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Research Article

Chronic granulomatous disease (CGD) is a rare genetic disorder in which phagocytes fail to produce superoxide because of defects in one of several components of the NADPH oxidase complex. As a result, patients develop recurrent life-threatening bacterial and fungal infections. The organisms to which CGD patients are most susceptible produce catalase, regarded as an important factor for microbial pathogenicity in CGD. To test the role of pathogen-derived catalase in CGD directly, we have generated isogenic strains of Aspergillus nidulans in which one or both of the catalase genes (catA and catB), have been deleted. We hypothesized that catalase negative mutants would be less virulent than the wild-type strain in experimental animal models. CGD mice were produced by disruption of the p47(phox) gene which encodes the 47-kD subunit of the NADPH oxidase. Wild-type A. nidulans inoculated intranasally caused fatal infection in CGD mice, but did not cause disease in wild-type littermates. Surprisingly, wild-type A. nidulans and the catA, catB, and catA/catB mutants were equally virulent in CGD mice. Histopathological studies of fatally infected CGD mice showed widely distributed lesions in the lungs regardless of the presence or absence of the catA and catB genes. Similar to the CGD model, catalase-deficient A. nidulans was highly virulent in cortisone-treated BALB/c mice. Taken together, these results indicate that catalases do not play a significant […]

Virulence of Catalase-deficient *Aspergillus nidulans* in p47$^{phox-/-}$ Mice

Implications for Fungal Pathogenicity and Host Defense in Chronic Granulomatous Disease

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Abstract

Chronic granulomatous disease (CGD) is a rare genetic disorder in which phagocytes fail to produce superoxide because of defects in one of several components of the NADPH oxidase complex. As a result, patients develop recurrent life-threatening bacterial and fungal infections. The organisms to which CGD patients are most susceptible produce catalase, regarded as an important factor for microbial pathogenicity in CGD. To test the role of pathogen-derived catalase in CGD directly, we have generated isogenic strains of *Aspergillus nidulans* in which one or both of the catalase genes (*catA* and *catB),* have been deleted. We hypothesized that catalase negative mutants would be less virulent than the wild-type strain in experimental animal models. CGD mice were produced by disruption of the p47^{phox} gene which encodes the 47-kD subunit of the NADPH oxidase. Wild-type *A. nidulans* inoculated intranasally caused fatal infection in CGD mice, but did not cause disease in wild-type littermates. Surprisingly, wild-type *A. nidulans* and the *catA*, *catB,* and *catA*/*catB* mutants were equally virulent in CGD mice. Histopathological studies of fatally infected CGD mice showed widely distributed lesions in the lungs regardless of the presence or absence of the *catA* and *catB* genes. Similar to the CGD model, catalase-deficient *A. nidulans* was highly virulent in cortisone-treated BALB/c mice. Taken together, these results indicate that catalases do not play a significant role in pathogenicity of *A. nidulans* in p47$^{phox-/-}$ mice, and therefore raise doubt about the central role of catalases as a fungal virulence factor in CGD. (*J. Clin. Invest.* 1998. 101:1843–1850.) Key words: catalase • *catA* • *catB* • CGD • p47^{phox}

Introduction

Generation of oxidative products by phagocytic cells is known to be one of the important host defense mechanisms directed toward killing of invading microorganisms (1). Chronic granulomatous disease (CGD)[1] is a rare inherited disorder in which phagocytes fail to produce superoxide and hydrogen peroxide due to defects in NADPH oxidase. Patients lacking this important host antimicrobial pathway are highly susceptible to catalase-producing bacteria and fungi (1–5) but do not appear to be at increased risk of infection with catalase negative organisms (1). The lack of virulence of some catalase negative organisms, e.g., streptococci, may be due to the pathogen producing appreciable amounts of hydrogen peroxide, which can substitute for the absence of reactive oxidant formation by CGD leukocytes (6, 7). In vitro studies of neutrophil function have shown that hydrogen peroxide effectively kills fungal hyphae (8), and neutrophil-mediated hyphal damage is strongly blocked by myeloperoxidase inhibitors and catalase (9). Based on these observations, microbial catalase has been thought to be critical for virulence in CGD patients.

