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Catecholamine Oxidative Products, but Not Melanin, Are Produced by *Cryptococcus neoformans* during Neuropathogenesis in Mice

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Melanin has been proposed as a virulence factor in *Cryptococcus neoformans*, but its presence has not been shown unambiguously in vivo. Validated methods used previously to show production of cryptococcal eumelanin pigment in vitro (P. R. Williamson, K. Wakamatsu, and S. Ito, J. Bacteriol. 180:1570–1572, 1998) were used to assess for production of laccase-derived products in mouse brain of the Lacc$^+$ strains, 2E-TUC, H99 (serotype A), and ATCC 34873 (serotype D), and the Lacc$^-$ strain, 2E-TU. Pyrrole-2,3,5-tricarboxylic and pyrrole-2,3-dicarboxylic acid, specific degradation products of catecholamine derivatives such as melanin, were found in all Lacc$^+$ strains, but not in the Lacc$^-$ strain, 2E-TU. However, the presence of melanin pigment itself could not be demonstrated in the same cells. Lack of the specific degradation products aminohydroxyphenylalanine and aminohydroxyphenylethylamine in Lacc$^+$ strains upon hydriodic acid hydrolysis showed that pheomelanin was also not produced by the fungus in vivo. These are the first data to support the generation of catecholamine oxidation products by *C. neoformans* in vivo, but they do not support postenzymatic polymerization of these products to form typical eumelanin, as previously proposed.

Melanin, believed to be produced by laccase oxidation of neurocatecholamines, has been thought to be a major virulence factor in *Cryptococcus neoformans* since the description of pigment in cells grown on agar containing *Guizotia abyssinica* extracts over 30 years ago by Staib (35). Melanin-like pigments have been described in a variety of pathogenic fungi. *Pyricularia oryzae* and *Wangiella dermatitidis* synthesize melanin from endogenous dihydroxynaphthalene substrates by the pentaketide pathway, while *C. neoformans* requires exogenous catecholamines (27). Extensive study of in vitro-produced cryptococcal melanin has shown its ability to act as an immune protectant, based on its ability to act as an efficient free radical scavenger (28). Numerous studies have shown increased survival of Mel$^+$ versus Mel$^-$ cryptococcal cells in the presence of nitrogen-derived (39) or oxygen-derived (16, 17) oxidants. Another mechanism proposed for cellular effector evasion by melanin is its ability to alter cell wall surface interactions by charged polymer side groups which can alter phagocytosis of *C. neoformans* (26). Other potential roles of melanin have been proposed, including interfering with protective T-cell responses such as tumor necrosis factor alpha secretion (9) and reducing susceptibility to the antifungal agent amphotericin B (41). Melanin has been shown by electron paramagnetic spectroscopy (40) and by chemical degradative and absorptive studies (44) to be produced in vitro by the enzyme laccase. Laccase has been shown to be important in virulence by using congenic *CNLAC1*-knockout strains of *C. neoformans* (32). However, such experiments cannot determine whether laccase itself or laccase products potentiate *CNLAC1*-associated virulence. Initial attempts to study laccase products in vivo employed the Masson-Fontana silver stain for melanin, which indeed labeled organisms in mouse brain and spleen and human lung and brain. However, the authors concluded that silver-reducing compounds in the cell wall were unlikely to be melanin, since unpigmented cells grown on agar also stained by the Masson-Fontana technique (19). Thus, while production of melanin by *C. neoformans* has been an attractive hypothesis for the last 35 years, further study is required to determine if laccase-derived catecholamine oxidation products (COPs) are formed during pathogenesis.

Microanalytical high-performance liquid chromatography methods were used to quantify and characterize cryptococcal laccase products from fungal cells obtained in high yield during experimental murine meningoencephalitis. These microanalytical methods have been critical for the accurate structural determination of mammalian melanin (10, 11) and have recently been used as a sensitive assay of eumelanin pigments produced by *C. neoformans* in vitro (44).

MATERIALS AND METHODS

Strains. *C. neoformans* ATCC 34873 (B-3501, serotype D) was a gift of K. J. Kwon-Chung, and *C. neoformans* H99 (serotype A) was a gift of J. Perfect. 2E-TUC and 2E-TU are congenic *CNLAC1* (Lacc$^+$) and *cnlac1* (Lacc$^-$) strains, respectively, and have been described previously (32). The mouse strains used in this study were Swiss Albino and C.B.-17/ICrHsd-scid-bg (Harlan Bioproducts, Indianapolis, Ind.). Mice were housed under specific-pathogen-free conditions in enclosed filter-top cages. Clean food and water were given ad libitum. The mice were handled and maintained by using microisolator techniques and were monitored by the Unit for Laboratory Animal Medicine at the University of Illinois.

