[Frontiers in Bioscience 2, e99-107, November 1, 1997]

LACCASE AND MELANIN IN THE PATHOGENESIS OF *CRYPTOCOCCUS NEOFORMANS*

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1. ABSTRACT

Cryptococcosis, caused by an encapsulated fungus, *Cryptococcus neoformans*, has emerged as a life threatening infection in HIV positive individuals and other immunocomporomised hosts. The present review describes laccase and its product melanin as an important virulence factor of *Cryptococcus neoformans* and illustrates the approaches used in elucidating the pathogenesis of cryptococcosis. Characterization of the biochemical pathways leading to melanin synthesis is summarized using biochemical and biomolecular approaches. Melanin synthesis is dependent on a single copper-dependent enzyme, laccase. Since the mammalian host does not contain this enzyme, laccase is an attractive candidate for the study of fungal pathogenesis, as well as a drug target. The cloning of the *CNLAC1* gene and construction of *CNLAC1* gene knock-out strains has confirmed its role in the virulence of *Cryptococcus*. Also described is the role of melanin in the host-pathogen interactions. Melanin may protect *Cryptococcus* cells by a variety of methods including anti-oxidant or cell wall surface effects thereby offering protection against numerous effectors of cellular immunity.

2. INTRODUCTION

Cryptococcus neoformans is a major opportunistic pathogen in immuno-compromised hosts and accounts for approximately 6% of AIDS-related infections (1). Death from meningitis is common despite antifungal therapy and all patients need life-long therapy to prevent relapse (2). The species *C. neoformans* is the only major pathogen among about 20 species described in the genus *Cryptococcus* (3). The organism is unique because it grows well at 37^{o} C, and possesses a laccase (4) which forms a melanin-like pigment when grown on substrates containing polyphenolic or polyaminobenzene compounds (5, 6). The melanin synthetic pathway, which has the potential to use neurologic catecholamines, has been associated with virulence (7, 8, 9) and is thought to provide an explanation for neurotropism of the organism (10).

The melanin synthetic pathway is a potential target for pharmacological intervention or protection against *C. neoformans* . Precedents for the fungicidal role of melanin inhibitors can be found in the compounds tricyclozol, fthalide and pyroquilon which are used to inhibit pentaketide melanin and thereby prevent rice blast disease caused by *Pyricularia oryzae* (11). The need for a search of novel pharmacological targets has been highlighted by the recent emergence of resistance in *Cryptococcus* to the newer azole antifungals (12, 13, 14). An inhibitory agent against melanin synthesis would be expected to shut down melanin synthesis in *Cryptococcus*, since catecholamines are oxidized by a single enzyme, laccase. Since humans do not possess the laccase enzyme, inhibitors to laccases may not affect mammalian systems significantly to cause serious side effects.

3. MELANIN BIOSYNTHESIS OF *C. NEOFORMANS*

Staib first described pigmentation in *Cryptococcus neoformans* more than 35 years ago (6). In spite of considerable effort by several investigators, many aspects of the structure of melanin in Cryptococcus are not known. *In vitro*, electron paramagnetic resonance has been used to show that the free radical population within the *C. neoformans* pigment is similar to that found in mammalian eumelanin (15). In addition, Cryptococcal pigment has been found to resemble mammalian pigment in its ability to reduce reactive oxygen and nitrogen metabolites (16, 17, 18). However, elemental analysis of a melanin preparation produced *in vitro* from L-DOPA led one author to suggest that the pigment produced was quite different from mammalian or fungal melanins (19). Since

