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# Superoxide Dismutase Influences the Virulence of *Cryptococcus neoformans* by Affecting Growth within Macrophages

Gary M. Cox,[1]* Thomas S. Harrison,[2] Henry C. McDade,[1] Carlos P. Taborda,[3] Garrett Heinrich,[1] Arturo Casadevall,[3] and John R. Perfect[1]

*Departments of Medicine and Molecular Genetics and Microbiology, Duke University Medical Center, Durham, North Carolina[1]; Department of Infectious Diseases, St. George's Hospital Medical School, London, United Kingdom[2]; and Departments of Medicine and Microbiology and Immunology, Albert Einstein College of Medicine of Yeshiva University, Bronx, New York[3]*

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**Superoxide dismutase (SOD) is an enzyme that converts superoxide radicals into hydrogen peroxide and molecular oxygen and has been shown to contribute to the virulence of many human-pathogenic bacteria through its ability to neutralize toxic levels of reactive oxygen species generated by the host. SOD has also been speculated to be important in the pathogenesis of fungal infections, but the role of this enzyme has not been rigorously investigated. To examine the contribution of SOD to the pathogenesis of fungal infections, we cloned the Cu,Zn SOD-encoding gene (*SOD1*) from the human-pathogenic yeast *Cryptococcus neoformans* and made mutants via targeted disruption. The *sod1* mutant strains had marked decreases in SOD activity and were strikingly more susceptible to reactive oxygen species in vitro. A *sod1* mutant was significantly less virulent than the wild-type strain and two independent reconstituted strains, as measured by cumulative survival in the mouse inhalational model. In vitro studies established that the *sod1* strain had attenuated growth compared to the growth of the wild type and a reconstituted strain inside macrophages producing reduced amounts of nitric oxide. These findings demonstrate that (i) the Cu,Zn SOD contributes to virulence but is not required for pathogenicity in *C. neoformans*; (ii) the decreased virulence of the *sod1* strain may be due to increased susceptibility to oxygen radicals within macrophages; and (iii) other antioxidant defense systems in *C. neoformans* can compensate for the loss of the Cu,Zn SOD in vivo.**

Invasive fungal infections in humans are increasing in prevalence in parallel with the growing population of immunocompromised patients. There is a need for new antifungal drugs to treat these infections since the drugs currently available are either excessively toxic or lack broad fungicidal properties. Studies on the pathogenesis of fungal infections should provide insights that can help with the diagnosis and treatment of these important human diseases. *Cryptococcus neoformans* is a basidiomycetous yeast that has been used successfully as a model pathogenic fungus in a variety of molecular pathogenesis studies. We used *C. neoformans* to evaluate the contribution of superoxide dismutase (SOD) to the pathogenesis of fungal infections.

SODs are metalloenzymes that detoxify oxygen radicals through the conversion of superoxide to hydrogen peroxide and oxygen (20). These enzymes are present in virtually all cells, and this very high degree of conservation is testament to their importance in cellular homeostasis. The primary role of SODs is to protect cells from endogenously generated superoxide anion, which is a by-product of normal aerobic respiration. SODs can be complexed with iron, manganese, and copper plus zinc. The iron and manganese SODs are genetically similar to each other, whereas the Cu,Zn SOD exhibits no significant homology with the other two enzymes (16, 20, 21, 32). Eukaryotic cells generally contain an Mn SOD in the mitochondrial matrix and a Cu,Zn SOD which is located predominantly in the cytoplasm and to a lesser extent in peroxisomes (9, 28).

In addition to superoxide resulting from endogenous production, human-pathogenic organisms are exposed to reactive oxygen species generated by phagocytic cells. After phagocytosis by polymorphonuclear cells or macrophages, pathogens in the phagolysosomes are exposed to a variety of toxins, including superoxide. Superoxide anions are generated via the oxidative burst in activated immune cells by enzymes that transfer electrons from cytosolic NADPH to molecular oxygen. For some bacteria, SOD has been shown to play a role in virulence when the organisms have been tested in animal models, and it has been thought that the decreased virulence of SOD mutant strains was due to increased susceptibility to host phagocytic cells (32, 35). The role of SOD in the pathogenesis of fungal infections is not clear. Biochemical characterization of the *C. neoformans* Cu,Zn SOD has been done (23), and the Cu,Zn SOD gene has been cloned from three *C. neoformans* varieties (10). There has also been a suggestion that the cryptococcal SOD has antioxidant properties (23, 24, 26). We initially identified the Cu,Zn SOD in a screening for genes differentially regulated by temperature in *C. neoformans*, and we decided to study the contribution of this gene to pathogenesis using a molecular approach. The rationale for studing the Cu,Zn SOD instead of the Mn SOD is that the Cu,Zn SOD is the much more abundant form of the enzyme, and the cytoplasmic location was thought to be more relevant for possible protection against phagocyte-derived reactive oxygen species. We made specific mutants using targeted gene disruption, and we show

* Corresponding author. Mailing address: Box 3281, Duke Medical Center, Durham, NC 27710. Phone: (919) 681-5055. Fax: (919) 684-8902. E-mail: gary.cox@duke.edu.

below that one of the mutants is less virulent than both the wild-type and reconstituted strains. (Portions of this work were presented at the 101st American Society for Microbiology General Meeting, May 2001, and at the Fifth International Conference on Cryptococcus and Cryptococcosis, March 2002.)