Aspergillus spp. are a major cause of morbidity and mortality in patients with CGD. In a mouse model of X-linked CGD, intratracheal challenge with *Aspergillus fumigatus* resulted in high rates of mortality but did not cause disease in wild-type littermates (10–12). These findings demonstrate the importance of phagocyte-generated oxidants in murine host resistance to *A. fumigatus* infection. *Aspergillus nidulans* is the second most common *Aspergillus* species found to cause life-threatening infections in CGD (13, 14). Unlike *A. fumigatus*, however, rarely has it been documented to cause systemic aspergillosis in other high risk groups such as patients with prolonged neutropenia secondary to myelotoxic chemotherapy (13). Because of the existence of a sexual cycle and extensive information on *A. nidulans* genetics (15–17), *A. nidulans* provides an excellent system which can be used to determine whether catalase is an important factor for pathogenicity of *Aspergillus* species.

Catalases are ubiquitous metalloenzymes among aerobic organisms that protect cells from oxidative damage by converting hydrogen peroxide to water and oxygen. In *A. nidulans*, two catalase genes, *catA* and *catB,* have been identified recently (18, 19). The *catA*$^{+}$ gene encodes the major catalase of conidia and is not expressed during vegetative growth. The *catB*$^{+}$ gene is mainly expressed during hyphal growth and formation of conidial structures, but is barely expressed in conidia.

Previously, we generated a murine knockout model of CGD by disruption of the p47^{phox} gene which encodes a necessary component of the NADPH oxidase complex (20). p47$^{phox-/-}$ mice are susceptible to a spectrum of spontaneous and experimental infections similar to CGD patients (20). Here, we report the virulence of genetically engineered catalase-deficient strains of *A. nidulans* in experimental infection

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1. *Abbreviations used in this paper:* CGD, chronic granulomatous disease; GMS, Gomori methenamine silver; H&E, hematoxylin and eosin.

Table I. A. nidulans Strains

Strain	Genotype	Source
FGSC 26	*biA1; veA1*	Fungal Genetics StockCenter
RMS011	*pabaA1, yA2; ΔargB::trpCΔB; veA1, trpC801*	M.A. Stringer
TYCC224	*pabaA1, yA2; ΔargB::trpCΔB; ΔcatB::argB; veA1, trpC801*	This study
TYCC227	*pabaA1, yA2; ΔargB::trpCΔB; ΔcatA::argB*; *veA1, trpC801*	This study
RYC11	*biA1; ΔargB::trpCΔB; ΔcatB::argB; veA1**	This study
RYC12	*ΔargB::trpCΔB; ΔcatB::argB; veA1**	This study
RYC13	*veA1*	This study
RYC16	*ΔargB::trpCΔB; ΔcatA::argB; ΔcatB::argB, veA1**	This study
RYC17	*ΔargB::trpCΔB; ΔcatA::argB; veA1**	This study

*Partial genotype; may also contain *trpC801*.

with $p47^{phox-/-}$ mice as well as cortisone-treated immunosuppressed mice with an intact $p47^{phox}$ gene.

Methods

Strains and growth conditions. The *A. nidulans* strains are described in Table I. Strains were maintained on supplemented minimal-nitrate medium (21) or malt extract agar (0.1% peptone, 2% glucose, 2% malt extract, 2% agar). To monitor the spore germination rate, conidia of each strain were plated on different sectors of the same malt agar plate and incubated at 37°C for 8 h. The ratios of germinated versus nongerminated conidia were recorded and growth by hyphal extension was monitored under a microscope at 1-h intervals for an additional 4 h. The growth rates of the different strains were compared by measuring the rates of hyphal extension. Conidia were point inoculated on malt extract agar, incubated at 37°C, and the diameter of the colony was recorded. Hydrogen peroxide sensitivity of conidia was tested by incubating conidial suspensions with different H_2O_2 concentrations for 30 min at room temperature. After appropriate dilutions, H_2O_2-treated conidial suspensions were plated on malt extract agar. The number of colonies formed was determined after 24 h of incubation at 37°C. Hydrogen peroxide sensitivity of hyphae was determined as described previously (19).