Received 10/20/97 Accepted 10/24/97
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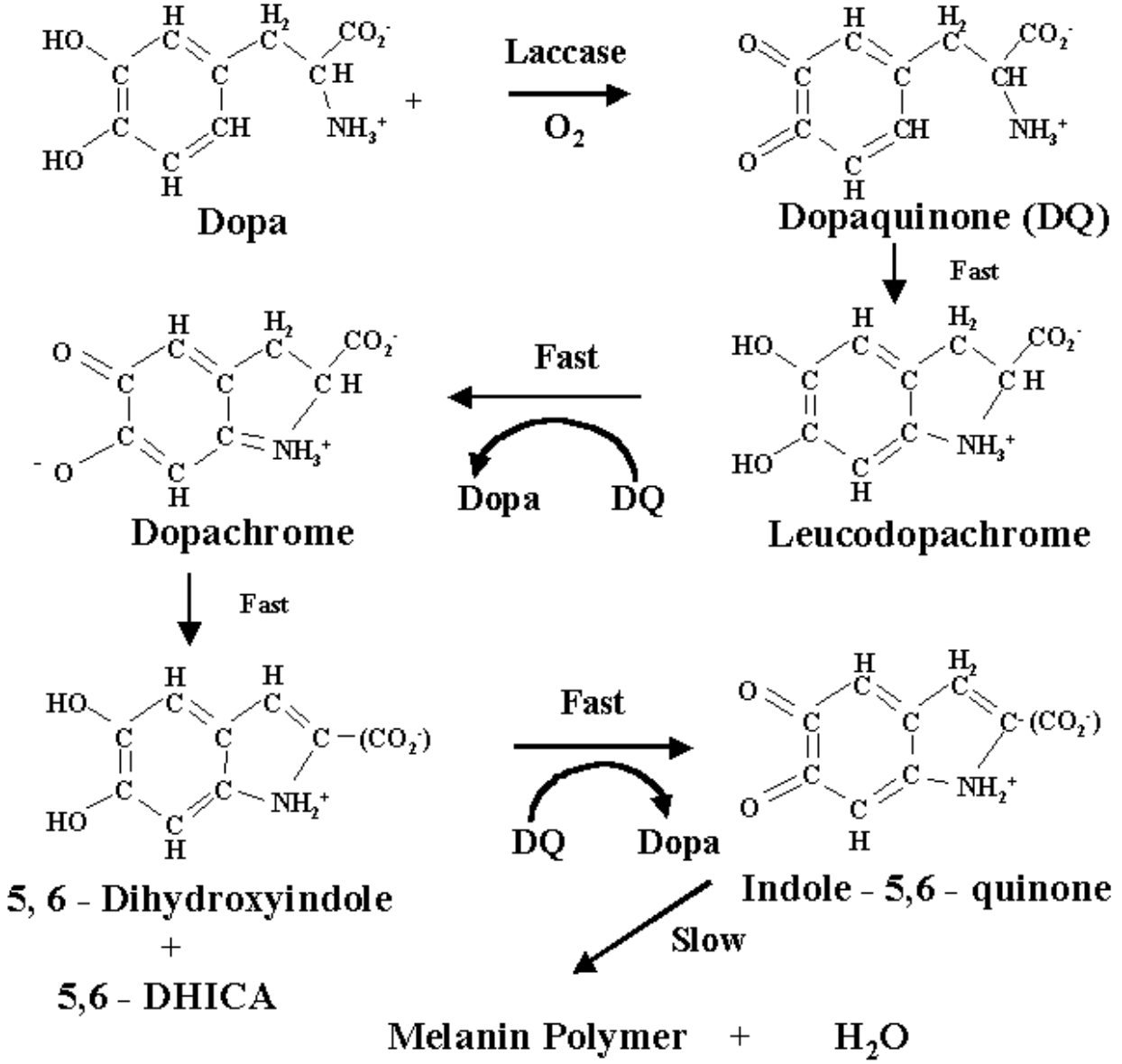

Fig. 1. Proposed melanin synthesis scheme in *Cryptococcus neoformans* adapted from the Mason-Raper model as modified by Ito (5, 6, 22).

chemical analyses of *Cryptococcus* melanin have not yet been made, any comparison to mammalian melanin is still tentative. Mammalian studies of melanin have emphasized the heterogeneity of melanin, whose structure is strongly dependent on substrates and the microenvironment in which it is synthesized (20). This would suggest that future structural and chemical studies will need to be directed toward analyzing melanin synthesized during pathogenesis or within organism's environment just prior to infection.

In vitro, pigment can be produced by *C. neoformans* using a wide variety of compounds including ortho- and para diphenols, amino phenols, and diaminobenzenes and catecholamines including 3-4, dihydroxyphenyl alanine (DOPA) and dopamine (5, 21). Based on this substrate specificity, and the well-known neurotropism of *Cryptococcus neoformans*, the Mason-Raper scheme for mammalian eumelanin synthesis has been adapted using the neurologic substrate DOPA. (10, 11, 22). According to this scheme (figure 1) DOPA undergoes a four electron oxidation by a phenoloxidase enzyme through a series of steps to form the somewhat stable intermediate, dopachrome. This intermediate then non-enzymatically decarboxylates to form 5,6-dihydroxyindole (DHI) and 5,6 dihydroxyindole carboxylic acid (DHCI), which then undergoes first a two electron oxidation to indole-5,6-quinone followed by a sequential polymerization to melachrome and then to melanin.