## MATERIALS AND METHODS

**Strains and media.** *C. neoformans* strain H99 (serotype A, Matα) and strain H99R (a spontaneous *ura5* auxotroph derived from H99 by plating on 5-FOA agar) were recovered from 15% glycerol stocks stored at −80°C prior to use in the experiments described below. The strains were maintained on YPD media (1% yeast extract, 2% peptone, 2% dextrose) and were tested on minimal media (YNB media without amino acids and 0.5% dextrose). Transformants were selected on ura dropout media containing 1 M sorbitol (14, 15), and reconstituted strains were selected on YPD media supplemented with 100 μg of nourseothricin (clonNAT; Werner Bioagents, Jena, Germany) per ml as described previously (33). Strains were tested on YNB media containing 1 to 50 mM *tert*-butyl hydroperoxide, 1 to 50 mM paraquat (methyl viologen), 100 to 1,000 μg of oxytetracycline per ml, 10 to 50 mM $FeSO_4$, and 1 mM $CuSO_4$ (all obtained from Sigma). Dopamine agar (15) and egg yolk agar (15) were made as described previously. Urease activity was measured grossly after growth on Christensen's agar as described previously (14).

**Isolation of the SOD gene.** A subtractive cDNA library with differential PCR amplification (PCR Select; Clontech) was used to select for cDNA preferentially expressed at 37°C versus 25°C. Briefly, yeast strain H99 was grown in either YPD or YNB broth for 1, 4, 8, and 24 h at either 25 or 37°C in a shaking incubator. Total RNA from yeast grown under each type of conditions was isolated by using Trizol reagent (Life Technologies), and the RNA from preparations incubated at each temperature was pooled and used for differential PCR amplification performed according to the manufacturer's protocol. Clones from the pool of cDNAs preferentially expressed at 37°C versus 25°C were screened for the intensity of hybridization by using labeled total RNA from H99 cells grown at the two temperatures and pulsed with [$^{32}$P]dATP. One of the cDNA clones that exhibited approximately threefold-greater hybridization with the labeled RNA from the yeast grown at 37°C than with the labeled RNA from yeast grown at 25°C was sequenced, and the sequence was used to search the GenBank database. The cDNA clone was found to have significant homology with Cu,Zn isoforms of SOD and was used to probe genomic and cDNA libraries to obtain the entire locus. A 3.1-kb *Kpn*I genomic fragment was cloned into a plasmid and sequenced. The sequence of the genomic fragment was compared to the sequences of the cloned cDNAs in order to locate the coding sequence and introns. The gene was designated *SOD1*.

**Disruption and reconstitution of *SOD1*.** The 3.1-kb genomic fragment was used to make a disruption construct by digestion with *Bst*EII and insertion of a 1,950-bp genomic fragment containing *URA5* into this single site after a fill-in reaction with DNA polymerase (Fig. 1A). The plasmid containing the disruption construct was used to transform *ura5* strain H99R by using biolistic delivery as described previously (14, 15). Stable prototrophic transformants were analyzed by using colony PCR and primers flanking the *URA5* insertion site (Fig. 1A). Disruption of the native *SOD1* gene was confirmed by using Southern blots probed with a labeled *SOD1* cDNA fragment. A reconstitution construct was created by inserting the nourseothricin resistance cassette into a *Not*I site in the plasmid containing the 3.1-kb *SOD1* genomic fragment. The reconstitution construct was used to transform one of the *sod1* mutant strains by using selection with nourseothricin. Both PCR and Southern analyses were used to confirm restoration of the wild-type *SOD1*.

**SOD assay.** SOD activity was assayed by using a standard colorimetric assay in which xanthine oxidase serves as a free radical generator and causes the reduction of nitro blue tetrazolium (NBT). The reduced NBT can be assayed by absorbance at 560 nm. SOD inhibits the reduction of NBT by scavenging the free radicals generated by the xanthine oxidase. After validation of the assay by using purified bovine erythrocyte SOD (Sigma), protein extracts from cryptococci were tested for SOD activity and compared to controls containing equivalent amounts of bovine serum albumin. Approximately $10^8$ yeast cells were vortexed for 5 min in 1 ml of ice-cold 50 mM potassium phosphate (pH 7.8) containing 0.5 g of 500-μm-diameter glass beads. The homogenates were centrifuged at 4°C, and the supernatants were assayed for protein content by the Lowry method (Sigma); 10 and 100 μg of total of protein were used in the assays. The homogenates were immediately assayed for SOD activity by mixing them with xanthine, xanthine oxidase, and NBT in a 3-ml (total volume) reaction mixture. Absorbance at 560 nm was monitored for 30 min. The data are expressed below as percentages of the absorbance of the control sample. All assays were repeated twice with three independent protein homogenates, and the data were pooled for analysis with Student's *t* test.

