INFECTION AND IMMUNITY, Aug. 1995, p. 3131–3136
0019-9567/95/$04.00+0

Vol. 63, No. 8

Cryptococcus neoformans Melanin and Virulence: Mechanism of Action

YULIN WANG,[1] PHILIP AISEN,[2] AND ARTURO CASADEVALL[1,3]*

Department of Microbiology and Immunology,[1] Department of Physiology and Biophysics,[2] and Department of Medicine,[3] Albert Einstein College of Medicine, Bronx, New York 10461

Received 23 March 1995/Returned for modification 12 May 1995/Accepted 18 May 1995

Black melanin-like pigments are produced by several neurotropic fungi, including *Cryptococcus neoformans*. Pigment production is associated with virulence. In media containing phenolic substrates such as L-dopa, *C. neoformans* cells become black as a result of pigment accumulation. Pigmented and nonpigmented *C. neoformans* cells were studied with transmission electron microscopy and electron spin resonance (ESR) spectroscopy. Transmission electron microscopy showed electron-dense cell walls, and ESR spectroscopy revealed a stable free-radical population in pigmented cells. The ESR signals of pigmented cells were increased by light, alkaline pH, and Zn^{2+} and decreased by acid pH, indicating that the black pigment was a type of melanin. A mutant deficient in melanin synthesis (*mel*) generated by UV radiation lacked ESR-detectable radicals, was less virulent for mice, was more susceptible to killing by nitrogen- and oxygen-derived radicals, and had 100-fold-less phenoloxidase activity than the parent strain. The interaction of melanized *C. neoformans*, nonmelanized *C. neoformans*, and the hypomelanotic *mel* mutant with J774.16 murine macrophage-like cells was studied. Melanized cells were more resistant to antibody-mediated phagocytosis and the antifungal effects of murine macrophages than nonmelanized cells. Small increases in the intensity of the ESR signals of melanized cells in solutions containing chemically generated oxygen- and nitrogen-derived radicals indicated electron transfer to or from melanin. Melanin appears to contribute to virulence by protecting fungal cells against attack by immune effector cells.

Melanin pigments are ubiquitous in nature and are produced by a variety of organisms, including bacteria, fungi, plants, and animals (12). The structure and function of melanins are poorly understood (12). Melanins are insoluble polyanionic molecules which contain stable populations of free radicals (8). Melanin-like pigments appear to be important for the virulence of several pathogens, including *Cryptococcus neoformans* (18, 27), *Wangiella dermatitidis* (6), and *Mycobacterium leprae* (33). Fungal melanins are complex pigments which are produced by at least two different synthetic pathways, depending on the organism (9, 33). *C. neoformans* produces black melanin-like pigments in media containing phenolic substrates such as L-dopa. In *C. neoformans*, synthesis of melanin-like pigments is catalyzed by a phenoloxidase which is a laccase (*CNLAC1*) (36). *C. neoformans* pigment production has been associated with virulence (17, 18, 27), and histochemical staining suggestive of melanin has been described for human brain tissue (16, 28). However, the mechanism by which pigments enhance virulence is poorly understood. Pigmented cells are less susceptible to free-radical killing, suggesting that the melanin-like pigment protects against oxidants produced by host effector cells (14, 31). Pigmented cells are also less susceptible to the antifungal agent amphotericin B, and this may contribute to persistence of infection in humans (32). In this study, the black pigment of *C. neoformans* is shown to be a type of melanin, melanized cells are shown to be more resistant to murine macrophages than nonmelanized cells, and electron spin resonance (ESR) spectroscopy is used to demonstrate electron transfer to or from melanin in fungal cells.

(The data in this report are from a thesis to be submitted by Y. Wang in partial fulfillment of the requirements for a Ph.D. in the Sue Golding Graduate Division of Medical Science, Albert Einstein College of Medicine, Yeshiva University, Bronx, N.Y.)

* Corresponding author. Mailing address: Department of Medicine, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461. Phone: (718) 430-4260. Fax: (718) 597-5814.

MATERIALS AND METHODS

C. neoformans. Strain 24067 (American Type Culture Collection, Rockville, Md.) was maintained in Sabouraud dextrose agar (Difco Laboratories, Detroit, Mich.). Pigmented and nonpigmented cells were obtained by growing yeast cells in defined minimal medium (15 mM glucose, 10 mM $MgSO_4$, 29.4 mM KH_2PO_4, 13 mM glycine, 3.0 μM vitamin B_1 [Sigma Chemical Co., Cleveland, Ohio]) with or without 1.0 mM L-dopa (Sigma), respectively, as described previously (31).

ESR. ESR spectra of whole *C. neoformans* cells and purified black pigment were obtained with a model ER 200D EPR/ENDOR spectrometer with ESP300 upgrade (Bruker Instruments, Inc., Billerica, Mass.) operating at X-band (9 GHz). The microwave frequency was measured with a Systron-Donner counter. Parameters for ESR were as follows: modulation frequency, 100 KHz; modulation amplitude, 1.433 G; center field, 3310.0 G; sweep width, 100.0 G; microwave frequency, 9.2995 GHz; microwave power, 15.9 mW; and temperature, 77 K. The intensity of the melanin ESR signal was estimated by double integration. A solution of 5×10^{-4} M $CuSO_4$ and 1×10^{-3} M EDTA in 3 M $NaClO_4$ (pH 7.0) was used as a standard for calculating melanin free-radical density. The ESR spectra of melanized *C. neoformans* cells in alkaline pH, acid pH, the presence of Zn^{2+} ions, and after visible light illumination from a 300-W slide projector bulb for 15 min were studied. These conditions were used because they result in changes to the intensity of melanin ESR spectra which have been used to define melanins (8).