The above scheme is limited by assumptions which may or may not be true for *C. neoformans* within the mammalian host. For example, the Mason-Raper scheme is based on the mammalian enzyme, tyrosinase using only tyrosine as substrate. In contrast, phenol oxidase activity in *C. neoformans* has been shown to be a laccase by its copper content, broad substrate specificity, absorbance spectrum and sequence homology of its gene, *CNLAC1* (4). Laccases have a much broader substrate specificity than tyrosinase, oxidizing a multitude of phenolic and aminophenolic substrates. At least four principal diphenolic substrates are believed to be present in significant quantities in brain tissue (DOPA, norepinephrine, epinephrine and dopamine) as well as a variety of aldehyde and acid metabolites (23, 24). All of these could potentially be oxidized by laccase from *C. neoformans*. A mixture of laccase products, could polymerize to produce an extremely heterogeneous and unpredictable melanin structure within the mammalian host. Since melanin is thought to exert virulence through anti-oxidant properties (16-18) and this property varies with the type of polymerized substrate (25), differences in virulence could be expected, depending on variables (i.e. animal strain, nutritional status, drugs) that affect catecholamine metabolism in the host. Furthermore, some critical steps in melanin synthesis of *C. neoformans* may be significantly different from the mammalian model, and elucidation of these differences may provide additional targets for pharmacological intervention.

4. PHENOL OXIDASE ACTIVITY OF *C. NEOFORMANS*

Early studies of phenol oxidase activity of *Cryptococcus* focused on whole-cell assays relying on detection of visible pigment. Staib (6) first described this pigment to be unique to *Cryptococcus neoformans*, requiring a substrate found in *Guizotia abysinica* extracts. The wide substrate specificity of the enzyme was first described by Strachen *et al* (26) who isolated caffeic acid from Niger seed and found that ortho-diphenols produced colored products, but that tyrosine did not. Pulverer and Korth (27) found that para diphenols also produced this pigment. Only one of three m-diphenolic compounds were found to produce pigment, occurring only in the presence of iron. This enhancement by iron was also found with other substrates and has led to the routine incorporation of iron into ferric citrate agar used for the identification of *C. neoformans* (28, 29, 30, 31). Shaw and Kapica (21) found that oxygen was also a co-substrate and that the enzyme activity was inhibited by the copper chelators phenylthiourea and diethyldithiocarbamate which led the investigators to conclude the enzyme was copper dependent. They were also able to partially purify the enzyme by ammonium sulfate precipitation of glass bead-fractured cell lysates. However, the phenoloxidase activity was not further purified and could have contained a number of enzymes with oxidative activity although the inclusion of catalase precluded any contaminating peroxidase activity. Chaskes and Tyndall (5) widened the substrate specificity to include amino phenols and diaminobenzenes. Polacheck *et al* (32) isolated phenoloxidase activity from digitonin-solubilized *Cryptococcus*. In contrast to earlier data, copper chelators were not found to inhibit phenol oxidase activity. Instead, inhibition by the iron-chelators hydroximide and 8-hydroxyquinoline suggested the enzyme might be iron dependent. For a better understanding of melanin synthesis, Ikeda *et al*. (33) and Williamson (4) purified the enzyme from *C. neoformans*. Using purified enzyme to assess substrate specificity and enzyme inhibition patterns in the reaction mechanism is essential, since impurities in a crude preparation can unpredictably inhibit or stimulate a given reaction. Enzymatic studies may, in turn, identify important core chemical groups useful in the design of transition state or ground state inhibitors with pharmacological potential or may shed light on essential differences between melanin synthesis of humans and *Cryptococcus*. For example, while tyrosinase produces dopachrome by a sequential oxidation via dopaquinone and leucodopachrome, fungal laccases from other organisms such as *Coriolus versicolor* have been shown to oxidize phenolic substrates by a free-radical sequential one electron oxidation (34). Thus, development of anti-fungal agents directed against melanin in *C. neoformans* will require laccase derived from this pathogen.