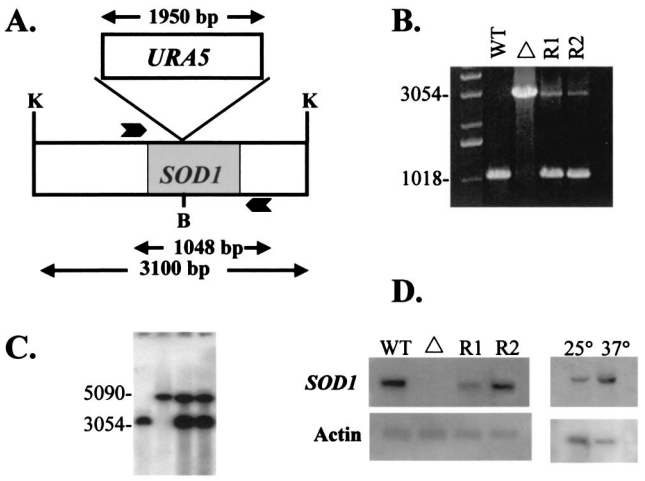


FIG. 1. (A) Map of the *Kpn*I (K) genomic fragment containing *SOD1*. The *URA5* gene was inserted into the *Bst*EII site (B) of *SOD1* in order to create a disruption construct. Sites of the PCR primers used to verify disruption are indicated by solid arrowheads, and the sizes of the relevant pieces of DNA are also indicated. (B) PCR analysis of genomic DNA from the wild-type strain (WT), the *sod1* strain (△), and two independent reconstituted strains (Rec1 [R1] and Rec2 [R2]) performed with primers indicated in panel A. Disruption of the native *SOD1* was indicated by the single amplicon at approximately 3 kb for the *sod1* strain, and ectopic reconstitution for the Rec1 and Rec2 strains was indicated by amplification of both the native and disrupted versions of *SOD1.* (C) Southern blot of genomic DNAs from the same four strains (in the same order as in panel B) that were digested with *Kpn*I and probed with a labeled *SOD1* cDNA. The results show the expected displacement of the native gene to 5,050 bp in the *sod1* strain and restoration of the wild-type loci at 3,100 bp in the two reconstituted strains. (D) Northern blot of total RNA from the wild-type (WT), *sod1* (△), Rec1 (R1), and Rec2 (R2) strains grown in YPD broth overnight at 30°C and total RNA from wild-type yeast after growth in YPD broth at 24 and 37°C for 3 h. Labeled actin and *SOD1* cDNA fragments were used to probe the blot. Quantitation of the *SOD1* signal with a phosphoimager and with actin hybridization as a control confirmed that there was a 3.1-fold increase in the intensity of the hybridization signal at 37°C compared to the intensity of the hybridization signal at 24°C.

**Phenotypic assays.** Quinacrine staining of the yeast was performed as described previously (13) by pelleting logarithmically growing cells and suspending them in YPD broth containing 50 mM $NaHPO_4$ and 200 μM quinacrine with the pH adjusted to 7.5. The cells were incubated for 5 min at 30°C, washed in phosphate-buffered saline (PBS), and viewed on slides with a fluorescent microscope. Freeze-thaw sensitivity was analyzed as described previously (34). Briefly, logarithmically growing cells were washed in PBS, and the cell density was adjusted to $10^5$ cells/ml in PBS. The cell suspensions were frozen at −20°C for 24 h, thawed at 4°C for 40 min, and then diluted to prepare quantitative cultures on YPD medium plates. The experiments were done in triplicate, and the results were compared to the results for quantitative cultures obtained from the same samples just prior to freezing. Cells were grown in the presence of 100% $O_2$ by suspending them in PBS in open tubes which were then placed in an air-tight container hooked up to a vacuum pump. After vacuum evacuation of the air, pure oxygen was released into the container via a one-way valve from an oxygen tank, and the cycle of vacuum evacuation and replacement with oxygen was repeated every 3 to 4 days. The cultures were kept under these conditions for 4 weeks, as described previously (31), and then quantitated by spreading aliquots onto YPD medium plates. Cells were tested for sensitivity to oxygen radicals with a cell-free assay in which epinephrine was used as an electron donor (36). Cells were grown in YPD medium overnight, washed three times in PBS, and then

suspended in 50 mM sodium acetate (pH 5.5)–1 mM $MgSO_4$. Ferric ammonium sulfate, hydrogen peroxide, and epinephrine bitartrate were sequentially added to final concentrations of 0.5 mM, 0.0002%, and 1 mM, respectively, to a 1-ml (final volume) reaction mixture. Aliquots were removed at various times for quantitative culture on YPD agar. Superoxide levels in the cell-free assay mixtures were measured, as described previously (39), by adding 200 μl of the reaction mixture to 1.8 ml of HEPES-cytochrome *c* buffer (17 mM HEPES [pH 7.3], 120 mM NaCl, 5 mM glucose, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 100 mM cytochrome *c*). The mixture was incubated at 37°C for 30 min, and the absorbance at 550 nm and the absorbance at 540 nm were determined with a spectrophotometer. The amount of superoxide anion generated was calculated by using the following formula: $(A_{550} - A_{540}) \times 1{,}000/19.1$.

**Murine model.** Cryptococci were used to infect 4- to 6-week-old female A/Jcr mice (NCI/Charles River Laboratories) by nasal inhalation. Ten mice were infected with $5 \times 10^4$ yeast cells of the H99, *sod1,* Rec1, and Rec2 strains in a 50-μl dose via nasal inhalation as described previously (14, 15). The mice were fed ad libitum and were monitored by inspection twice daily. Mice that appeared moribund or in pain were sacrificed by $CO_2$ inhalation. The protocol was approved by the Duke University Animal Use Committee. Survival data from the mouse experiments were analyzed by a Kruskal-Wallis test.