Transmission electron microscopy. Pigmented and nonpigmented *C. neoformans* cells (day 8 of growth) were washed twice in 0.02 M phosphate-buffered saline (PBS) and fixed in 2% glutaraldehyde for 2 h. The cells were then incubated overnight in 4% formaldehyde–1% glutaraldehyde–0.1% PBS; 1.5 h of postfixation in 2% osmium followed. Dehydration was accomplished by serial washing in graded ethanol solutions of 50 to 95% for 10 min, followed by two final washes in 100% ethanol for 15 min. Cells were embedded in Spurr's resin, and transmission electron microscopy pictures were obtained with a model 102 electron microscope (Siemens, Berlin, Germany).

Macrophage experiments. J774.16 is a mouse macrophage-like cell line which has been used to study the *C. neoformans* interaction with macrophages (23). In the presence of capsule-binding monoclonal antibodies (MAbs), J774.16 cells reduce *C. neoformans* CFU by 2 h through a combination of fungicidal and fungistatic effects (23). Phagocytosis and CFU assays for melanized and non-

melanized *C. neoformans* cells were performed in the presence and absence of MAb 2H1 as described previously (23). Macrophage assays were done with *C. neoformans* cells grown in minimal medium for 6 days in the presence (melanized) or absence (nonmelanized) of 1 mM L-dopa. The ratios of macrophages to *C. neoformans* cells were 1:1 and 20:1 for phagocytosis and CFU assays, respectively. Percent survival of *C. neoformans* was obtained by comparing the number of CFU after macrophage coincubation with the number of CFU in a suspension not exposed to macrophages. To determine whether ingestion of melanized cells was toxic to macrophages, J774.16 cells were incubated with MAb 2H1 opsonized heat-killed melanized *C. neoformans* cells for 36 h, and macrophage cell viability was determined by trypan blue staining.

Surface hydrophobicity. Surface hydrophobicity was determined by Octyl-Sepharose CL-4B (Sigma) hydrophobic interaction chromatography (15). Briefly, pigmented or nonpigmented *C. neoformans* cells were mixed with 1 ml of the gel for 30 min at room temperature in 20 ml of attachment buffer [1 M $(NH_4)_2SO_4$ in 0.01 M phosphate buffer (pH 6.8)] poured onto 20- to 25-μm-pore-size Whatman no. 4 filter paper (Fisher Scientific, Pittsburgh, Pa.), and the filtrate containing unbound cells was collected. The cells were counted with a hemacytometer, and hydrophobicity was calculated as the number of unbound cells relative to the number of cells added to the gel.

Experiments with chemically generated nitrogen- and oxygen-derived radicals. *C. neoformans* killing after exposure to nitrogen- and oxygen-derived reactive species was determined as described previously (32), except that the H_2O_2 concentration used in the epinephrine oxidative system was 1 mM. The interaction of chemically generated reactive species and cell wall pigment was studied by comparing the intensities of the ESR signal in the presence and absence of chemically generated reactive species. For the interaction with oxygen-derived radicals, equal numbers of pigmented cells (10-day growth) were suspended in 25 mM MOPS (morpholinepropanesulfonic acid; pH 7.01; Sigma) containing the reagents of the epinephrine oxidative system (31) with and without H_2O_2, respectively, and the ESR spectra were recorded. For the interaction with nitrogen-derived oxidants, equal numbers of pigmented cells (10-day growth) were suspended in 25 mM succinic acid (pH 4.17) with 0.5 mM $NaNO_2$ and distilled water (pH 4.17 adjusted with HCl), respectively, and ESR spectra were recorded. Control experiments with *C. neoformans* suspended in either succinic acid or 0.5 mM $NaNO_2$ revealed no effect of succinic acid or $NaNO_2$ on the intensity of the pigment ESR spectra. Double integration was used to quantify pigment ESR signals. Fourier transformation was used to smooth ESR spectra.

Pigment-deficient mutant. UV irradiation was used to generate a mutant deficient in pigment production. Briefly, cells were grown in Sabouraud dextrose agar (Difco) plates for 72 h at 30°C, collected, washed, and suspended in PBS (10^7 cells per ml). The suspension was placed in a petri dish (100 by 15 mm; Becton Dickinson Labware, Lincoln Park, N.J.), irradiated with 60 to 65 mJ of UV light (245 nm) per cm^2 in a Stratalinker 1800 (Stratagene, La Jolla, Calif.), plated on Sabouraud dextrose agar (Difco), and incubated at 30°C for 2 days. Plates with 100-fold killing were replica plated to L-dopa plates (1.0 mM L-dopa in minimal medium and 1.5% agar). The pigment-deficient mutant (*mel*) was identified as a white colony on L-dopa plates. The parental strain and the *mel* mutant did not differ by electrophoretic karyotyping, urea agar hydrolysis (Becton Dickinson Microbiology Systems, Becton Dickinson and Co., Cockeysville, Md.), growth at 37°C, capsule size (by India ink), and growth on minimal plates. Electrophoretic karyotyping was done with a CHEF-DR III (Bio-Rad Laboratories, Hercules, Calif.) with 0.5× Tris-borate-EDTA at 6 V/cm with a 60-s switching time for 16 h followed by a 90-s switching time for 9 h with minor modifications of existing protocols (24). Southern blot analysis of parental and mutant DNA was done as described previously (3) by using as a probe a phenoloxidase gene DNA fragment amplified by the PCR with primers (5′ CCTCTCGCGCACCTCTACGTT and 5′ AGCTGTGCAGTTTGTTTGGCC) designed from the published sequence (35).

