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Catecholamines and Virulence of *Cryptococcus neoformans*

ITZHACK POLACHECK,[1]* YEHUDIT PLATT,[1] AND JACOB ARONOVITCH[2]

Department of Clinical Microbiology, The Hebrew University-Hadassah Medical Center, P.O. Box 12000, Jerusalem 91120,[1] and Department of Molecular Biology, The Hebrew University–Hadassah Medical School, Jerusalem 91010,[2] Israel

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***Cryptococcus neoformans* was unable to utilize catecholamines (epinephrine, norepinephrine, or dopamine) as sole carbon or nitrogen sources. Therefore, catecholamines are not essential growth factors for this fungus and the brain is not a preferred nutritional niche for its growth with regard to catecholamines. To establish whether the brain is a survival niche for *C. neoformans* and to explain the role of phenoloxidase as a virulence factor, a wild-type strain that had phenoloxidase activity and mutants which lacked it were exposed to an epinephrine oxidative system, and the survival of both strains was tested. The oxidative system contained epinephrine as an electron donor, Fe^{3+} as the catalytic transition metal ion, and hydrogen peroxide as an electron acceptor. The wild-type strain was found to be resistant to this oxidative system, whereas under the same conditions the mutant strain was susceptible and its survival decreased at a rate of 4 logs per h. Damage to high-molecular-weight DNA seems to be a causative factor of cell death after exposure of the mutants to the oxidative system. These results suggest that *C. neoformans* may survive in the brain because of its ability to utilize catecholamines for melanogenesis and thus neutralize the harmful effects of catecholamines which are manifested in the presence of hydrogen peroxide and transition metal ions. The role of phenoloxidase in resistance to the epinephrine oxidative system is also discussed.**

Cryptococcus neoformans is a pathogenic fungus which grows preferentially in the brains of humans and experimental animals (5). Recent reports have shown that in the United States, 8.6% of patients with acquired immunodeficiency syndrome developed cryptococcosis (28) and that in African acquired immunodeficiency syndrome cases, it is one of the most commonly observed opportunistic infections (20). The factors which enhance the growth of this fungus in the brain remain unknown. *C. neoformans* has an efficient system for the utilization of catecholamines that comprises a specific transport system and the membrane-bound enzyme phenoloxidase, which can produce melanin from these compounds through a series of oxidation-reduction reactions (17, 18). Melanin formation was demonstrated to be an essential virulence factor in *C. neoformans* (12, 13, 21). Melanin-negative mutants which lacked phenoloxidase activity lost their virulence, and coreversion of the melanin phenotype and virulence has been observed (12, 13, 21). A special histopathological stain (11, 22) was used to show that melanin is produced by this fungus in the brain.

In the present study, we tried to establish whether catecholamines are used by this fungus as essential nutrients or whether their utilization permits survival of the fungus under conditions that might develop in the brain. In order to explain the role of phenoloxidase as a virulence factor in *C. neoformans*, we describe an oxidative system containing catecholamine that can efficiently kill yeast cells, and we present data which suggest that phenoloxidase may play a protective role against oxidative damage.

MATERIALS AND METHODS

Strains, media, and growth conditions. The following strains were used: wild-type strain *C. neoformans* B-3501 (mel^+) (12), which has phenoloxidase activity, a catecholamine uptake system, and the ability to produce melanin; the mutant strains *C. neoformans* Sb-26 (mel^-) and W-5 (mel^-) (21), which are melanin negative and avirulent and lack phenoloxidase activity; and the mutant strain Sb-7 (mel^-) (12), which is melanin-negative and nonvirulent and lacks phenoloxidase activity and the catecholamine uptake system. These cultures were maintained on Sabouraud dextrose agar (SDA).