**Macrophage assays.** The MH-S murine alveolar macrophage cell line (American Type Culture Collection, Manassas, Va.) was maintained in RPMI 1640 containing 10% fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 4.5 g of glucose per liter, 1.5 g of bicarbonate per liter, 0.05 mM 2-mercaptoethanol, and penicillin-streptomycin at 37°C in the presence of 5% $CO_2$. Macrophages were harvested from monolayers by using 0.25% trypsin–0.03% EDTA, and the numbers of viable cells were determined by trypan blue exclusion and counting with a hemacytometer. The macrophage concentration was adjusted to $10^5$ cells/ml, and in experiments in which activated macrophages were used, the cells were primed with 100 U of murine gamma interferon (Sigma) per ml and stimulated with 0.3 μg of lipopolysaccharide (LPS) per ml just prior to mixing with yeasts. One hundred microliters of a macrophage suspension was put into each well of 96-well plates. Cryptococci that had been washed three times in PBS were counted with a hemacytometer, the concentration was adjusted to $10^5$ cells/ml by using cell culture media, and 100 μl was added to the MH-S cells at a multiplicity of infection (ratio of effectors to targets) of 1:1. Some macrophages were treated with an irreversible inhibitor of inducible nitric oxide synthase by using 1 mM L-*N*-monomethyl arginine (Cayman Chemical, Ann Arbor, Mich.). Control wells containing only macrophages and only yeast cells were included in all experiments. In all experiments 10 μg of 18B7 (immunoglobulin G1 anti-GXM monoclonal antibody) per ml was added to the yeast inocula as an opsonin. The macrophage-yeast mixtures were incubated for 1 h before they were washed with two changes of PBS to get rid of the extracellular yeast cells. At different times, quantitative cultures were prepared by aspirating the medium from each well and then lysing the remaining macrophages with two changes of 100 μl of 0.5% sodium dodecyl sulfate in water. The aspirated media and sodium dodecyl sulfate solutions were combined and cultured for quantitation on Sabouraud agar containing chloramphenicol. In all experiments three duplicate wells per reading were used, and all experiments were repeated three separate times. Fifty microliters of the supernatant was immediately frozen for nitrite assays. The nitrite assays were done in 96-well plates with equal volumes of Griess reagent and supernatant, and absorbance at 540 nm was determined with a plate reader. The absorbance values were compared to a standard curve obtained by using sodium nitrite dilutions. The phagocytic index was determined as described previously by counting 600 macrophages for each yeast strain in preparations containing stimulated, unstimulated, and L-NMMA-treated macrophages (19). After 4 h of incubation, the wells were washed with three changes of PBS, and the macrophages were stained with Giemsa stain as described previously (19). The phagocytic index was calculated by dividing the number of attached and ingested cryptococci by the number of macrophages (19). The quantitative culture data were combined, and the three groups were compared to each other by using a one-way analysis of variance with a Bonferroni correction posttest. All other analyses were performed with the unpaired Student *t* test. Primary macrophages were derived from peripheral blood monocytes obtained by elutriation of buffy coat cells from normal human donors as described previously (25). After culture for 1 week, $7 \times 10^4$ macrophages were infected with $10^4$ cryptococci in the presence of 5% pooled human serum as an opsonin. After 48 h of culture at 37°C in the presence of 5% $CO_2$, the cells were lysed, and the number of CFU was determined by quantitative culture. The results were expressed as percentages of growth compared to the inoculum.

**Nucleotide sequence accession number.** The sequence of the *SOD1* gene has been deposited in the GenBank database under accession number AF324862.

## RESULTS

We used a PCR subtraction technique in a genetic screening analysis to identify cDNA preferentially expressed at 37°C versus 25°C and obtained a 435-bp partial cDNA fragment that was identical to a *C. neoformans* Cu,Zn SOD gene (*SOD1*) sequence in the GenBank database. Southern blot analysis of genomic DNA digested with a variety of restriction enzymes demonstrated that *SOD1* existed as a single copy within the genome. The genomic locus was cloned, and the entire Cu,Zn SOD gene was contained in a *Kpn*I genomic fragment (Fig. 1A). By comparison of the genomic sequence with the sequences of the cloned full-length cDNA fragments, *SOD1* was determined to be 884 bp long and to contain four introns. The predicted amino acid sequence contains 154 residues that exhibit 63% homology with the Cu,Zn SODs from *Aspergillus fumigatus* and *Neurospora crassa* and 61% homology with the Cu,Zn SOD from *Candida albicans*.

Northern analysis confirmed that *SOD1* had threefold-increased expression at 37°C compared with the expression at 25°C (Fig. 1D). No differences in expression were found when yeasts were grown in the presence of pure oxygen and exposed to 0.5 mM $Cu^{2+}$ or when they were exposed to the oxidative stressors *tert*-butyl hydroperoxide and paraquat (data not shown).

In our initial attempts to disrupt the gene, we performed a phenotypic screening analysis with minimal media containing 5 mM paraquat, but no transformants with impaired growth were identified among the 288 transformants tested. This result made us wary of assays that relied on phenotypic screening to identify the mutants. We then used colony PCR performed with primers flanking the *URA5* insertion in the disruption construct as a genotypic screening technique and found that 11 of 48 transformants (23%) had amplification of only the disrupted version of the gene (Fig. 1B). Southern blotting of these 11 mutant strains demonstrated that in all of them the native band was displaced to the expected position of the disrupted version, and 9 of them appeared to have single insertions in the genome (Fig. 1C). By gross inspection, all nine of these mutant strains appeared to be equivalent to each other and to the wild type in terms of growth at 37°C on YPD medium, melanin production on dopamine agar, capsule size in the presence of 5% $CO_2$, extracellular phospholipase activity, and urease production. Thus, mutation of *SOD1* does not affect any of these cryptococcal phenotypes that have been associated with virulence. One of the mutant strains was chosen for further analysis and designated *sod1*.

Reconstitution of *sod1* to the wild-type phenotype was accomplished by ectopic integration of the *Kpn*I genomic fragment containing *SOD1*. Reconstituted strains were verified by performing PCR and Southern analyses (Fig. 1B and C). The reconstituted strains were screened for growth at 37°C, melanin production on dopamine agar, and capsule size in the presence of 5% $CO_2$, and two strains that had phenotypes that were grossly similar to the phenotypes of both the wild-type and *sod1* strains were designated Rec1 and Rec2.