Purified Cryptococcal laccase resembled that of the earlier crude enzyme preparations by its broad substrate specificity with the addition of hydroquinone that was not found to be oxidized in earlier studies (4). This may be explained by the fact that the oxidative product of hydroquinone has a very low molar absorptivity and may have been missed in absorbance measurements of earlier studies (32), whereas the more recent study followed consumption of oxygen as a measure of enzyme activity. Purified laccase was also shown by high performance liquid chromatography and mass spectroscopy to produce dopachrome from dopamine by (4). Physical characterization showed the enzyme to contain 4 mol/mol of copper by atomic absorption measurements and to have an absorbance at 610 nm and 320 nm, characteristic of type I and III copper of laccases. An important difference between Cryptococcal laccase and other fungal laccases is in its activity toward catechols having various side chains. While other fungal laccases such as that from *Agaricus* show a lack of activity toward catecholamines relative to catechol itself (35), the Cryptococcal enzyme is much more active toward catecholamines such as dopamine and DOPA than catechol. This activity is consistent with the role of Cryptococcal laccase in processing neurologic catecholamines during pathogenesis.

The structural gene of laccase, *CNLAC1*, has been cloned (4). Sequence analysis found a single copy gene located on chromosome 5-6 containing 14 introns which encodes a putative polypeptide of 624 amino acids. The large number of introns is a distinguishing feature of basidiomycetes like *Cryptococcus*, which is also consistent with its close relationship by rRNA gene sequences (36) to mushrooms like *Coriolus hirsutus* (37) and *Agaricus bisporus* (38), whose laccase also contains 10 and 14 introns, respectively. In contrast, ascomycete fungi like *Aspergillus* and *Candida* and *Histoplasma* rarely have introns in their genes. An other feature of cryptococcal genes (39, 40, 41) was exhibited by *CNLAC1* in that it does

not contain typical polyadenylation or termination signals. This is in contrast to the highly conserved polyadenylation and termination sequences of *S. cerevisiae* (42). The amino acid sequence of the Cryptococcal laccase shows a large amount of sequence diversity typical of fungal laccases with homology to other laccases only in its copper-binding regions (4). Sequence divergence may have allowed the evolution of binding sites necessary for the unique catecholamine substrate preference for laccase from this neurotropic pathogen. Sequence diversity in laccase may also be useful in the study of evolutionary similarity between closely related serotypes of *Cryptococcus neoformans*.

5. *CRYPTOCOCCUS NEOFORMANS* AND THE ROLE OF MELANIN IN VIRULENCE

Melanin production has been associated with virulence in a number of studies using classically derived mutants. In elegant studies by Kwon-Chung *et al*. (7), a mel$^-$ 92t-1 strain was produced from the Mel$^+$ strain, B-3502 by ultraviolet irradiation and then crossed to B-3501 to produce 3 mel$^-$ strains, sb7,8 and 31 and three Mel$^+$ strains, sb2,58 and 45. All six mel$^-$ strains were inoculated into Swiss Albino mice at inocula of 10^6 and 10^5 organisms. All three Mel$^+$ strains (sb2, 58 and 45) killed mice with a mean survival of 23, 46 and 29 days respectively for the 10^5 inocula. In contrast, none of the mice died after inoculation with either 10^5 or 10^6 cells of the Mel$^-$ strains. Clearance of *C. neoformans* from spleen, liver and brain was also evident with the Mel$^-$ sb8 strain, but not with the B-3501 Mel$^+$ strain. For example, a total of 10^8 organisms/g of brain tissue was found 24 days after inoculation with B-3501, while all sb8 organisms were cleared from the brain within 4 days. All strains tested appeared to have identical morphology and growth rates in rich media, but growth rate was significantly slower for all mel$^-$ mutants in minimal media.