A loopful of cells cultured on SDA for 24 to 48 h at 30°C was suspended in 50 ml of yeast nitrogen base broth (YNB; Difco Laboratories, Detroit, Mich.) containing 20 g of glucose and 1 g of L-asparagine (as a nitrogen source) per liter and 10 mM phosphate buffer (pH 7). After growth for 24 h on a shaker (100 oscillations per min) at 30°C, 0.2 ml of cells was transferred to a fresh medium for further growth for 20 h. An inoculum from this culture was transferred to a fresh medium to give a yeast concentration of 10^4 cells per ml. This culture yielded 2×10^7 to 3×10^7 cells per ml after 20 h at 30°C (early exponential phase).

Catecholamines as a nutrient source for *C. neoformans*. *C. neoformans* B-3501 was grown for 48 h on SDA and was then washed three times in double-distilled water (1,800 × *g*, 5 min at room temperature). An inoculum of 5×10^5 cells per ml was suspended in 20 ml of yeast carbon base broth (YCB; Difco) containing 10 g of glucose per liter and 10 mM phosphate buffer (pH 7). The catecholamines epinephrine bitartrate, norepinephrine bitartrate, and dopamine (all purchased from Sigma Chemical Co., St. Louis, Mo.) in a final concentration of 1 g/liter were tested for the ability to serve as a nitrogen source. The experiment was performed in glass tubes incubated on a rotary shaker at 30°C. The cultures were tested after 24 h. If no visible growth was detected, they were incubated for a further 24 h for confirmation before being called negative. The positives were reinoculated into a fresh medium of the same composition for an additional 24 h. The same catecholamines in a final concentration of 10 g/liter were also tested for the ability to serve as a sole carbon source. The growth medium was YNB-asparagine agar. When epinephrine was used, it was dissolved in

* Corresponding author.

0.5 N HCl and the medium contained 100 mM phosphate buffer (pH 7). After being washed as described above, cells were inoculated on plates and incubated for 48 h at 30°C.

Oxidative system. The oxidative system contained 1 mM epinephrine bitartrate which was freshly prepared from a 10×-concentrated stock solution in triple-distilled water, 1 mM H_2O_2 (Aldrich Chemical Co., Inc., Milwaukee, Wis.) taken from a freshly prepared 100×-concentrated stock solution, and 0.5 mM Fe^{3+} in 50 mM sodium acetate (pH 5.5) containing 1 mM $MgSO_4$. The ferric ammonium sulfate was prepared as 100×-concentrated stock solutions in 50 mM sodium acetate buffer (pH 5.5).

In addition to free ferric ions, we tested the activities of the following Fe^{3+} complexes: (i) Fe^{3+}-EDTA, (ii) Fe^{3+}-ADP, and (iii) ferric nitrilotriacetic acid (Fe^{3+}-NTA). Complexes 2 and 3 were prepared by dissolving the chelators in water to a final concentration of 11 mM. Ferric ammonium sulfate [$Fe^{3+}NH_4(SO_4)_2$] in a concentration of 10 mM was then dissolved in 1 mM sulfuric acid. Equal volumes of the two solutions were mixed together until the molar ratio 1:1.1 of Fe^{3+} to chelator was achieved and the final concentration of the ferrum was 5 mM. This solution was 10 times the concentration of the final reaction mixture (0.5 mM).

Survival of yeast cells in the oxidative system. Yeast cells grown under the conditions described above were washed twice (10 min, 17,000 × *g* at 4°C), and suspended in 50 mM sodium acetate (pH 5.5) containing 1 mM $MgSO_4$ that was used as the reaction buffer. The cells were kept on ice until used. They were then incubated for 10 min at room temperature and then for a further 7 to 10 min at 37°C before the addition of the reagents. The final volume of the reaction mixture was 2 ml. The reagents were then added in the following order: transition metal, hydrogen peroxide, and reducing agent. Samples (100 μl) were then taken periodically and transferred into 0.9 ml of dilution buffer (1 mM phosphate buffer [pH 7.4] containing 1 mM $MgSO_4$, 0.1 mM EDTA, and 5 μg of catalase per ml) at 4°C to stop the reaction. A sample taken before the reagents were added was used as a control. Each sample was serially diluted in 10-fold dilutions to 10^{-5}, and from each dilution, 3 drops of 10 μl each were inoculated on SDA medium. The plates were incubated for 48 h at 30°C, and CFUs were determined by a colony counter.