Plasmid construction. The plasmid pREN3, which contains the *catA* gene of *A. nidulans*, was a gift from J. Aquirre. The *catA* deletion construct (pYCC227) was created as follows. The 3.0-kb XbaI fragment of pREN3 was subcloned into pBC KS vector (Stratagene Inc., La Jolla, CA) to give pYCC226. The 1.5-kb HpaI/SphI region of pYCC226 was replaced by 1.9-kb BamHI fragment of pSalArgB which contains the *argB2* gene to give pYCC227 (Fig. 1 *A*). The plasmid was linearized with XbaI before *A. nidulans* transformation.

The *catB* deletion construct (pYCC224) was generated as follows. The *catB* gene from the standard wild-type *A. nidulans* strain FGSC26 was amplified by PCR using the oligonucleotide primers (TCGATTCGATTCGTTCGCGCCTTCC and ATCTAGGCTTATGTGGTGTGA). The resulting PCR product, pYCC222, was cloned and sequenced. The 1.2-kb StyI region of pYCC222 was replaced by the 1.9-kb BamHI fragment of pSalArgB to give pYCC224 (Fig. 1 *B*). The plasmid was linearized with PstI before transformation of *A. nidulans*.

Strain construction. To delete either *catA* or *catB*, pYCC227 and pYCC224 were transformed separately into strain RMS011 by standard PEG methods (22). The resulting transformants were screened by PCR and confirmed by Southern blot analysis. For PCR screening, conidia from transformants were heated at 94°C for 15 min in the PCR reaction buffer (Boehringer Mannheim, Indianapolis, IN) and standard PCR reactions were performed with appropriate primer sets. The transformants TYCC224 and TYCC227 correspond to *catB* and *catA* mutants, respectively. TYCC224 (*p*-aminobenzoic acid auxotroph) was crossed to FGSC26 (biotin auxotroph). The offspring derived from the cross were screened for the *catB* genotype by PCR and were confirmed by Southern blot analysis. RYC13 and RYC12 were the prototroph progenies from the cross and were a $catB^+$ strain and a *catB* mutant, respectively. One of the biotin auxotrophs of the *catB* deletion strain (RYC11) was crossed with a *catA*-deleted transformant (TYCC227). The resulting nutritional prototroph progenies were screened by PCR and by Southern blot analysis as described above. RYC17 and RYC16 were the *catA* mutant and *catA*/*catB* double mutant, respectively.

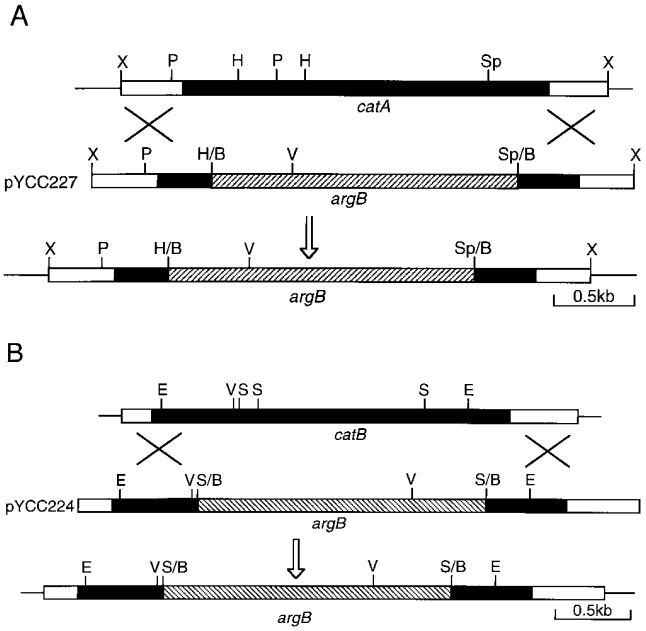

Figure 1. Deletion of catalase genes. (*A*) Plasmid pYCC227 was constructed by replacing a major portion of the *catA* coding region (*black box*) with the *argB* gene (*hatched box*). Linearized pYCC227 was used to transform the *argB*-deleted strain RMS011. The *catA*-deleted transformants were screened by PCR and confirmed by Southern blot analysis. (*B*) Plasmid pYCC224 was constructed by replacing nearly 50% of the *catB* coding region (*black box*) with the *argB* gene (*hatched box*). The *catB*-deleted transformants were screened as in the *catA* deletion. *B*, BamHI; *E*, EagI; *V*, EcoRV; *H*, HpaI; *P*, PstI; *S*, StyI; *Sp*, SphI; *X*, XbaI.