Phenoloxidase activity. Phenoloxidase activity was measured by a modification of published protocols (13). Briefly, *C. neoformans* was grown in 10 ml of 2% glucose–2% yeast extract at 30°C overnight, after which 145 ml of minimal medium was added and the culture was grown for another 24 h. The cells were collected and grown again for 5.5 h in minimal medium without glucose. The cells were collected, washed, and suspended in 50 mM NaH_2PO_4–0.5 M NaCl (pH 6.3) buffer with 100 μg of the protease inhibitor phenylmethylsulfonyl fluoride per ml. The cells were broken on ice by sonication at 40% power (300 W) and a frequency of 20 kHz for 10 min in a Sonic Dismembrator (model 300; Fisher Scientific). The lysate was clarified at 0°C by centrifugation at 47,807 × *g* in an SS-34 rotor (Dupont Co., Wilmington, Del.) for 3 h and concentrated with a Centricon-3 spin column (Amicon, Inc., Beverly, Mass.). Protein concentration was determined with the Bio-Rad protein assay. Equal amounts of concentrated protein extract from 24067 and Mel^- strains were separated by 10% polyacrylamide gel electrophoresis without sodium dodecyl sulfate. Gels were stained with 1 mM L-dopa in 0.1 M citric acid–0.2 M Na_2HPO_4 (pH 6.0) buffer. Phenoloxidase catalyzes the production of melanin pigment which is deposited in the gel at the position of the phenoloxidase protein. The gel was screened in a computing densitometer (Molecular Dynamics, Sunnyvale, Calif.) to measure the amount of pigment made.

Mouse studies. The virulence of parent and *mel* mutant strains was studied in A/J mice (Jackson Laboratories, Bar Harbor, Maine) by determination of organ colony counts at 4 and 7 days after intraperitoneal infection with 5×10^6 yeast cells as described previously (22).

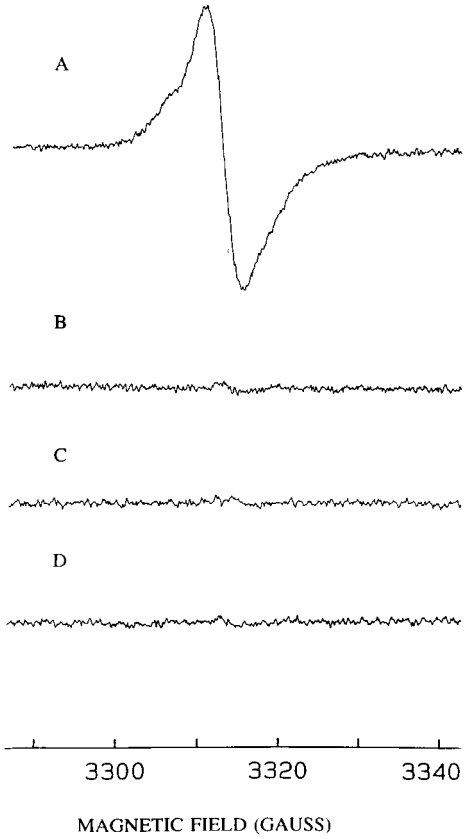

FIG. 1. ESR spectra of *C. neoformans* cells. (A and B) Strain 24067 grown for 6 days in minimal medium with and without L-dopa, respectively; (C and D) *mel* mutant strain grown for 6 days in minimal medium with and without L-dopa, respectively. Eight-day-old melanized *C. neoformans* cells and purified pigment from melanized strain 24067 cells had ESR spectra like that shown in panel A.

Statistics. Comparisons between groups were done by *t* test or chi-square statistics by use of Primer of Biostatistics 3/e version 3.0 Software (McGraw-Hill, Inc., New York) or Excel (GreyMatter International, Inc., Cambridge, Mass.).

RESULTS

Identification of the pigment in *C. neoformans* as a melanin. ESR spectroscopy is valuable for studying melanins because these pigments contain stable populations of free radicals (8). ESR spectra of 8-day-old *C. neoformans* cells grown with L-dopa (pigmented) revealed a signal with a g value of 2.004 and a line width of 4.69 G (Fig. 1A). Purified *C. neoformans* pigment produced identical spectra (data not shown). No ESR signal was present for nonpigmented *C. neoformans* cells (Fig. 1B, C, and D). Apart from having characteristic ESR spectra, the intensity of melanin ESR signals has been shown to change with the following conditions: boiling (no change), low pH (decrease), high pH (increase), illumination with visible light (increase), and addition of Zn^{2+} (increase) (8). The ESR intensity of purified *C. neoformans* pigment was not affected by boiling (data not shown). Illumination of pigmented *C. neoformans* cells with visible light increased the intensity of the ESR spectra 2.8-fold, suspension in solutions of pH 2.0 or 12.0 changed the intensity of pigmented *C. neoformans* cell ESR spectra to 0.76- and 1.9-fold, respectively, of that measured at pH 7.0, and addition of 0.1 M Zn^{2+} ($ZnCl_2$) to pigmented cell