Genomic DNA preparation. Genomic DNA was prepared from protoplasts by our recently published method for chromosomal DNA extraction (19), modified as follows. After protoplast lysis, the DNA was extracted with phenol and then treated with RNase followed by proteinase K. After an additional phenol extraction, the DNA was stored at 4°C in Tris-EDTA buffer.

Electrophoresis of DNA in agarose gel. DNA in loading solution (running buffer containing bromphenol blue, xylenecyanol, and 30% glycerol) was run on a 1% agarose gel. The running buffer was TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA [pH 8.0]), and the running conditions were a 25-mA constant current overnight. The gel was stained with TBE buffer containing ethidium bromide (10 μg/liter) for 15 min.

RESULTS

Catecholamines as nutrients for the growth of *C. neoformans* B-3501. No growth was observed after 5 days in any of the media containing one of the catecholamines (epinephrine, norepinephrine, or dopamine) as a source of carbon. A control culture in a medium containing glucose as the sole carbon source was positive. No growth was detected after 48 h in the media containing these catecholamines as a nitrogen source, but a control containing asparagine was positive.

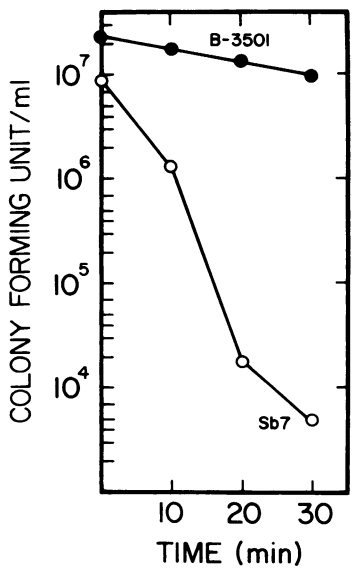

FIG. 1. Survival of the *C. neoformans* wild-type strain (●) and the melanin-negative mutant strain (○) in the epinephrine oxidative system.

Survival of *C. neoformans* in the epinephrine oxidative system. In order to establish whether the brain is a survival niche for *C. neoformans*, the wild-type strain that had phenoloxidase activity and the mutants which lacked this activity were exposed to epinephrine oxidative systems with various Fe^{3+} complexes. The wild-type strain *C. neoformans* B-3501, the melanin-negative mutants W-5 and Sb-26 (both lacking phenoloxidase activity), and the mutant Sb-7 (which, besides lacking phenoloxidase activity, lacks the catecholamine transport system) were exposed for 1 h to the oxidative systems. In all systems, except that which contained Fe^{3+}-EDTA, a decrease in viability was observed in the mutant strains, whereas the wild-type strain was resistant. When ferric ammonium sulfate was used, a decrease of 1 order of magnitude was observed with the wild type. The decrease was of 2 orders of magnitude with the mutant strains W-5 and Sb-26 and 3 orders of magnitude with Sb-7. In the system containing Fe^{3+}-ADP, this decrease was observed within 30 min in the mutant strain Sb-7 (Fig. 1). All three components were necessary for killing, and no killing was found in the controls that contained only two reagents.

Effect of the oxidative system on *C. neoformans* DNA. *C. neoformans* B-3501 and Sb-7 were treated for 1 h in an oxidative system containing epinephrine, hydrogen peroxide, and ferric ammonium sulfate. High-molecular-weight DNA was prepared from these cells. The DNA from the wild-type strain appeared after agarose gel electrophoresis as a single clear band representing high-molecular-weight DNA (Fig. 2). The smear which was obtained with Sb-7 DNA (Fig. 2, right lane) indicated a high extent of degradation of the mutant DNA. Appropriate controls of DNA isolated from the wild-type and mutant strain cells that had not been exposed to the oxidative system looked uncut and appeared similar to the band from B-3501 DNA (Fig. 2). When extracellular DNA was exposed to this system, total degradation of both wild-type DNA and mutant DNA was observed (data not shown).