Isolation of *C. neoformans* from mouse brains. The mouse meningoencephalitis model of Lim et al. (21), as modified by Salas et al. (32), was used to obtain brain organisms. Briefly, *C. neoformans* cells were suspended in saline after 48 h of growth on 1% yeast extract–2% Bacto Peptone–2% glucose (YPD) agar at 30°C. Cell counts were estimated by hemocytometer and confirmed by colony counts on YPD agar. Six- to ten-week-old female Swiss Albino mice were inoculated with 0.1 ml of yeast cell suspensions containing 2×10^7 organisms in the lateral tail vein and monitored daily for survival. At various times, between onset of hydrocephalus up to the time the animal was unable to reach food or water, animals were sacrificed. The mouse brain was exposed, removed intact, weighed, and immediately homogenized in 0.25% sodium dodecyl sulfate to which was added 2 mg of proteinase K per ml (Life Technologies, Bethesda, Md.). A portion was removed and plated in serial dilutions on YPD for colony counts.

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TABLE 1. Degradative products of *C. neoformans* from mouse brains

Strain	Time of harvest [mean days (range)]	Amt harvested (CFU/g of brain)	No. of cells recovered	Alkaline oxidation (ng/million cells)		Hydriodic acid hydrolysis (ng/million cells)	
				PTCA	PDCA	AHPEA	AHP
2E-TUC							
Group 1	10 (0)	NP[a]	2.6×10^6	2.0	0.4	NP	NP
Group 2	16.3 (1.1)	1.5×10^6	1.7×10^7	11.2	1.9	<0.2	1.8
2E-TU							
Group 1	28 (0)	2.4×10^6	1.9×10^6	<0.2[b]	<0.2[b]	<0.2[b]	1.1[b]
Group 2	43 (0)	1.8×10^6	3.0×10^6				
H99							
Group 1	20.5 (0.5)	6.2×10^5	2.4×10^6	1.3	0.2	NP	NP
Group 2	52 (1)	NP	2.4×10^6	8.6	8.2	0.5	4.1
ATCC 34873 (B-3501)							
Group 1	21 (0)	4.2×10^5	8.0×10^5	3.8	0.7	NP	NP
Group 2	31 (2)	6.9×10^6	4.4×10^6	0.3	0.2	0.7	1.1

[a] NP, not performed.
[b] Groups 1 and 2 combined.

The remainder of the suspension was incubated at 37°C for 30 min, at which time, an equal volume of 0.25% sodium dodecyl sulfate was added, and the suspension was layered on top of a discontinuous 60 to 80% sucrose gradient and centrifuged at 1,000 × *g* for 30 min. Cryptococcal cells were obtained at the 60 to 80% interface and heated at 70°C for 30 min. Control experiments were performed in which 2×10^7 in vitro-pigmented cells (produced by incubation of Lacc$^+$ 2E-TUC on asparagine salts [32] containing 100 mg of dopamine [DA] per liter for 48 h) were injected into a sacrificed uninfected mouse brain. The brain was then processed as described above to recover cryptococcal cells.

Analysis of in vivo cryptococcal products. Cells were disrupted in water with an ultrasonic cell disrupter. The results summarized in Table 1 are expressed per 1 million cells. Alkaline hydrogen peroxide oxidation was performed by the method of Napolitano et al. (24), as modified by Ito and Wakamatsu (15). Hydriodic acid hydrolysis was performed as described previously (12, 15). Adaptations for analysis of *C. neoformans* were described previously (44). Oxidation and hydrolysis products were analyzed by reverse-phase high-performance liquid chromatography (HPLC), and eluent was monitored at 269 nm or +400 mV versus an Hg/Hg_2Cl_2 reference electrode as previously described (15).

Quantitation of yeast and histology of *C. neoformans* in brain. Brains of mice were removed aseptically, weighed, and placed in a tissue homogenizer with 0.5 ml of sterile distilled water. The number of CFU in the specimens was determined by a plate dilution method on Sabouraud dextrose agar. Colonies were counted after 48 h at 30°C, and results are expressed as the number of CFU per gram. Paraffin-embedded transverse brain sections were used for periodic acid-Schiff staining performed as described previously (1).