A standard biochemical assay established that the *sod1* mutant strain had significantly lower SOD activity than either the wild-type strain or the reconstituted strains (Fig. 2A). There were not significant differences in the SOD activities of the

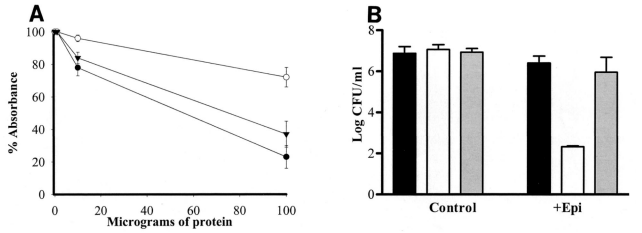


FIG. 2. (A) SOD assay in which xanthine was used as a source of superoxide. The superoxide reduced NBT, which could be quantified by absorbance at 560 nm. The action of SOD decreased the amount of NBT that was reduced, thus decreasing the absorbance. Various amounts of whole-protein extracts from the H99 (●), *sod1* (○), and Rec1 (▼) strains were added to reaction mixtures. Each value represents the results of three independent readings, and the results are expressed as percentages of the control absorbance (bovine serum albumin only); the error bars indicate standard errors. The *sod1* protein extracts had significantly higher absorbance with both amounts than the wild-type, Rec1, and Rec2 strains had. The Rec2 data were not included for clarity. (B) Recovery of yeast cells after incubation for 24 h in a cell-free system in which oxygen radicals were generated from the electron donor epinephrine. The results are expressed in log CFU per milliliter (mean ± standard deviation) and represent three independent readings. A total of $10^6$ yeast cells of the wild-type strain (solid bars), the *sod1* strain (open bars), or the Rec1 strain (gray bars) were inoculated into a reaction mixture containing epinephrine (+Epi) and a control mixture containing all of the components except epinephrine (Control). The number of *sod1* yeast cells recovered was significantly lower than the numbers of wild-type and Rec1 yeast cells recovered ($P < 0.0001$).

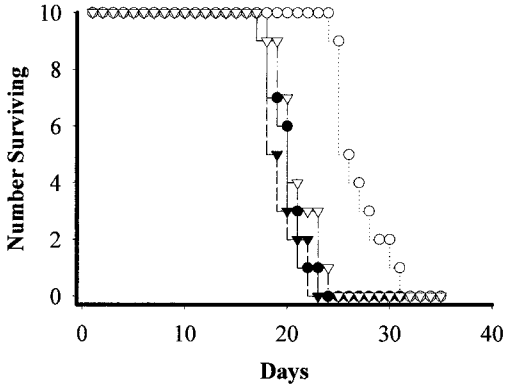


FIG. 3. Survival of mice infected with equal numbers of yeast cells via nasal inhalation. Symbols: ●, H99 (wild type); ▼, Rec1; ▽, Rec2; ○, *sod1*. The *sod1*-infected mice lived significantly longer than the mice in the other three groups ($P < 0.003$), and there were not significant differences in survival among the wild-type, Rec1, and Rec2 strains.

wild-type, Rec1, and Rec2 strains, demonstrating that there was full phenotypic reconstitution in Rec1 and Rec2 (Fig. 2A). The residual SOD activity in the *sod1* strain compared to the activities in control assay mixtures containing no SOD were presumed to be due to the manganese isoform of SOD. The *sod1* strain was found to be much more sensitive to oxygen radicals generated in a cell-free system than the wild-type and Rec1 strains were (Fig. 2B). Significantly lower numbers of yeast cells were recovered after 24 h of incubation in the reaction mixture containing the electron donor epinephrine and the *sod1* strain (2.4 ± 0.01 log CFU/ml) than in the reaction mixtures containing epinephrine and the wild-type and Rec1 strains (6.6 ± 0.28 and 6.4 ± 0.47 log CFU/ml, respectively) ($P < 0.0001$). In the control mixtures that contained all the same constituents except epinephrine, there were not significant differences in the numbers of yeast cells when the three groups were compared.

These strains were also tested for various phenotypes that have been observed with Cu,Zn SOD mutants of *Saccharomyces cerevisiae*. When quantitative cultures from liquid media were used, there were not significant differences among the *sod1*, wild-type, Rec1, and Rec2 strains in terms of growth on minimal media, prolonged stationary phase in the presence of 100% oxygen, or growth on YPD agar with either *tert*-butyl hydroperoxide (1 to 50 mM), paraquat (1 to 50 mM), oxytetracycline (20 to 2,000 μg/ml), or iron (1 to 10 mM). Furthermore, there was no loss of viability of the *sod1* strain compared to the viabilities of the wild-type strains after repeated cycles of freezing at −20°C and thawing. No differences in gross vacuole morphology were apparent among these strains after quinacrine staining of the vacuoles. Thus, the *sod1* strain did not appear to have many of the in vitro phenotypes that have been seen with Cu,Zn SOD mutants of *S. cerevisiae*.

The four strains were tested in vivo by using the mouse inhalational model (Fig. 3). Mice infected with the *sod1* strain lived significantly longer than mice infected with the wild-type, Rec1, and Rec2 strains. In fact, all of the mice infected with the strains carrying a wild-type copy of *SOD1* died before any of the mice infected with the *sod1* strain succumbed to infection (Fig. 3). The average survival time for mice infected with the *sod1* strain was 27 days, compared with 20 days for the group infected with H99 ($P = 0.001$), 19 days for the group infected with Rec1 ($P = 0.001$), and 21 days for the group infected with Rec2 ($P < 0.003$). There were not significant differences in survival between the wild-type and reconstituted groups. Therefore, the *sod1* mutant was significantly less virulent than the wild-type strain as assessed by cumulative survival.