Determination of catalase activity. Native gel electrophoresis was performed on protein extracts from conidia as described (18). Protein concentrations in cell extracts were estimated by the method of Bradford (23) using BSA as a standard. 50 μg of protein was loaded onto 8% Tris-glycine gel and run at 125 V at 4°C in Xcell Mini-Cell (Novex, San Diego, CA). Catalase activity was detected as described previously (24, 25).

Nucleic acid isolation and analysis. Total DNA was isolated as described by Timberlake (26). DNA samples were digested with restriction enzymes and fractionated in agarose gels. DNA was transferred to Hybond-N nylon membranes and hybridized as suggested by the manufacturer (Amersham Corp., Arlington Heights, IL). Random hexamer priming was used to label the DNA probes to specific activities $> 10^8$ dpm/μg (27).

Animal model studies. Each fungal strain was grown for 7 d at 37°C on minimal nitrate medium. Conidia were harvested as a suspension in 10 ml of PBS containing 0.01% Tween 20 (PBST) and washed twice in the same buffer. The concentration of conidia in each sample was determined with a hemocytometer and confirmed by quantitative culture on agar media.

CGD mice were generated by targeted disruption of the $p47^{phox}$ gene (20). Age of mice used for fungal infection ranged from 8 to 11 wk. For the drug-induced immunosuppressed mouse model, BALB/c mice were administered 125 mg/kg cortisone acetate (Merk, Westpoint, PA) subcutaneously, on days −4, −3, −2, −1 and on the day of *A. nidulans* challenge, and on days +2, +4, +6, and +8. All mice were maintained in sterilized microisolator cages in a pathogen-free environment. As prophylaxis against bacterial infection, trimethoprim-sulfamethoxazole (Bactrim; 600 mg/liter water) was administered in the drinking water for both animal models.

After induction of anesthesia with ketamine HCl and xylazine, mice were inoculated intranasally with either a high inoculum (10^5 conidia per mouse in 50-μl volume) or a low inoculum (4×10^3 conidia per mouse in 30-μl volume) of wild-type or catalase-deficient *A. nidulans*. Survival analysis was the principal end point in the high inoculum experiments. In low inoculum experiments, mice were killed at 2, 4, and 9 wk after inoculation, and lungs were evaluated for extent of pulmonary inflammation and hyphal invasion. The control mice that received PBST remained healthy throughout the entire course of experiments in both animal models.

Histopathology. Lungs were removed and fixed in 10% neutral buffered formalin. Sections were embedded in paraffin, and stained with hematoxylin and eosin (H&E) and Gomori methenamine silver (GMS) (performed by American Histolabs, Gaithersburg, MD). All histopathologic analysis was performed in a blinded fashion by one of us (G.F. Miller).