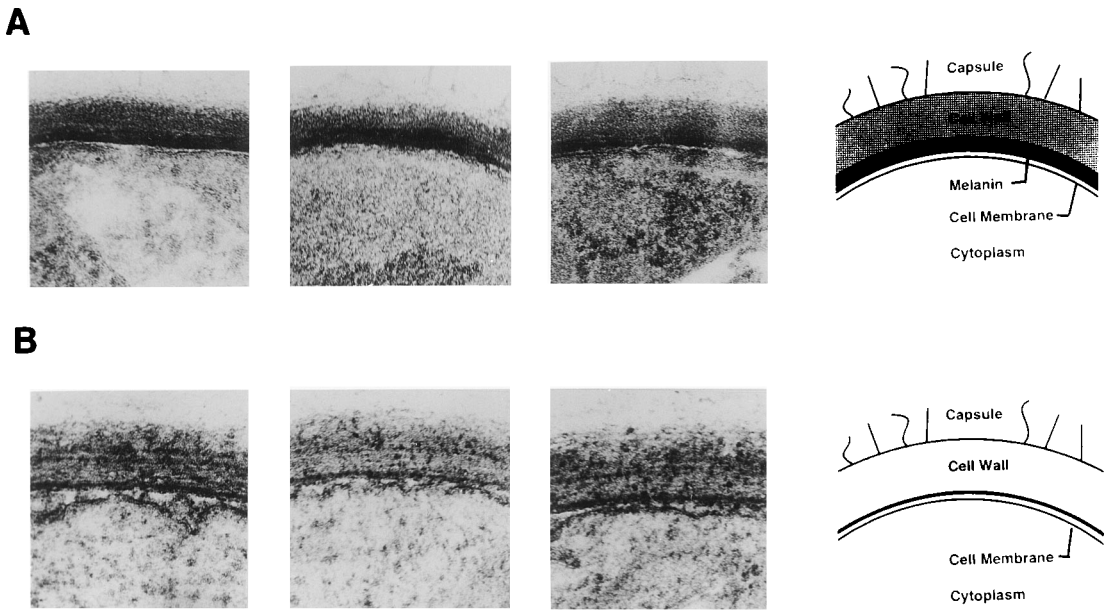

FIG. 2. Transmission electron microscopy of strain 24067 cells grown with (A) and without (B) L-dopa. The diagrams highlight the structures. Cells grown with L-dopa have more electron-dense cell walls.

suspensions increased the ESR signal more than 3.6-fold. These ESR features are indicative of a stable free-radical population in pigmented *C. neoformans* cells like that described for melanins and define the black pigment of *C. neoformans* as a melanin (8). Pigmented cells are henceforth referred to as melanized. No signal was detected for cells grown in the absence of L-dopa or for a melanin-deficient mutant in the presence or absence of L-dopa (Fig. 1B, C, and D). Electron micrographs of melanized and nonmelanized cells revealed that the electron density of the cell wall was increased in melanized cells (Fig. 2).

Interaction of nitrogen- and oxygen-derived free radicals with cell-associated melanin. ESR spectroscopy was used to study whether chemically derived free radicals affected the intensity of the free-radical signal of cell-associated melanin. Sodium nitrate in succinic acid produces nitrogen-derived radicals, including nitric oxide (1). The intensity of the melanin ESR signal from melanized cells in a solution of 0.5 mM $NaNO_2$ in 25 mM succinic acid increased 48% relative to that of an equal number of melanized cells suspended in water (electron spin concentrations increased from 1.53×10^{-7} to 2.23×10^{-7} M, spectra B and A, respectively) (Fig. 3). Suspension of melanized cells in either 25 mM succinic acid or 0.5 mM $NaNO_2$ solutions had no effect on melanin ESR signal intensity (data not shown). A solution of 0.5 mM $NaNO_2$ in 25 mM succinic acid had no detectable signal in this region of the ESR spectra (data not shown). Hydroxyl radicals are generated in the epinephrine oxidative system (26). The intensity of melanin ESR spectra from melanized cells increased 5% when the cells were suspended in a solution containing all of the components of the epinephrine oxidative system relative to that of equal number of melanized cells suspended in the same solution without H_2O_2 (electron spin concentrations increased from 1.15×10^{-6} to 1.21×10^{-6} M, spectra C and D, respectively) (Fig. 3). A solution containing all components of the epinephrine oxidative system without melanized cells had no signal in this region of the ESR spectra (data not shown). The increase in the intensity of the melanin ESR signal after exposure to nitrogen- and oxygen-derived radicals indicates transfer of unpaired electrons of oxygen- and nitrogen-derived radicals to or from melanin in melanized cells.

Characterization of the melanin-deficient mutant (*mel*). A melanin-deficient mutant which produced white colonies in L-dopa plates was selected. After growth in L-dopa agar for more than 1 week, *mel* colonies turned brown, suggesting partial melanization. Analysis of 2.8×10^8 cells in L-dopa agar