RESULTS

Recovery of *C. neoformans* from infected mouse brains. To characterize laccase products of *C. neoformans* in vivo, a new method was developed to separate fungal cells from brain by a simple discontinuous sucrose gradient. Examination of cells by microscopy showed only budding yeast cells without other cellular debris. Two sets of analyses were performed for each strain of *Cryptococcus* tested (Table 1), each using pooled fungal cells from two mice (three mice for group 2 of 2E-TUC). Recovered cells were found to exhibit viabilities of 9% for 2E-TUC, 8% for 2E-TU, 28% for ATCC 34873, and 50% for 2E-TUC recovered from immunosuppressed scid/beige mice. As shown in Table 1, good recoveries of cryptococcal cells were achieved, often exceeding the CFU count from direct plating. As shown in Fig. 1 (row 3, lanes A and B), in vitro-melaninized cryptococcal cells injected into sacrificed mice retained pigment through the recovery procedure. (The shape of the cell pellet appears different in row 3, lane A, because these cells were spun in a microcentrifuge tube, and the bottom of the tube was placed into the well of the microtiter plate.)

Microanalysis of in vivo *C. neoformans* catecholamine oxidation products. A previously validated microanalytical method (15) was used to characterize catecholamine oxidative products in cryptococcal cells obtained from mouse brain. These methods have been used to identify and characterize melanin pigment in *C. neoformans* in vitro (44). Alkaline hy-

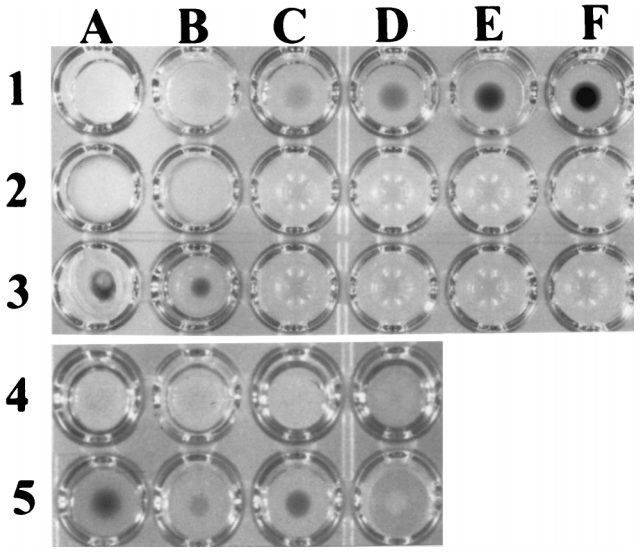

FIG. 1. Pigmentation of *C. neoformans* 2E-TUC (Lacc$^+$) obtained in vitro and in vivo as shown in microtiter wells. Lane 1, fungal cells (10^7 cells in each well) incubated in vitro on asparagine agar for 48 h containing DA at 0 (A), 1 (B), 3 (C), 10 (D), 30 (E), or 100 (F) mg/liter. Lane 2, 10^7 cells recovered from mice (column A, Swiss Albino; B, scid/beige). Lane 3, cells grown on asparagine agar containing 100 mg of DA per liter for 48 h, washed, and inoculated into the brain of a sacrificed Swiss Albino mouse and recovered (10^6 cells in each well [column A, postinoculation; column B, preinoculation). Lane 4, *C. neoformans* cells directly recovered from brains of two Swiss Albino mice (A, 3501 [3.2×10^6]; B, 2ETUC [3.7×10^6]; C, H99 [7.2×10^6]; D, 2ETU [2.0×10^6]). Lane 5, equivalent amounts of each strain recovered from mouse brain and grown on asparagine agar with norepinephrine at 100 mg/liter for 48 h.

drogen peroxide oxidation of cryptococcal cells showed that all Lacc$^+$ strains tested produced pyrrole-2,3,5-tricarboxylic acid (PTCA) and pyrrole-2,3-dicarboxylic acid (PDCA) (Table 1), specific degradative products of catecholamine derivatives of 3,4-dihydroxyphenylalanine (DOPA) and DA, respectively (15). In contrast, the Lacc$^-$ 2E-TU strain produced no detectable degradative products. The relatively high PTCA/PDCA ratio in the 2E-TUC strain (group 2) suggests the presence of DOPA-derived products, whereas comparable amounts of PTCA and PDCA in strain H99 (group 2) implicate DA-derived products (15). It was difficult to assess these relationships in the group 1 cells of both strains, since smaller quantities of total oxidation products in this group made such comparisons less reliable. While the complexity of the analytical technique did not allow sufficiently large sample sizes to permit strong inferences, greater amounts of degradation products tended to be obtained from cryptococcal cells later in the course of infection.