To investigate the mechanisms for the reduced virulence of the *sod1* strain, yeast cells were tested for growth within macrophages. Phagocytic cells generate oxygen radicals to kill ingested microorganisms, and our data obtained with the cell-free system that generated oxygen radicals revealed that the *sod1* strain was much more sensitive to these radicals than the wild-type strain was. Therefore, we hypothesized that *SOD1* was important for the survival of *C. neoformans* within macrophages. Both primary human macrophages and murine macrophage cell lines were used in this study. In the MH-S cell line, there were not significant differences in the phagocytic indices among the wild-type, *sod1*, and Rec1 strains with stimulated, unstimulated, and L-NMMA-treated macrophages (data not shown). Thus, there were not differences in the abilities of the three strains to be taken up by macrophages with an anti-GXM monoclonal antibody serving as an opsonin. However, once the cryptococci were taken up, the *sod1* strain was associated with significantly slower growth within the macrophages. In the MH-S cell line, the *sod1* strain exhibited significantly slower growth within unstimulated macrophages than the wild-type and Rec1 strains exhibited (Fig. 4A). Significantly fewer *sod1* yeast cells ($8.93 \times 10^4$ CFU/ml) than wild-type and Rec1 yeast cells ($1.25 \times 10^5$ and $1.39 \times 10^5$ CFU/ml, respectively) were recovered from the unstimulated

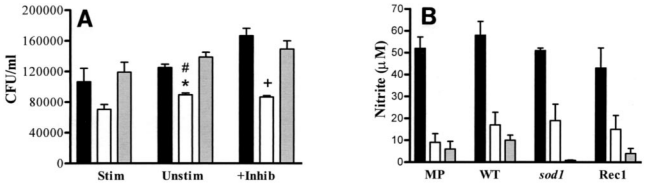


FIG. 4. (A) Number of yeast cells (CFU per milliliter; mean ± standard deviation) recovered from MH-S macrophages incubated with yeast after 24 h. The wild-type (solid bars), *sod1* (open bars), and Rec1 (gray bars) strains were incubated with cells stimulated with both gamma interferon and LPS (Stim), cells with no stimulation (Unstim), or cells stimulated with both gamma interferon and LPS and treated with the nitric oxide synthase inhibitor L-NMMA (+Inhib). A number sign indicates that the *P* value was <0.002 compared with the results for the wild-type and Rec1 yeasts in unstimulated macrophages. An asterisk indicates that the *P* value was 0.024 compared with the results for *sod1* yeast in stimulated macrophages. A plus sign indicates that the *P* value was <0.010 compared with the results for the wild-type and Rec1 yeasts in stimulated macrophages treated with inhibitor. There were not significant differences for the comparisons of any other groups. (B) Nitric oxide levels in cell culture supernatants in the experiments shown in panel A were determined by measuring nitrite levels. Each bar and error bar indicate the average + standard deviation for nine samples from three different experiments. MP, macrophage control (no yeast); WT, wild type; *sod1*, *sod1* mutant strain; Rec1, reconstituted strain. Solid bars, macrophages stimulated with gamma interferon and LPS; open bars, unstimulated macrophages; gray bars, stimulated macrophages treated with the nitric oxide synthase inhibitor L-NMMA. Significantly higher levels of nitrite were measured for each strain in the stimulated macrophages than under the other two conditions. There were not significant differences between the unstimulated and inhibitor-treated conditions for any of the groups, and there were not significant differences in the nitric oxide levels of the three strains compared to the levels of the control containing no yeast for each of the three conditions.

macrophages at 24 h ($P = 0.0008$ and $P = 0.001$, respectively). The number of *sod1* yeast cells recovered from macrophages stimulated with gamma interferon and LPS ($7.03 \times 10^4$ CFU/ml) was also lower than the numbers of wild-type and Rec1 cells recovered from such macrophages ($1.07 \times 10^5$ and $1.19 \times 10^5$ CFU/ml, respectively), but the difference did not quite reach statistical significance at the $P = 0.05$ level for the wild-type strain ($P = 0.089$ and $P = 0.049$, respectively). The number of *sod1* yeast cells recovered from the unstimulated macrophages ($8.93 \times 10^4$ CFU/ml) was significantly higher than the number recovered from the stimulated macrophages ($7.03 \times 10^4$ CFU/ml) ($P = 0.024$). Thus, the *sod1* strain was more susceptible than the wild-type strain to growth inhibition in the unstimulated macrophages but was not more susceptible in the stimulated macrophages. There were not significant differences in the colony counts from the macrophages at the 4-h time point, and there were not differences in either the number or the viability of the macrophages from any of the groups as assessed by trypan blue exclusion (data not shown).