In another study, Rhodes *et al* (8) used a spontaneous mel$^-$ mutant of B-3502 which was crossed to the Mel$^+$ B-3501 to produce 3 Mel$^+$ (Br 1, 2 and 3) and 3 mel$^-$ (w-2,3,5) isolates. the growth rates varied between isolates, but, as a whole, the average doubling time was similar between the two groups. When injected into mice, the cumulative mortality was greater in the Mel$^+$ than the mel$^-$ group. However, some mice did die with a large inoculum of 10^6 mel$^-$ organisms toward the end of the experiment. Perhaps the most interesting finding, however, was that a mixture of Mel$^+$ and mel$^-$ *Cryptococci* were recovered from the dead mice that were injected only with mel$^-$ strains. When two of these Mel$^+$ revertants were again injected into mice, virulence had been restored.

A subsequent study (9) examined the virulence in mice of a set of mutants derived from a cross of an acapsular, melanin deficient strain, B-4131, with the Cap$^+$, Mel$^+$ B-3502. Doubling time was similar between the two groups, ranging between 160 and 190 minutes, and again, all mel$^-$ mutants were less virulent after I.V. injection of 10^6 cells; most of the mice died within 35 days in the Mel$^+$ group but only 30% of the mel$^-$ group died by 70 days. Again, a spontaneous Mel$^+$ revertant, B-4181 (with an identical doubling time to its parent) had its virulence restored, killing all mice within 35 days.

The above studies, while highly suggestive of the role of melanin in virulence, were limited by a lack of bio-molecular characterization of the Cryptococcal strains. Virulence is a complex interplay of innumerable host and pathogen phenotypes dependent upon basic housekeeping functions such as growth and substrate utilization as well as features peculiar to the host-pathogen interaction such as melanin deposition. Multiple mutations introduced into structural genes during non-selective mutagenesis may be responsible for decreased virulence of Mel$^-$ strains. In addition, multiple undetected phenotypes may result if a mutation is within a single regulatory gene. These difficulties require that organisms used for virulence comparisons or studies of host-pathogen interactions be as genetically identical as possible except for a gene involved in producing only the phenotype of interest. Towards that end, recent developments in biomolecular techniques have furnished powerful tools to produce and characterize congenic sets. The recent isolation of the structural gene for laccase, *CNLAC1*, has allowed the construction and characterization of congenic sets of *C. neoformans*, differing only in the functional activity of *CNLAC1*. Salas *et al*. (43) produced a deletant *cnlac1* mutant, 10S derived from a pathogenic strain of *Cryptococcus*, B-4476. 10S was found to contain a 4 kb. deletion of the 5' end of the *CNLAC1* gene, resulting in undetectable transcripts and laccase activity. Complementing 10S with either a functional *CNLAC1* gene or the selection marker alone, *URA5* , produced a set of mutants, 10S-BUC (Mel$^+$) and 10S-BU (mel$^-$), respectively. In order to control for possible inadvertent mutations introduced during electroporation transformation, both isolates were backcrossed four times with the parent B-4476 (or its isogenic mate, B-4500) to remove any unintended mutations and produce the congenic Mel$^+$ and mel$^-$ set, 10S-BUC4 and 10S-BU4, respectively. Chromosomal patterns and growth rates of the two organisms were virtually identical. The mutant 10S-BU4 was found to have lost significant virulence in an I.V. mouse model, which was restored by complementation with functional *CNLAC1*. An additional set of congenic Mel$^+$ and mel$^-$ mutants were constructed by complementing a classically-derived mel$^-$ mutant produced by Torres-Guerrero *et al*. (44) with either intact *CNLAC1* or a selection marker alone, followed by backcrossing. Virulence of the Mel$^-$ strain was dramatically and significantly reduced from that of the Mel$^+$ strain, corroborating the results from the 10S series. These data strongly support the concept that laccase and/or its enzyme product(s) are responsible for the pigment-associated virulence demonstrated in earlier studies.

6. MOLECULAR MECHANISMS OF PATHOGENESIS

Mechanisms by which *Cryptococcus neoformans* organizes and regulates processes involved in virulence are not known. Melanin synthesis in *Cryptococcus* is an ideal model system for the study of molecular mechanisms of pathogenesis in eukaryotes, because virulence appears to depend on the product of a single structural gene

(*CNLAC1*) and the production of colored pigment by transcription of this gene makes screening for enhancer and repressor mutants facile.