Neutrophil-induced damage of hyphae. The damage to hyphae of wild-type and catalase mutants caused by neutrophils from CGD mice and wild-type littermates was assessed using the 2,2-bis-(1-methoxy-4-nitro-5-sulphenyl)-(2H)-tetrazolium-5-carboxanilide (XTT) colorimetric assay (28). This assay takes advantage of the fact that viable hyphae convert the XTT salt to a yellow/orange formazan derivative which can be measured colorimetrically. Neutrophil-mediated hyphal damage can thus be evaluated by the magnitude of loss of hyphal metabolic activity. Conidia were suspended in RPMI 1640 with 10 mM Hepes and 50-μl aliquots (10^6 spores) were inoculated onto 24-well plates (Costar Corp., Cambridge, MA) which contained 0.8 ml 1% agarose with RPMI 1640 and 10 mM Hepes. The bottoms of wells were coated with an agarose layer because unlike *A. fumigatus*, *A. nidulans* hyphae were poorly adherent to the plastic surface. The plates were incubated at 30°C for 15 h to allow hyphae formation. Neutrophils were obtained by thioglycollate-induced peritonitis (20). Briefly, mice were injected intraperitoneally with 3% thioglycollate. After 4.5–5 h, peritoneal lavage was performed with 10 ml PBS without Ca^{2+} or Mg^{2+}. Cells were spun and resuspended in PBS with Ca^{2+} and Mg^{2+} at a concentration of 2×10^6 PMN/ml (effector/target ratio = ∼ 10:1) immediately before use. White blood cell viability was ≥ 95% as determined by trypan exclusion. This method consistently yields ∼ 90% neutrophils with small amounts of macrophages and lymphocytes. 1 ml of either PBS or wild-type or CGD neutrophils was added to each well, and plates were incubated at 37°C for 2 h. Wells were aspirated, and 0.3 ml of ice-cold distilled H_2O was added per well to lyse the remaining white blood cells. H_2O was aspirated after 10 min. 0.4 ml of a solution containing XTT (0.5 mg/ml) and coenzyme Q (40 μg/ml) in PBS was added to each well and incubated for 1 h at 37°C (28). 100-μl aliquots from each well were then transferred to a 96-well U-bottom plate (Costar Corp.). Absorbance (450 nm) was determined by an MRX spectrophotometer (Dynatech Laboratories, Inc., Chantilly, VA). Percent absorbance was determined as follows: [(mean absorbance of aliquots from wells with added neutrophils − absorbance of XTT:coenzyme Q solution)/(mean absorbance of aliquots from wells with added PBS − absorbance of XTT:coenzyme Q solution)] × 100%. Each experiment was performed using duplicate wells and repeated three to four times for each *A. nidulans* strain. Data from separate experiments were pooled and mean percentage absorbance was calculated for each *A. nidulans* strain challenged with neutrophils of wild-type and CGD mice.

Statistical analysis. Kaplan-Meier analysis of survival was performed using JMP software for Macintosh (SAS Institute, Cary, NC). Student's *t* test was used to analyze the results of XTT assay.

Results

Construction of catA and catB deletion strains of A. nidulans. Recently a *catA* mutant of *A. nidulans* was constructed by gene disruption (18). Because this strain contains a duplication of part of the *catA* gene, reversion might occur by recombination between the duplicated regions. To preclude such an event, we deleted the major portion of the *catA* coding region (amino acids 118–617) by gene replacement. Fig. 1 *A* depicts the event expected upon gene replacement at the *catA* locus. We cloned *catB* by PCR and deleted nearly 50% of the *catB* gene (amino acids 195–532 deleted) by gene replacement (Fig. 1 *B*).

To compare the virulence of the different catalase mutants, it is important that the strains have a similar genetic background except for the catalase genes. Prototrophic, nearly isogenic strains of wild-type, *catA*, *catB*, and *catA*/*catB* mutants were subsequently constructed by sexual crossing (Table I; see Methods). Fig. 2 shows the Southern blot analysis of genomic DNA isolated from these strains. When the *catA* gene probe was hybridized to the blot, RYC16 and RYC17 showed the loss of the 3.5- and 0.7-kb bands present in wild-type (FGSC26), RYC12, and RYC13 and a new 4.2-kb band appeared in these two strains (Fig. 2 *A*). This indicated that RYC16 and RYC17 are *catA* deletants. When a probe of the *catB* gene was hybridized to the blot, the 2.1-kb band present in FGSC26, RYC13, and RYC17 was replaced by 1.6- and 1.7-kb bands in RYC16 and RYC12 (Fig. 2 *B*). This indicated that RYC16 and RYC12 are *catB* deletants. These results further indicated that RYC16 is a *catA* and *catB* double mutant and RYC13 contains the wild-type copy of *catA* and *catB*.

Protein extracts were prepared from conidia of various catalase mutant strains, fractionated in native polyacrylamide gels, treated with H_2O_2, and stained to detect catalase activity. The wild-type *A. nidulans* conidia contained abundant catalase A and small amounts of catalase B, whereas only the catalase A band existed in RYC12 and the catalase B band in RYC17. No enzyme activity was detectable in RYC16 (Fig. 3). Thus, the catalase activity in these strains was consistent with the results of Southern blot analysis of genomic DNA.