In order to assess the presence of pheomelanins, hydriodic acid hydrolysis was performed with selected samples (Table 1). Aminohydroxyphenylalanine (AHP), a specific indicator of the cysteinyldopa oxidation product pheomelanin (12), was found only in trace quantities from all strains tested, including the Lacc$^-$ strain, 2E-TU. These very low AHP values, which do not appear laccase dependent, show that pheomelanin production in *C. neoformans* is not significant. Trace amounts of aminohydroxyphenylethylamine (AHPEA) were found in Lacc$^+$ cryptococcal cells. AHPEA has been proposed as an indicator of cysteinyldopamine oxidation products (2, 38), although recent studies have shown that DA oxidation products produce AHPEA as well (15).

Pigment production by *C. neoformans* in vivo. Previous studies have shown that incubation of the same strains of Lacc$^+$ cryptococcal cells in the presence of the catecholamines DA and DOPA produces dark eumelanin pigments in vitro (44). As demonstrated in Fig. 1, row 1, 10^7 2E-TUC organisms incubated on agar for 2 days containing 1, 3, 10, 30, and 100 mg (lanes B to F, respectively) of DA per liter show increasing amounts of visible melanin pigment. Pigment production was proportional to quantities of the degradation product of DA-melanin, PDCA. Amounts of recovered PDCA per 1 million cells grown in the presence of the indicated substrate concentrations were as follows: 100 mg/liter, 1.08 ng; 30 mg/liter, 0.48 ng; 10 mg/liter, 0.26 ng; 3 mg/liter, 0.06 ng; 1 mg/liter, nondetectable; and 0 mg/liter, nondetectable. In contrast, all strains obtained from mice were completely white (Fig. 1, row 4, lanes A to D). Incubation of equivalent numbers of brain-recovered Lacc$^+$ cells with catecholamines resulted in dark pigment production (Fig. 1, row 5, lanes A to C), indicative of a retained ability to produce melanin in vitro. Recovery of larger numbers (10^7) of 2E-TUC cells obtained from eight mice (Fig. 1, row 2, lane B) were still indistinguishable from equivalent quantities of cells grown on asparagine agar without catecholamines (Fig. 1, row 1, lane A). Soluene 350 solubilization was performed with these cells which showed insignificant absorbance for all strains from mouse brain tested ($A_{500}/10^6$ cells for 2E-TUC, 0.003; 2E-TU, 0.000; and H99, 0.000). Soluene 350 solubilization was previously used to show pigmentation of cryptococcal melanin produced in vitro (44). The presence of viable cells obtained by these methods argues against bleaching of melanin by brain phagocytes, since previous studies indicate that it takes approximately 100× the lethal concentration of oxidants to bleach cryptococcal melanin (40). Nevertheless, in order to control for possible immune oxidation of melanin during infection, scid/beige mice, which do not mount a significant cellular response to *C. neoformans* (33), were subjected to infection by 2E-TUC. A lack of brain cellular response was confirmed in the present study by serial histology sections of one of the three mice injected with 2E-TUC, which showed cystic clusters of organisms without significant cellular infiltrate. As shown in Fig. 1 (row 2, lane A), 2E-TUC cells obtained from scid/beige mice were also unpigmented, indistinguishable from cryptococcal cells grown in vitro without catecholamines. Soluene 350 solubilization also showed negligible absorbance ($A_{500}/10^6$ cells for 2E-TUC, 0.006).

To determine the fate of COPs in the presence of a high-protein environment, such as in the brain, 100 μM DA and 200 μM bovine serum albumin (BSA; Sigma, St. Louis, Mo.) were incubated in the presence or absence of 5,000 U of recombinant cryptococcal laccase, obtained as described previously (43). At time periods of 5, 10, and 20 min, aliquots of each reaction mixture were precipitated with trichloroacetic acid (TCA), washed, and subjected to alkaline oxidation as described above. A parallel reaction was performed with a trace label of [2,5,6-^{3}H]DA (New England Nuclear, Boston, Mass.) to determine TCA-precipitable conjugate formation by liquid scintillation. DA-BSA conjugation was found to be laccase dependent, maximizing at 5 min, and yielding 235 ± 8 ng of DA/mg of TCA-precipitated BSA in the presence of laccase and 40 ± 3 ng of DA/mg of BSA in the absence of laccase (mean of four determinations ± standard error). Alkaline oxidation yielded 39.5 ng of PDCA/mg of BSA in the presence of laccase and 0.7 ng of PDCA/mg of BSA in the absence of laccase (mean of two determinations). No pigmentation was observed in either reaction, despite large amounts of DA oxidation product being produced.