We reasoned that the differences in growth of the *sod1* strain between the stimulated and unstimulated macrophages at the 24-h time point may have been due to the expanded fungistatic repertoire of the macrophages resulting from gamma interferon and LPS stimulation. Nitric oxide was considered to be the most likely fungistatic candidate in the stimulated macrophages, and nitrite levels in the macrophage supernatants were measured (Fig. 4B). As expected, the stimulated macrophages made significantly more nitric oxide than the unstimulated macrophages made (Fig. 4B). There were not significant differences in the nitric oxide levels of the three strains compared to the levels in control samples containing no yeast cells for each type of conditions. Inhibition of the inducible nitric oxide synthase in the stimulated macrophages with the L-arginine analog L-NMMA not only resulted in a significant decrease in the nitric oxide levels (Fig. 4B) but also resulted in a significant increase in the average number of *sod1* yeast cells recovered compared to the number in stimulated macrophages not treated with the inhibitor ($8.66 \times 10^4$ and $7.03 \times 10^4$ CFU/ml, respectively) ($P = 0.04$) (Fig. 4A). The number of *sod1* yeast cells recovered from the unstimulated macrophages ($8.93 \times 10^4$ CFU/ml) was similar to the number recovered from the stimulated macrophages treated with the inhibitor ($8.66 \times 10^4$ CFU/ml) ($P = 0.23$). Therefore, the reason that there were not significant differences in the numbers of *sod1* and wild-type yeast cells recovered from the stimulated macrophages was because of the fungistatic effects of nitric oxide.

The slower intracellular growth of the *sod1* strain was also demonstrated in human macrophages. In four independent triplicate experiments, the percentages of growth compared to the size of the inoculum were 39% ± 10% and 156% ± 42% (means ± standard errors) for the *sod1* and wild-type strains, respectively ($P = 0.01$).

## DISCUSSION

We first identified the *C. neoformans SOD1* gene in a screening for genes regulated by temperature. The regulation of *SOD1* by temperature was confirmed by a Northern analysis that showed that there was a threefold increase in *SOD1* expression at 37°C compared with *SOD1* expression at 25°C. The expression of many genes involved in resistance to oxidative damage increases in other fungi in response to stresses such as temperature (8), and our data are the first data which show that there is temperature-related expression of a SOD-encoding gene in a human-pathogenic fungus. The increased expression of *SOD1* in *C. neoformans* may be part of a generalized stress response, but it may also be a response to increased intracellular stresses related to higher rates of oxidative metabolism. Interestingly, the increased expression of *SOD1* has been independently confirmed by Jim Kronstad, who also found higher levels of *SOD1* mRNA in *C. neoformans* strain JEC21 grown at 37°C than in the same strain grown at 24°C (Jim Kronstad, personal communication).

We were able to create *sod1* mutants using targeted disruption, and the *sod1* mutants clearly had decreased SOD activity, as measured by a standard assay. In vitro comparison demonstrated that the *sod1* strain was largely killed in the presence of oxygen radicals, whereas both the wild-type and reconstituted strains were able to survive with no appreciable cell death. Hence, *SOD1* is critically important in the yeast defense against extracellular oxygen radicals generated by epinephrine in a cell-free system. However, despite this striking phenotype, we were unable to find in the *sod1* mutants any of the phenotypes thought to be due to an excess of intracellular oxygen radicals, such as those that have been described for *S. cerevisiae* SOD mutants. For example, mutation of the Cu,Zn SOD in *S. cerevisiae* leads to sensitivity to oxytetracycline (3), iron (17),

paraquat (21), 100% oxygen (21), freeze-thaw stress (34), age (4), and auxotrophy for methionine and lysine (21). The differences in such disparate phenotypes associated with the same gene in these two fungi are striking and could be due to intrinsic differences between the two fungi that are unrelated to resistance to oxidative damage. However, the differences may also reflect the fact that *C. neoformans* has other redundant systems that can detoxify superoxide radicals. We believe that part of this redundancy can be explained by the ability of *C. neoformans* to produce two powerful free radical quenchers, mannitol and melanin. Both of these products are made by *C. neoformans* but not by *S. cerevisiae*, and both have been postulated by other investigators to be oxygen radical scavengers in *C. neoformans* (11, 12, 24, 26, 29). The presence of such redundant scavenger systems may explain why the *C. neoformans sod1* strain does not have some of the phenotypes that have been found in *S. cerevisiae*. However, these redundant systems cannot fully compensate for the loss of *SOD1* since the *sod1* strain is much more sensitive than the wild type to oxygen radicals, as demonstrated in our cell-free assay, and it clearly has a decreased ability to detoxify superoxide, as shown in the SOD assays. Therefore, we believe that the postulated redundant systems can only partially compensate for the loss of *SOD1*.

There have been suggestions that the virulence of *C. neoformans* strains may be related to an individual strain's resistance to oxidative stress. One study correlated the virulence of three different clinical isolates of *C. neoformans* in the murine model with in vitro resistance to reactive oxygen and nitrogen species (41). The most virulent strain was also the strain that was most resistant to oxidative damage. In our studies, we showed that the *sod1* mutant was significantly less virulent than the wild-type strain in the murine inhalational model. The fact that in two independent reconstituted strains virulence was restored strongly supports the claim that the virulence defect was due to the *sod1* mutation itself rather than to some unspecified mutation that occurred during the transformation process. The virulence defect was not due to any obvious, known virulence phenotype, such as growth rate at 37°C or production of melanin, extracellular phospholipase, urease, and the polysaccharide capsule. Therefore, we reasoned that the decreased ability of the *sod1* strain to cause infection is due to increased susceptibility to oxygen radical attack within phagocytic cells.

One of the mechanisms by which human phagocytic cells kill ingested microorganisms is by selective production of oxygen radicals, including superoxide, in phagolysosomes. The importance of superoxide in human immune defenses is illustrated by the susceptibility of patients with chronic granulomatous disease to a variety of bacterial and fungal infections. These patients have defects in the NADPH oxidase system and suffer from recurrent infections due to both bacteria and fungi (27). The importance of superoxide in the killing of cryptococci is reflected by the fact that neutrophils from patients with chronic granulomatous disease exhibit decreased killing of *C. neoformans* in vitro (18). In some bacteria, SODs have been shown to be important for survival within macrophages and for virulence in animal models. For these microbes, it has been postulated that the mechanism for decreased virulence was increased susceptibility of the SOD mutant strains within macrophages (32, 35). We reasoned that *SOD1* might play a similar role in cryptococcosis.