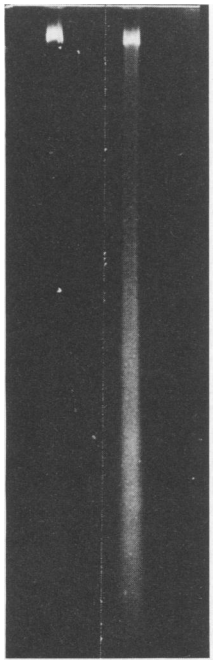

FIG. 2. High-molecular-weight DNA from *C. neoformans* after 1 h of treatment in the epinephrine oxidative system. Results of agarose gel electrophoresis of DNA from the wild-type strain B-3501 (left) and from the melanin-negative mutant Sb-7 (right) are shown.

DISCUSSION

We tried to establish whether catecholamines are used by *C. neoformans* as an essential nutrient source or if catecholamine utilization permits survival of this fungus under conditions that might develop in the brain which is rich in catecholamines (17), i.e., whether the brain constitutes a preferential nutritional niche for growth or a survival niche for this fungus.

The finding that *C. neoformans* was unable to utilize catecholamines (epinephrine, norepinephrine, or dopamine) as a sole carbon or nitrogen source negates the possibility that the brain is a preferred nutritional niche for *C. neoformans* growth, with regard to catecholamines.

To establish whether the brain is a survival niche for *C. neoformans*, the wild-type strain that had phenoloxidase activity and mutant strains which lacked this enzymatic activity were exposed to an oxidative system containing epinephrine. The oxidative system was analogous to other oxidative systems which produce oxygen-derived free radicals by the metal-catalyzed Haber-Weiss reaction (1, 24). Fe^{3+} was chosen as a transition metal ion because of its physiological importance, its high level in cells, and its availability for in vivo redox reactions (9). When Fe^{3+} was substituted for Cu^{2+}, no activity was observed under the same conditions with the catecholamine-driven Fenton reaction (I. Polacheck, Y. Platt, and J. Aronovitch, submitted for publication). However, production of hydroxyl radicals in other catecholamine oxidative systems catalyzed by copper ions caused erythrocyte lysis (14) and bacterial death (J. Aronovitch, D. Godinger, and G. Czapski, Free Radicals Res. Commun. in press). Fe^{3+} precipitates at physiological pH; therefore, soluble complexes such as Fe^{3+}-EDTA were used in order to increase Fe^{3+} solubility.

Wild-type strain *C. neoformans* B-3501, which can produce melanin on bird seed agar (17), and melanin negative mutant strains Sb-26, W-5, and Sb-7 (12, 21) were tested for susceptibility to oxidative systems containing catecholamines. The mutant Sb-7, which lacks phenoloxidase activity and the specific catecholamine uptake system (12), was the most susceptible to the systems described above; its survival decreased at a rate of 4 logs/h (Fig. 1). The mutant strains Sb-26 and W-5, which lack phenoloxidase activity, were less susceptible (a decrease in cell survival of 2 logs/h) and the wild-type strain B-3501 was relatively resistant to the oxidative system. All the strains were resistant when Fe^{3+}-EDTA was used as a ferric complex, although EDTA is known to facilitate the Haber-Weiss reaction (8, 10, 15, 23). When Fe^{3+}-NTA and Fe^{3+}-ADP were used, high levels of fungicidal activity were observed. ADP and NTA are chelators known for their high specificities to Fe^{3+} ions, although the linkage to Fe^{3+} is weak, and the ferric ion is easily released (15, 25). When these two complexes were used, strain B-3501 was almost totally resistant after 1 h of treatment, but strain Sb-7 was highly susceptible. The greatest effect was observed with Fe^{3+}-ADP (Fig. 1).

