrates. Differences in substrate uptake by the various strains could have also contributed to differences in strain melanization.

In summary, we describe the isolation of melanin from melanized *C. neoformans* cells treated with denaturing agents and acid. Melanin may contribute to fungal survival by promoting cell wall integrity. Hypochlorite treatment of melanized cells abolished the melanin spectrum, consistent with destruction or modification of the pigment. Levels of melanization at 30 and 37°C were comparable and increased with time. ESR spectroscopy was used to demonstrate differences in apparent melanin content for different *C. neoformans* strains. The results demonstrate the usefulness of ESR spectroscopy in studying diverse aspects of melanin and melanization in a fungal pathogen.

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