DISCUSSION

The present study used recently developed microanalytical techniques to provide insights into the presence and identity of cryptococcal laccase products in vivo, first proposed to be associated with virulence over 30 years ago by Staib (35). The congenic Lacc$^+$ strain 2E-TUC and Lacc$^-$ strain 2E-TU were isolated from brains of infected Swiss Albino mice by a new, yet simple method, and alkaline peroxide oxidation was performed which yielded the catecholamine-derived degradation products PTCA and PDCA only in the Lacc$^+$ 2E-TUC strain. The absence of these products in Lacc$^-$ 2E-TU confirms that laccase-dependent catecholamine oxidation was required to obtain these products. Upon alkaline peroxide oxidation of in vitro-grown cryptococcal cells incubated in the presence of catecholamines, PTCA is produced from 5,6-dihydroxyindole-2-carboxylic acid- (DHICA) melanin, formed from polymerized oxidative products of DOPA, and PDCA is derived from 5,6-dihydroxyindole (DHI) melanin, formed from polymerized oxidative products of DA (44). Evidence of COPs was corroborated by analysis of two additional representative Lacc$^+$ cryptococcal strains, H99 (serotype A) and B-3501 (serotype D). A predominance of PTCA versus PDCA from the 2E-TUC strain grown in vivo was unexpected, considering that DA is the predominant catecholamine in rodent brain (6) and that DA yields predominantly PDCA in vitro (44). Quantities of degradative products varied substantially between the groups of cells analyzed, which may be a result of variations in the brain environment due to differences in the immune responses of individual mice to cryptococcosis (23). Nevertheless, degradation products appeared in most cases to be greater in quantity in groups harvested later in infection, presumably a function of longer exposure to endogenous catecholamine substrate.

These data provide the first identification of laccase-derived COPs from cryptococcal cells isolated from mammalian tissue.

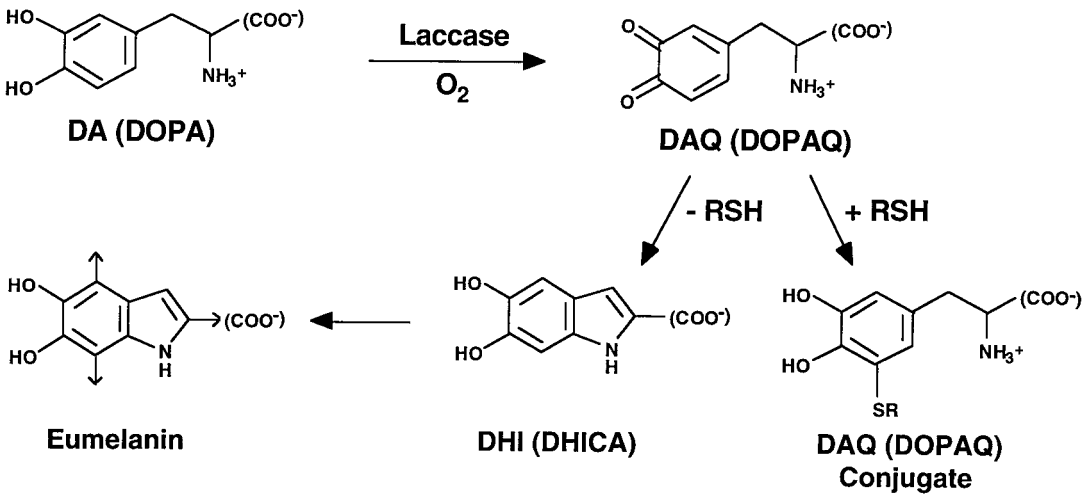

FIG. 2. Production of catecholamine oxidation products by laccase of *C. neoformans*.