This difference in susceptibilities to the oxidative system was also observed at the DNA level. After exposure of the mutant Sb-7 to the system described above, extensive DNA degradation occurred, whereas most of the DNA in the wild type could still be seen as high-molecular-weight DNA after 1 h of treatment (Fig. 2). The rate of DNA damage, monitored as single-strand DNA breaks and measured after alkaline denaturation of the DNA, was higher than that of killing (data not shown), which suggests that DNA damage is one of the causes of cell death rather than the result of cell death. When isolated soluble DNA was used, total degradation was also observed, indicating that DNA damage was caused by catecholamine oxidation products and not by nuclease activation in the yeast cells. The chromosomal DNA seems therefore to be susceptible to in vivo cleavage by the oxidative system, and this probably explains the observed difference in susceptibilities between the wild-type and the mutant strains, as expressed in cell death.

These findings indicate a relationship between the production of melanin because of phenoloxidase activity and resistance to the catecholamine oxidative system. The enzyme phenoloxidase played a role in this resistance, probably by one or a combination of the following mechanisms: (i) phenoloxidase may have used the catecholamines for melanin synthesis, so that catecholamines were not available for the oxidative reaction in the presence of hydrogen peroxide; (ii) melanin, which is a final product of the enzyme reaction, may have served as a scavenger of the free radicals produced by the oxidative system (7).

It is clear that melanin production is a very important virulence factor in *C. neoformans*, as demonstrated by in vivo experiments (12, 13, 21). Specific staining showed that melanin can be synthesized by this fungus in the brain (11, 22). Whether the oxidative system which we have used can operate in the brain in vivo is an open question. All the components of the system are available in the brain. Hydrogen peroxide can be produced by oxidation of catecholamines in the presence of oxygen or via oxidative burst during inflammation; Fe^{3+}, in its bound form, is found under physiological conditions. Moreover, there is a dynamic equilibrium between bound and free forms of Fe^{3+}, which allows its availability for redox reactions (9). The physical conditions used in vitro can be found in vivo, i.e., temperature of 37°C and low pH, which is found under inflammatory conditions (3). It is therefore possible that the oxidative conditions similar to the conditions described above occur in

the brain. The wild-type strain of *C. neoformans* could therefore survive under these oxidative conditions, whereas melanin-lacking mutant strains could not.

Catecholamines may serve either as sources of damaging radicals or as radical scavengers, depending on the conditions (2). It is therefore plausible that the relatively high resistances of the phenoloxidase mutants which retained the catecholamine transport system (Sb-26, W-5), compared with that of the transport-deficient double mutant (W-5), were due to the protective effect of intracellular catecholamines. The accumulation of catecholamines increased the reductive capacity of cells and enhanced their resistance to oxidative stress.

If the DNA damage observed is caused by a localized site-specific mechanism, then permeation by the components into the cell followed by binding of at least one component to the DNA must occur. Permeation by Fe^{3+} can take place via siderophores or other iron uptake systems which are present in fungi (4, 26, 27). It is also known that metal ligands enable the transfer of metal through the cell wall or the membrane (6). The uptake of epinephrine in the yeast *C. neoformans* is mediated by a specific uptake system (12, 17). Hydrogen peroxide diffuses easily through the cell membrane (9). We do not yet know whether Fe^{3+} or epinephrine is linked to the DNA. However, intermediates in melanin biosynthesis which have a structure similar to that of catecholamines can bind to DNA and modify it (16), and epinephrine-copper forms ternary complexes with DNA (J. Aronovitch, D. Godinger, and G. Czapski, Free Radicals Res. Commun., in press). It is therefore possible that epinephrine-iron forms tertiary complexes with DNA.

The results presented suggest that *C. neoformans* may survive in the brain because of the ability to utilize catecholamines for melanogenesis, and thus *C. neoformans* neutralizes or prevents the harmful effects of catecholamines when the catecholamines are oxidized in the presence of metal ions and hydrogen peroxide.

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