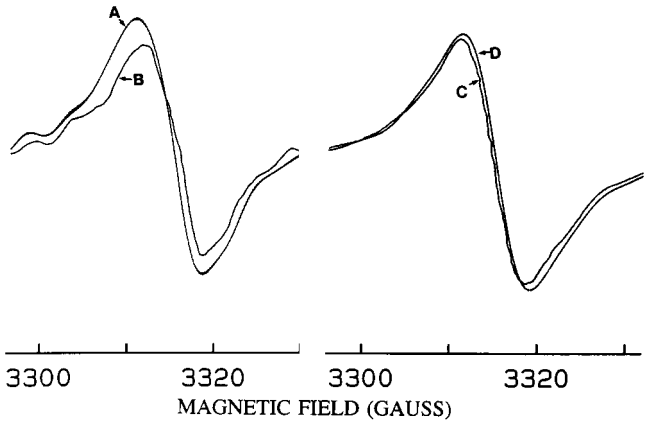

FIG. 3. Melanin interaction with chemically generated nitrogen- and oxygen-derived free radicals. Spectra: A, ESR signal from melanized cells suspended in 25 mM succinic acid containing 0.5 mM $NaNO_2$ (pH 4.17); B, ESR signal from melanized cells suspended in distilled water (pH 4.17); C, ESR signal from melanized cells suspended in 25 mM MOPS (pH 7.01) with the epinephrine oxidative system without 1 mM H_2O_2; D, ESR signal from melanized cells suspended in 25 mM MOPS (pH 7.01) containing the epinephrine oxidative system (with 1 mM H_2O_2). For the nitrate experiment, three independent experiments performed on different days revealed increases in the ESR signal intensity ranging from 34 to 48% of that observed in the absence of 0.5 mM $NaNO_2$ in 25 mM succinic acid. For the epinephrine oxidative system, two independent experiments performed on different days revealed increases in ESR signal intensity in the presence of H_2O_2 ranging from 5 to 18% of that obtained in the absence of H_2O_2.

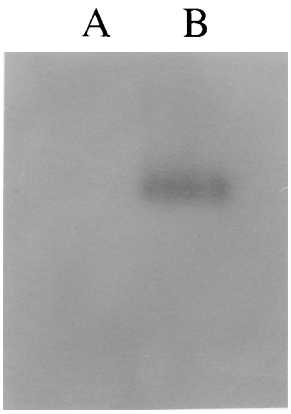

FIG. 4. Melanin synthesis by phenoloxidase enzyme after nondenaturing polyacrylamide gel electrophoresis. Melanin synthesis occurs in situ in the gel after incubation with the substrate L-dopa. (A) Concentrated protein extract from the *mel* mutant strain; (B) concentrated protein extract from strain 24067. The same amounts of protein were loaded into each well. Scanning of this gel revealed that the *mel* lysate produced approximately 1.5% of that measured for strain 24067.

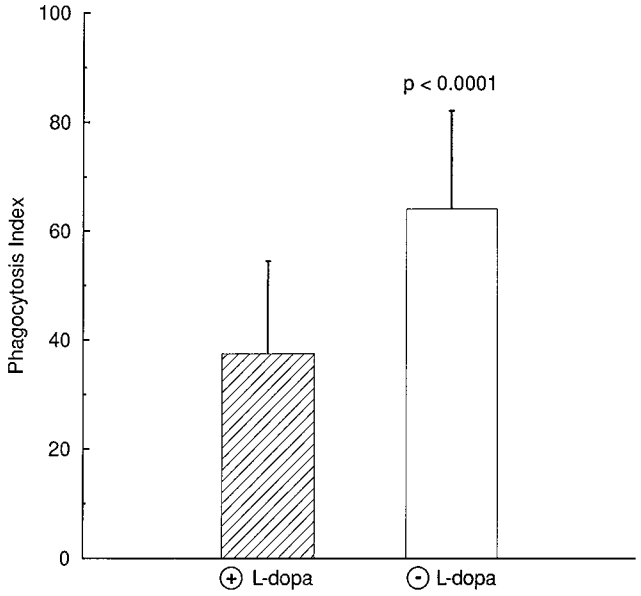

FIG. 5. Phagocytosis of melanized and nonmelanized *C. neoformans* cells by J774.16. The phagocytic index is the [(number of attached and internalized yeast cells)/(number of macrophages)] × 100. Melanized and nonmelanized cells were obtained by growing cultures for 6 days in minimal medium with and without L-dopa, respectively. The experiment was done in the presence of 5 μg of MAb 2H1 per ml. Similar results were observed in three independent experiments.

revealed no spontaneous revertant. The *mel* mutant had only 1.5% phenoloxidase activity of that found in the parent strain as measured by melanin synthesis in nondenaturing polyacrylamide gels (Fig. 4). No difference was noted in the apparent sizes of strain 24067 and *mel* strain phenoloxidase genes by Southern blot analysis of *Eco*RI-, *Ssp*I-, and *Sal*I-digested genomic DNA. ESR spectra of 6-day-old *mel* mutant cells grown on media containing L-dopa revealed no signal at a g value of 2.0, indicating no detectable melanin (Fig. 1). No differences in karyotype, ability to grow on minimal medium or at 37°C, capsule size, or urease activity were detected between the *mel* mutant and parental strains (data not shown). *mel* mutant cells were more susceptible to oxygen- and nitrogen-derived free radicals than parental cells were (Table 1). Growth of *mel* cells in media containing L-dopa reduced their susceptibility to nitrogen-derived radicals but not oxygen-derived radicals (Table 1). Infection of A/J mice (five in each group) with 5×10^6 yeast cells resulted in lung and brain infection in 100% (five of five) of mice infected with strain 24067 but only 20% (one of five) of mice infected with the *mel* mutant strain ($P = 0.048$; chi-square test) at day 7 of infection. At day 4, the average ($n = 10$) lung colony counts for mice infected with 5×10^6 yeast cells were 78,410 ± 93,063 (mean ± standard deviation) and 5,179 ± 8,380 per g for strain 24067 and the *mel* mutant, respectively ($P = 0.017$; *t* test with unequal variances).