*C. neoformans* is known to reside in macrophages during many stages of experimental and human infections (19), and resistance to macrophage-mediated killing may be important for virulence in this fungus. In this study, we demonstrated that the *sod1* strain was significantly more susceptible to oxygen radicals in a cell-free system, and consequently, we wanted to see if this strain was more susceptible to the fungistatic mechanisms within macrophages. Our data established that the *sod1* strain had decreased growth rates compared with the growth rates of the wild type within macrophage cell lines and primary macrophages. Within the cell lines, the differences in growth between the *sod1* and wild-type strains were not as marked in the stimulated macrophages as in the unstimulated macrophages. We feel that the intracellular growth defect of the *sod1* strain was more apparent in the unstimulated macrophages because of the limited fungistatic repertoire in the unstimulated cells. Unstimulated macrophages do not produce significant amounts of nitric oxide, and in these cells the oxygen radicals can be expected to have a more important role in fungistasis. Therefore, the protective effects of *SOD1* against superoxide anions in the macrophages are more obvious when nitric oxide is not being produced. We were able to show that this effect is specific to nitric oxide by inhibiting nitric oxide production using L-NMMA. In stimulated macrophages treated with this drug, nitric oxide production was almost completely eliminated, and this resulted in a significant decrease in the number of *sod1* yeast cells recovered compared to the number of wild-type cells recovered.

The fact that the *sod1* mutants still exhibited significant growth within macrophages and were still pathogenic suggests that there are other mechanisms in addition to *SOD1* that are important in the resistance to the oxidative stresses encountered in the host. In the acidic conditions of the phagolysosome, superoxide becomes protonated and reacts with itself to form hydrogen peroxide ($H_2O_2$). Hydrogen peroxide is a more reactive oxidant than superoxide and can cause oxidation of proteins, DNA, membrane lipids, and components of the respiratory chain. Hydrogen peroxide can interact with superoxide via the Haber-Weiss reaction to form hydroxyl radical ($\cdot OH$), which is considered to be the most destructive of the reactive oxygen species. SOD is not thought to affect the levels of these other reactive oxygen species. Catalase, glutathione peroxidase, and thioredoxin peroxidase have each been shown to break down hydrogen peroxide in *S. cerevisiae* (5, 22), and it is possible that these enzymes also have a significant role in the intracellular survival of *C. neoformans*. As mentioned above, both mannitol and melanin are postulated to be scavengers of oxygen radicals (11, 12, 24, 26, 29), and both have been shown to have some role in the protection of cryptococci within phagocytic cells (12, 29). Nitric oxide is another important part of the oxidative attack directed against *C. neoformans* in the macrophage phagolysosome. The important fungistatic role of nitric oxide against *C. neoformans* in macrophages has been demonstrated both in vitro and in vivo (1, 2). Mice deficient in the inducible nitric oxide synthase also had higher burdens of infection with *C. neoformans* than wild-type mice had (37, 38). One research group has reported that *C. neoformans* reduces nitric oxide activity in macrophages and astrocytes via nitric

oxide consumption by some yeast factor (40). Two candidates for the cryptococcal yeast factor that may consume nitric oxide are the polysaccharide capsule and flavohemoglobin. The *S. cerevisiae* flavohemoglobin encoded by the *YHB1* gene has been shown to metabolize nitric oxide and to protect the yeast against nitrosative stress (30). Flavohemoglobins have been proposed to be a conserved protective mechanism in all microorganisms, and a flavohemoglobin-encoding gene is present in *C. neoformans* (Joseph Heitman, personal communication).

Since the *sod1* strain can grow in vitro and also kill mice, SOD does not fit the classical definition of a virulence factor as a trait that is dispensable for growth in vitro but is required for pathogenicity (6). However, this classical definition of a virulence factor is probably too restrictive and is not considered applicable to traits associated with virulence in many organisms, including some fungi (7). The definition of virulence factor has been recently modified to a microbial trait that promotes host damage (7). By this modified definition SOD qualifies as a virulence factor since its presence translated into increased host damage, as manifested by reduced survival time in the wild-type and complemented strains. SOD can function as a virulence factor by promoting the survival of yeast cells, which, in turn, translates into host damage as a consequence of microbe-mediated effects and the host response to infection.

We concluded that the Cu,Zn SOD contributes to the virulence of *C. neoformans* but is not required for pathogenicity. We believe that the role of *SOD1* in virulence is in resistance to oxygen radical-mediated damage within macrophage phagolysosomes and that there are other mechanisms by which *C. neoformans* can resist oxidative damage within the phagolysosomes. We also believe that in order for *C. neoformans* to be a successful intracellular pathogen, it must also have mechanisms for the detoxification of reactive nitrogen species. Further study of all these mechanisms should provide important insights into how this yeast can persist within macrophages and cause systemic infection. Given that *C. neoformans* has multiple mechanisms with which to resist oxidative damage, it may be very difficult to further investigate the role of oxidative resistance in pathogenesis by targeting one specific effector gene, such as the SOD or catalase gene. Some options include studying strains containing multiple gene disruptions or mutation of transcription factors that may control the levels of several different genes involved in the response to oxidative damage.

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