Regulation of laccase was initially studied to optimize the identification of pathogenic pigmented *Cryptococcus*. Whole cryptococcal cells were used in earlier studies to determine which growth media would maximize pigment production in the greatest number of pathogenic clinical isolates. Nuruden and Ahearn (45) found that glucose was a strong repressor of the enzyme at concentrations as low as 0.55 mM for all strains of *C. neoformans*, but that nitrogen catabolite repression was strain and amino acid dependent. Glutamine repressed phenoloxidase activity of all isolates except serotype B and ammonium sulfate repressed all isolates except those of serotype A. More recently, Jacobson and Emery (46) found that there was also temperature regulation of phenoloxidase activity in all strains tested. Growth at 37^{o}C decreased enzyme activity by five fold from cells grown at 25^{o}C. Since no antibody was available for quantitation of laccase protein, it is difficult to determine if decreased activity at elevated temperatures was due to a change in laccase synthesis or a change in the specific activity of the enzyme. Since Shaw and Kapica (21) showed that phenoloxidase activity of cell extracts is maximal at 37-40^{o}C, temperature regulation of laccase is most likely mediated via enzyme synthesis.

With the purification of laccase and cloning of *CNLAC1*, new tools have become available to study the regulation of melanin synthesis in *Cryptococcus*. For example, melanin glucose repression was shown to be due to transcriptional repression of *CNLAC1* transcript, with maximal transcription occurring 2 hours after derepression and maximal enzyme activity within three hours (4). Sequencing of the gene shows that a TATA box is present in the *CNLAC1* sequence which can be used to define and isolate the transcription factor II-D complex (TFII-D). This complex has been shown to be important for transcriptional regulation in diverse organisms; its isolation will allow identification of trans-regulators produced by genes on distant chromosomes. Presently, work is underway in our laboratory to design recombinant promoter plasmids for *Cryptococcus* which can be used to identify and study the role of putative DNA-binding sites of other enhancer and repressor elements. *CNLAC1* itself is an ideal reporter plasmid, similar to the luciferase gene used in other diverse sequences, in that its product can be measured by visible absorbance measurements of dopachrome at 475 nm (32). *CNLAC1* has the further advantage as a reporter gene in that possible DNA-binding sites within its 14 introns can also be identified and studied.

7. THE MECHANISM OF MELANIN IN PROTECTION AGAINST IMMUNE EFFECTORS

Cryptococcus has been found to be inhibited by a variety of cells including alveolar macrophages (47), microglial cells (48), neutrophils and monocytes (49), NK cells (50), and astrocytes (51). Most work concerning the role of melanin in protection against such effectors has been performed with macrophages. Alveolar macrophages are believed to be the first line of defense in the lung (52). Macrophages can be seen by electronmicroscopy to contact the cell wall of *C. neoformans* by means of large pseudopodia followed by phagocytosis (53). If encapsulated organism are too large to be ingested by macrophages, a host cell inflammatory exudate forms from "histiocytic rings" (54) into multinucleated giant cells which require the participation of CD_4^+ T-cells (55).

One putative mechanism for melanin's virulence is based on it's ability to act as an efficient free-radical scavenger (25). Numerous studies have shown increased survival of Mel^+ vs. mel^- cryptococcal cells in the presence of nitrogen- (15) or oxygen-derived oxidants (16, 56). A classically-derived mel^- mutant has been shown to have decreased survival in murine macrophage-like J774.16 cells; an effect attributed to the anti-oxidant properties of melanin (15). However, the importance of the respiratory burst in Cryptococcal-macrophage interactions has been questioned by some (47). Large amounts of oxygen metabolites have not been detected on incubation of *C. neoformans* with activated peritoneal macrophages as compared to that with stimulation and alveolar macrophage killing of encapsulated *C. neoformans* was not reduced by respiratory burst inhibitors or scavengers (47).

A second possible mechanism proposed for macrophage evasion by melanin is by alteration in surface effects of the cell wall of the organism by charged side groups of the melanin polymer. This may explain differences in phagocytosis between Mel^+ and mel^- organisms when the organisms are pre-treated with the charged substrate DOPA (15). Recently, cellular charge was determined from electrochemical zeta potentials which showed that melanin produced from DOPA combines with capsular polysaccharide to increase the negative charge of the organism (57). These cell-wall effects may be important in initial binding of macrophages to *C. neoformans* and phagocytosis which occur by penetrating pseudopodia (53).