Interaction of melanized and nonmelanized *C. neoformans* cells with macrophages. In the absence of capsule-binding MAb 2H1, there was no phagocytosis or difference in the numbers of CFU between melanized and nonmelanized cells after incubation with J774.16 cells (data not shown). Phagocytosis of melanized *C. neoformans* cells in the presence of MAb 2H1 was less than that of nonmelanized cells (Fig. 5). There was no significant difference in the phagocytosis of nonmelanized strain 24067 and that of the *mel* mutant. Growth of the *mel* mutant in the presence or absence of L-dopa had no effect on phagocytosis by J774.16 cells. Surface hydrophobicity of melanized and nonmelanized cells was measured by Sepharose CL-4B hydrophobic interaction chromatography. There was no

TABLE 1. Survival of parental (24067) and *mel* mutant strains after exposure to oxidants and activated J774.16 cells

Treatment	Strain 24067			*mel* mutant		
	% Survival[a]		*P* values[b]	% Survival[a]		*P* values[b]
	− L-dopa	+ L-dopa		− L-dopa	+ L-dopa	
Exposure to oxygen-derived oxidants[c]	49.4 ± 14.0	85.4 ± 10.2	<0.0001*	60.7 ± 12.5	63.1 ± 10.1	0.3386*; 0.0003**
Exposure to nitrogen-derived oxidants[c]	45.5 ± 5.2	68.5 ± 4.40	<0.0001*	35.5 ± 2.6	58.3 ± 10.8	0.0003*; 0.0180**
Incubation with J774.16[d]	87.2 ± 9.30	98.5 ± 14.01	0.005*	70.8 ± 7.02	67.7 ± 10.6	0.4480*; <0.0001**

[a] Percent survival was calculated relative to cells from the same culture not exposed to oxidants or J774.16 cells. Values are averages ± standard deviations.

[b] *P* values were calculated by the *t* test with correction for unequal variances. *, comparison between percentages of survival in the presence and absence of L-dopa for the strain 24067 and *mel* groups; **, comparison between percentages of survival of strain 24067 and *mel* in the presence of L-dopa. Under these conditions, only strain 24067 was melanized.

[c] Oxygen-derived oxidants were generated chemically with the epinephrine oxidative system (26). Nitrogen-derived oxidants were generated chemically by dissolving sodium nitrite in acidic buffer as described previously (1, 31). *C. neoformans* cells were incubated with oxygen-derived oxidants and nitrogen-derived oxidants for 1.5 and 2 h, respectively. For each experiment, *n* equals eight measurements. Each experiment was done twice with similar results.

[d] J774.16 macrophage CFU assays were done in the presence of MAb 2H1 with gamma-interferon- and LPS-activated cells as described previously (23). Values are averages ± standard deviations ($n = 10$). This experiment was done three times with similar results.

difference in the percentages of cells eluted from the gel (53.3% for melanized cells and 53.6% for nonmelanized cells). Melanized cells had consistently higher levels of survival than nonmelanized cells after coincubation with J774.16 macrophage-like cells in the presence of MAb 2H1 (Table 1). In contrast, the percent survival of *mel* mutant cells after exposure to J774.16 cells did not differ regardless of whether the cells were grown in the presence or absence of L-dopa (Table 1). To investigate whether the reduced efficacy of J774.16 cells against melanized *C. neoformans* cells was due to a toxic effect of fungal melanin for J774.16 cells, trypan blue exclusion was used to measure the viability of macrophages after ingestion of heat-killed *C. neoformans*. There was no difference between the viabilities of macrophages ingesting heavily melanized *C. neoformans* and of those ingesting nonmelanized *C. neoformans*.

DISCUSSION

The black pigment made by *C. neoformans* on media containing L-dopa has been assumed to be a melanin on the basis of fungal phenoloxidase activity (25) and histochemical staining with reducing silver stains (16). Melanins are difficult molecules to study because they are insoluble, heterogeneous, and resistant to enzymatic or chemical degradation (8, 12). Melanins are polyanions which contain stable populations of free radicals (8). The presence of unpaired electrons in melanins produces ESR spectra which can be used to identify and define them (8). The *C. neoformans* black pigment and melanized cells have identical ESR spectra characterized by a g value of 2.004 and line width of 4.69 G. The ESR signal of pigmented cells increased after visible light irradiation, suspension in alkaline solution, or addition of Zn^{2+}. The ESR signal decreased in intensity after melanized cells were suspended in acidic solution. These changes in intensity are characteristic of melanins and show that the black pigment of *C. neoformans* is a type of melanin (8). Electron micrographs revealed increased electron density in the cell walls of melanized cells, localizing the melanin to the cell wall. An electron-dense cell wall has been observed by electron microscopy of *C. neoformans* in human tissues (28) and after melanization in vitro (29). Melanin appears to be concentrated in the inner aspect of the cell wall. This is in contrast to *W. dermatitidis*, where increased electron density in melanized cells was in the outer aspect of the cell wall (34). The location of melanin in the cell wall places it in position to interact with substances in the extracellular milieu and prevent their penetration into cells.

Melanized cells are less susceptible to killing by oxygen- and nitrogen-derived radicals (14, 31). Melanins are efficient free-radical scavengers (2, 12, 19, 20), and *C. neoformans* melanization may protect fungal cells by scavenging fungicidal free-radical species (14, 31). The intensity of ESR signals of melanized *C. neoformans* increased when cells were suspended in solutions where oxygen- and nitrogen-derived radicals were generated. The increase in the intensity of melanin ESR spectra indicates an increase in the number of unpaired electrons and implies electron transfer between chemically generated free radicals and cell wall melanin. The changes in ESR signal intensity provide strong evidence for the interaction of melanin molecules with free radicals and support a model where melanins enhance virulence by protecting against free-radical fluxes generated by immune effector cells.