Their production in the brain is facilitated by a 10-fold-greater concentration of DA and DOPA within dopaminergic pathways (6) and has been proposed as an explanation for striatal localization of organisms in human infection (20). Oxidized catecholamines, such as dopamine-*o*-quinone (DAQ), are potent cytotoxic agents by virtue of their ability to condense with the sulfhydryl group of cysteine within proteins (13, 37). An oxidative intermediate preceding DAQ, DA semiquinone, is a potent inhibitor of mammalian vacuolar H^+-ATPase (36). COPs have been implicated as cytotoxic agents in neurotoxicity leading to the development of dementia in Parkinson's disease (3). In addition, if condensed with cysteine in the brain, these oxidative products form cysteinylcatechols which have been found to be neurotoxic to pyramidal neurons in organotypic cultures of hippocampus (22). The cytotoxic effects of laccase-produced COPs thus have the potential to interfere with cellular host defense or potentiate neurotoxicity, leading to the dementia described in chronic cryptococcal meningitis (18).

The dark color of melanin pigmentation is produced by optical dispersion by the polymeric lattice (31). This is a uniform phenomenon of polymeric melanin and has been described extensively in mammalian (29) and cryptococcal melanin (42). This pigmentation, easily seen in cells grown in vitro, was not present in any of the cells obtained in vivo and indicates a lack of polymerization of the catecholamine oxidation products described above. Melanoma cells producing pheomelanin may lack visible pigmentation (5). However, there can be no significant amounts of pheomelanin in *C. neoformans* in vivo, because hydriodic acid hydrolysis produced only trace amounts of AHP and AHPEA that were not laccase dependent. Other methods of determining polymerization, such as electron paramagnetic resonance spectroscopy, were not sufficiently sensitive for assessment of *Cryptococcus* cells in vivo (40). Lack of melanin formation upon catecholamine oxidation in vivo is most likely due to differences between the brain environment and that found on agar plates during cryptococcal melanin production in vitro. Melanin formation may be prevented in vivo by low concentrations of catecholamines and/or competition for catecholamine oxidative products by materials such as brain matrix proteins. In vitro, the pigment of *C. neoformans* is most easily produced at high substrate concentrations, such as the 100 mg/liter used by investigators studying possible immunologic roles of the fungal pigment (39). As shown in Fig. 2, DA is oxidized by cryptococcal laccase in vitro to DAQ, which then undergoes oxidative polymerization to eumelanin (44). Such a process involving the interactions of multiple short-lived intermediates would be expected to be dependent on relatively high concentrations of catecholamines. Indeed, in mammalian systems, melanin is formed at high substrate concentrations within specialized melanosomes of melanocytes. In contrast, within rodent brain, the highest level of catecholamines is found within the striatal tracts, but only reaches 1 to 7 mg/liter (6), a concentration not optimal for melanin polymerization. In addition, as shown in Fig. 2, the reactive *o*-quinone, DAQ, may undergo heterocondensation with other chemical moieties, including cysteine-containing proteins, as demonstrated previously by degradative techniques (7, 13). This possibility was illustrated in the present study, which showed the formation of a nonpigmented DA-BSA conjugate by cryptococcal recombinant laccase and the production of PDCA degradation products by alkaline oxidation of the conjugate. Since homopolymerization of DAQ is an oxidative process, an additional role for inhibition of melanin formation in normal brain by reductants such as glutathione and ascorbic acid may be operative, as has been proposed in the Parkinson's disease field, but this remains controversial (8, 30, 34, 45). Limited production of oligomeric homocondensation products may also occur in *C. neoformans* in vivo. It has been shown that alkaline peroxide oxidation of DHICA dimers produces PTCA, while both PTCA and PDCA are produced from DHI dimers (24, 25). In fact, DHICA oligomers have been found in the eyes of catfish (14). However, it is very unlikely that DHI oligomers are stable in nature, because of their extreme tendency to be oxidized to black eumelanin. The exact chemical structures of the final *o*-quinone condensation products of *C. neoformans* in the brain remain to be established and are most likely heterogeneous, similar to the myriad of products formed after hydroxyl radical generation in vivo (4). Highly reactive COPs are the most likely species to mediate laccase-dependent toxicity, forming condensation products with multiple mammalian and fungal nucleophilic moieties. Nevertheless, these data do not support paradigms of cryptococcal virulence that have been based on in vitro production of dark eumelanin pigments. It is doubtful, for example, that *o*-quinone–protein adducts would possess the extensive metal-trapping properties comparable to melanin's

unique antioxidant or free radical scavenger effects. Instead, these studies offer new directions in cryptococcal virulence research, by suggesting that laccase of *C. neoformans* may confer protection either by itself or by coopting the host's normal catecholamines and converting them into potentially cytotoxic *o*-quinones.

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