Other potential roles of melanin have been proposed including interfering with protective T-cell responses such as TNF-alpha secretion (58), reducing susceptibility to the antifungal agent Amphotericin B (59), and antibody-mediated phagocytosis (57). The laccase itself, or other enzyme products beside melanin, may also contribute to the pathogenesis of this organism.

8. MELANIN BIOSYNTHESIS AND THE ECOLOGICAL NICHE OF *CRYPTOCOCCUS*

C. neoformans consists of two varieties: *C. neoformans* var. *neoformans*, with serotypes A, D and AD and *C. neoformans* var. *gattii* with serotypes B and C (60-64). The two varieties differ in geographic distribution and habitat. *C. neoformans* var. *neoformans* has a worldwide distribution, whereas *C. neoformans* var. *gattii* occurs predominantly in the tropics and southern hemisphere. Virtually all U.S. AIDS-related *C. neoformans* infections are

caused by *C. neoformans* var. *neoformans* (65) with approximately 80% serotype A (66).

Establishing a predominant ecological niche for *Cryptococcus neoformans* var. *neoformans* may be important to strategies to control infection among AIDS patients. While recent data suggest that *C. neoformans* may colonize the respiratory epithelium of dogs and cats (67), the vast majority of isolates have been obtained from the environment. The pathogen is found in high titers from aged pigeon droppings and soil contaminated with avian excreta (68, 69) and nearly one half of AIDS-associated cases of one study of tropical Cryptococcosis in Burundi had an exposure to pigeon coops (70). In the restricted region of the Maltese Islands, LoPassa *et al* (71) showed that two clinical isolates resembled the majority of pigeon dropping environmental isolates by means of pulsed-filed gel electrophoresis (PFGE) and randomly amplified polymorphic DNA (RAPD) analysis. However, the role of pigeon exposure is believed by others to have been overemphasized (3). The lack of a consistent relationship between pigeon exposure and infection, either in endemic or epidemic outbreaks as well as the isolation of *C. neoformans* var. *neoformans* from other sources such as dairy products, soil, and rotting vegetables and fruit (72) raises questions regarding the most predominant source of pathogenic *C. neoformans*.

The presence of melanin in *C. neoformans* may also have a role in determining the environmental habitat and infectious origin of this organism since essentially all pathogenic *Cryptococci* are pigmented. Melanin is found in the lignolytic homo-basidiomycete *Coriolus hirsutus* which uses laccase to breakdown polyphenolic compounds during wood rotting (37); rRNA analysis relate *C. neoformans* closely to these lignolytic mushrooms (36). This role of laccase in wood pulp lignin degradation suggests that pigmented *C. neoformans* may be present as a tree-dweller and indeed, has been isolated from wood (72) and more recently from the moist hollowed-out rotting cavities of trees (73). In addition, the closely related *C. neoformans* var. *gatti* which causes Cryptococcal infections in non-AIDS patients in the Southern hemisphere, has been found in the flowering debris of red gum tree *Eucalyptus camaldulensis* (74). It is interesting that *Cryptococcus* is a common cause of meningitis in the tree-dwelling Kuala bear in Australia, accounting for 1% of all deaths (75). In addition, a recent outbreak of meningitis among tree shrews in the National Zoo in Washington, D.C. was attributed to *Cryptococcus neoformans* (76). Thus, while pigeon droppings may represent a focal source of high titers of the fungus, an additional reservoir may be a lower density of wood-borne organisms in wide distribution.

In addition, it is plausible that melanin protects *Cryptococcus* in its natural environment by its protective properties against U.V. irradiation (77) or from the oxidative stress of fungal aging (78). The anti-oxidative properties of melanin may also protect the organism in its natural environment from various fungus-eating free amoeboid organisms such as *Acanthamoeba polyphaga* which are thought to kill ingested organisms by oxidative mechanisms (79). Laccase and its product, melanin appears to have multiple roles in survival of the organism within the environment and in susceptible hosts, maintaining *C. neoformans* as as an important and continued threat to the immunologically impaired host.

9. ACKNOWLEDGMENTS

Appreciation is expressed to J.E. Bennett and K.J. Kwon-Chung for helpful discussions and R. Tewari for manuscript review. Supported, in part, by United States Public Health Service Grant NIH-AI38258-01A1 from the National Institute of Allergy and Infectious Diseases.

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