Melanin production by *C. neoformans* has been associated with virulence (17, 18, 27). The melanin-deficient mutant (*mel*) synthesized ~100-fold-less melanin and was less virulent than the parental strain. The *mel* mutant was more susceptible to oxygen- and nitrogen-derived reactive species than the parental strain 24067. Growth in media containing L-dopa did not reduce the susceptibility of *mel* to oxygen-derived radicals. However, growth in media with L-dopa reduced the susceptibility of *mel* cells to nitrogen-derived reactive species, suggesting that even a small amount of melanin may provide significant protection against reactive nitrogen species. These results provide additional support for a model in which melanin enhances virulence by protecting against free radicals produced by host effector cells.

Melanized *C. neoformans* cells were more resistant to the fungicidal and fungistatic effects of J774.16 cells than nonmelanized cells. The mechanism by which J774.16 cells reduce *C. neoformans* CFU in the presence of specific antibody probably involves fungal cell damage by oxygen- and nitrogen-derived free radicals (5) and fungicidal proteins (11) produced by activated cells (see reference 23). MAb 2H1-mediated phagocytosis can enhance nitrate production by J774.16 cells (21), and nitric oxide is an important antimicrobial molecule against *C. neoformans* (37). Macrophages mediate anti-*C. neoformans* activity extracellularly and intracellularly, and phagocytosis can enhance antifungal efficacy (10). Melanized cells were more resistant to antibody-mediated phagocytosis than nonmelanized cells. Phagocytosis of *C. neoformans* can be affected by the surface hydrophobicity and charge of microorganisms (15). No difference in surface hydrophobicity was detected by Sepharose CL-4B hydrophobic interaction chromatography. Since some melanins are charged polymers (35) and the phagocytic ability of neutrophils and monocytes is inversely correlated with cell charge (7, 30), melanized cells may resist phagocytosis by surface charge effects. Thus, the mechanism by which melanin enhances the resistance of *C. neoformans* to J774.16 cells may be a combination of reduced phagocytosis and protection against oxygen- and nitrogen-derived antimicrobial products produced by these cells after gamma interferon and lipopolysaccharide (LPS) activation (5).

In summary, *C. neoformans* synthesizes melanin. Melanin (or a melanin-associated agent) appears to contribute to virulence by enhancing resistance of fungal cells to attack by immune effector cells. Melanin may function in a manner analogous to that of mycobacterial glycolipids, which are efficient free-radical scavengers (4). These results suggest that inhibition of the *C. neoformans* phenoloxidase pathway is a potential strategy for drug discovery.

ACKNOWLEDGMENTS

We are grateful to Marcia Goldberg and Matthew D. Scharff for critical reading of the manuscript.

Y.W. and A.C. were supported by a James S. McDonnell Scholar Award and NIH grants RO1-AI33774 and RO1-AI13342. P.A. was supported by NIH grant DK15056. These supports are gratefully acknowledged.

ADDENDUM IN PROOF

Blasi et al. (E. Blasi, R. Barluzzi, R Mazzola, B. Tancini, S. Saleppico, M. Puliti, L. Pitzurra, and F. Bistoni, J. Neuroimmunol. **58:**111–116, 1995) have recently reported that melanized *C. neoformans* cells are more resistant to phagocytosis and the antifungal effects of murine microglia than nonmelanized cells.

REFERENCES

1. **Alspaugh, J. A., and D. L. Granger.** 1991. Inhibition of *Cryptococcus neoformans* replication by nitrogen oxide supports the role of these molecules as effectors of macrophage-mediated cystostasis. Infect. Immun. **59:**2291–2296.
2. **Bustamante, J., L. Bredeston, G. Malanga, and J. Mordoh.** 1993. Role of

melanin as a scavenger of active oxygen species. Pigment Cell Res. **6:**348–353.
3. **Casadevall, A., L. Freundlich, L. Marsh, and M. D. Scharff.** 1992. Extensive allelic variation in *Cryptococcus neoformans*. J. Clin. Microbiol. **30:**1080–1084.
4. **Chan, J., T. Fujiwara, P. Brennan, M. McNeil, S. J. Turco, J.-C. Sibille, M. Snapper, P. Aisen, and B. R. Bloom.** 1989. Microbial glycolipids: possible virulence factors that scavenge oxygen radicals. Proc. Natl. Acad. Sci. USA **86:**2453–2457.
5. **Chan, J., Y. Xing, R. S. Magliozzo, and B. R. Bloom.** 1992. Killing of virulent *Mycobacterium tuberculosis* by reactive nitrogen intermediates produced by activated murine macrophages. J. Exp. Med. **175:**1111–1122.
6. **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.
7. **Eggleton, P., N. Crawford, and D. Fisher.** 1992. Fractionation of human neutrophils into subpopulations by countercurrent distribution: surface charge and functional heterogeneity. Eur. J. Cell. Biol. **57:**265–272.
8. **Enochs, W. S., M. J. Nilges, and H. M. Swartz.** 1993. A standardized test for the identification and characterization of melanins using electron paramagnetic (EPR) spectroscopy. Pigment Cell Res. **6:**91–99.
9. **Geis, P. A., M. H. Wheeler, and P. J. Szaniszlo.** 1984. Pentaketide metabolites of melanin synthesis in the dematiaceous fungus *Wangiella dermatitidis*. Arch. Microbiol. **137:**324–328.
10. **Granger, D. L., J. R. Perfect, and D. T. Durack.** 1986. Macrophage-mediated fungistais *in vitro*: requirements for intracellular and extracellular cytotoxicity. J. Immunol. **136:**672–680.
11. **Hiemstra, P. S., P. B. Eisenhauer, L. S. Harwig, M. T. van den Barselaar, R. van Furth, and R. I. Lehrer.** 1993. Antimicrobial proteins of murine macrophages. Infect. Immun. **61:**3038–3046.
12. **Hill, Z. H.** 1992. The function of melanin or six blind people examine an elephant. BioEssays **14:**49–56.
13. **Ikeda, R., and E. S. Jacobson.** 1992. Heterogeneity of phenol oxidases in *Cryptococcus neoformans*. Infect. Immun. **60:**3552–3555.
14. **Jacobson, E. S., and S. B. Tinnell.** 1993. Antioxidant function of fungal melanin. J. Bacteriol. **175:**7102–7104.
15. **Kozel, T. R.** 1983. Dissociation of a hydrophobic surface from phagocytosis of encapsulated and non-encapsulated *Cryptococcus neoformans*. Infect. Immun. **39:**1214–1219.
16. **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.
17. **Kwon-Chung, K. J., I. Polacheck, and T. J. Popkin.** 1982. Melanin-lacking mutants of *Cryptococcus neoformans* and their virulence for mice. J. Bacteriol. **150:**1414–1421.
18. **Kwon-Chung, K. J., and J. C. Rhodes.** 1986. Encapsulation and melanin formation as indicators of virulence in *Cryptococcus neoformans*. Infect. Immun. **51:**218–223.
19. **Longuet-Higgins, H. C.** 1960. On the origin of the free radical property of melanins. Arch. Biochem. Biophys. **86:**231–232.
20. **Mason, H. S., D. J. E. Ingram, and B. Allen.** 1960. The free radical property of melanins. Arch. Biochem. Biophys. **86:**225–230.
21. **Mozaffarian, N., J. W. Berman, and A. Casadevall.** 1995. Immune complexes increase nitric oxide production by interferon-gamma-stimulated murine macrophage-like J774.16 cells. J. Leukocyte Biol. **57:**657–662.
22. **Mukherjee, S., S. Lee, J. Mukherjee, M. D. Scharff, and A. Casadevall.** 1994. Monoclonal antibodies to *Cryptococcus neoformans* capsular polysaccharide modify the course of intravenous infection in mice. Infect. Immun. **62:**1079–1088.
23. **Mukherjee, S., S. C. Lee, and A. Casadevall.** 1995. Antibodies to *Cryptococcus neoformans* glucuronoxylomannan enhance antifungal activity of murine macrophages. Infect. Immun. **63:**573–579.
24. **Perfect, J. R., B. B. Magee, and P. T. Magee.** 1989. Separation of chromosomes of *Cryptococcus neoformans* by pulsed-field gel electrophoresis. Infect. Immun. **57:**2624–2627.
25. **Polacheck, I., and K. J. Kwon-Chung.** 1988. Melanogenesis in *Cryptococcus neoformans*. J. Gen. Microbiol. **134:**1034–1041.
26. **Polacheck, I., Y. Platt, and J. Aronovitch.** 1990. Catecholamines and virulence of *Cryptococcus neoformans*. Infect. Immun. **58:**2919–2922.
27. **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.
28. **Ro, Y. J., S. S. Lee, and A. G. Ayala.** 1987. Advantage of Fontana-Masson stain in capsule-deficient cryptococcal infection. Arch. Pathol. Lab. Med. **111:**53–57.
29. **Shaw, C. E., and L. Kapica.** 1972. Production of diagnostic pigment by phenoloxidase activity of *Cryptococcus neoformans*. Appl. Microbiol. **24:**824–830.
30. **Walter, H., L. L. Graham, E. J. Krob, and M. Hill.** 1980. Correlation between phagocytic and membrane surface properties reflected by partitioning of human peripheral blood monocytes in two-polymer aqueous phases. Biochim. Biophys. Acta **602:**309–322.
31. **Wang, Y., and A. Casadevall.** 1994. Susceptibility of melanized and nonmelanized *Cryptococcus neoformans* to nitrogen- and oxygen-derived oxidants. Infect. Immun. **64:**3004–3007.
32. **Wang, Y., and A. Casadevall.** 1994. Growth of *Cryptococcus neoformans* in the presence of L-dopa decreases its susceptibility to amphotericin B. Antimicrob. Agents Chemother. **38:**2646–2650.
33. **Wheeler, M. H., and A. A. Bell.** 1988. Melanins and their importance in pathogenic fungi. Curr. Top. Med. Mycol. **2:**338–387.
34. **Wheeler, M. H., and R. D. Stipanovic.** 1985. Melanin biosynthesis and the metabolism of flaviolin and 2-hydroxyjuglone in *Wangiella dermatitidis*. Arch. Microbiol. **142:**234–241.
35. **White, L. P.** 1958. Melanin: a naturally occurring cation exchange material. Nature (London) **182:**1427–1428.
36. **Williamson, P. R.** 1994. Biochemical and molecular characterization of the diphenol oxidase of *Cryptococcus neoformans*: identification as a laccase. J. Bacteriol. **176:**656–664.
37. **Yoshida, K., T. Akaike, T. Doi, K. Sato, S. Ijiri, M. Suga, M. Ando, and H. Maeda.** 1993. Pronounced enhancement of NO-dependent antimicrobial activity by an NO-oxidizing agent, imidazolineoxyl *N*-oxide. Infect. Immun. **61:**3552–3555.