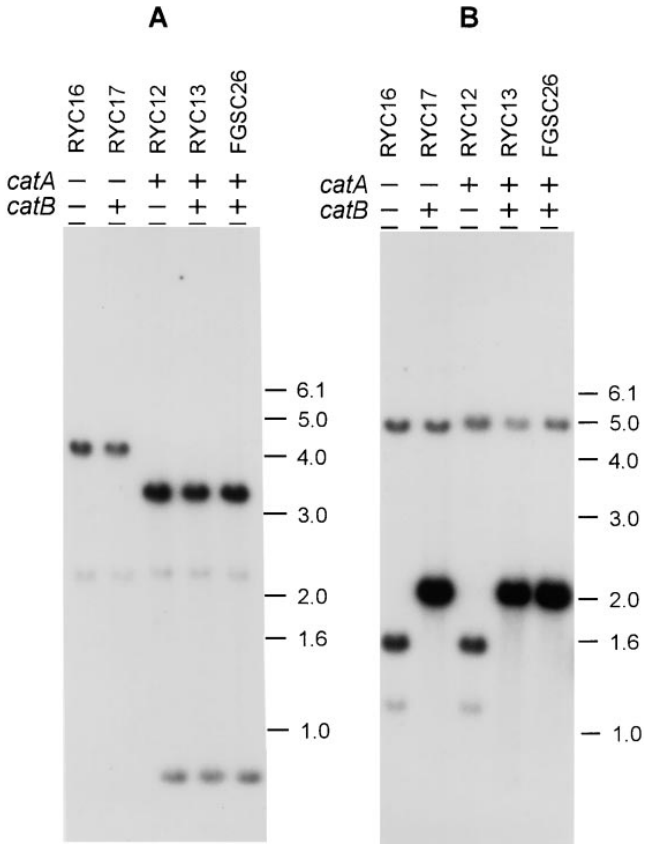

Figure 2. Southern blot analysis of the catalase mutants. (*A*) The genomic blots of PstI-digested DNA from wild-type (FGSC26 and RYC13), *catA* (RYC17), *catB* (RYC12), and *catA*/*catB* (RYC16) mutants were hybridized with a probe of pYCC226 containing the XbaI fragment of *catA* gene. (*B*) The EcoRV-digested genomic DNA blot was hybridized with a probe of the EagI fragment of *catB*. The blots hybridized with a probe of *argB* gave results consistent with the prediction (data not shown).

It was important to determine whether any difference in growth rate existed between wild-type and catalase-deficient strains before use in animal model studies. The growth rate was determined by the germination rate of conidia and hyphal extension (see Methods). No obvious growth rate difference was observed in vitro between wild-type and catalase-deficient strains at 37°C.

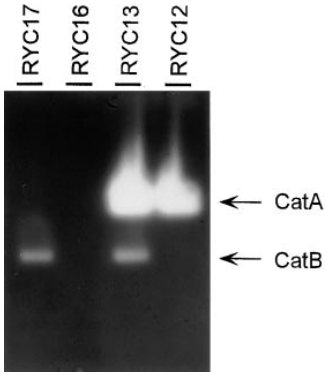

Figure 3. Catalase activity assay. Protein extracts from conidia of wild-type (RYC13), *catA* (RYC17), *catB* (RYC12), and *catA*/*catB* (RYC16) mutants were fractionated in native polyacrylamide gels and stained for catalase activity.

Sensitivity of wild-type and catalase mutants to H_2O_2 treatment. The *catA* gene is mostly expressed at the conidiation stage and the *catB* gene is highly expressed in the hyphal stage. Survival of conidia and hyphae after treatment with various concentration of H_2O_2 was examined. Conidia of RYC17 (*catA*) and RYC16 (*catA/catB*) were more sensitive to H_2O_2 treatment than the conidia of wild-type (RYC13) and RYC12 (*catB*) (Fig. 4 *A*). In contrast, RYC12 (*catB*) and RYC16 (*catA*/*catB*) showed increased sensitivity to H_2O_2 treatment during hyphal growth (Fig. 4 *B*). In both developmental stages, no obvious difference in H_2O_2 sensitivity was observed among the strains containing single or double deletions of catalase genes. Furthermore, survival of spores and hyphae in the absence of added H_2O_2 was similar between wild-type and catalase-deficient strains.

CGD mice are highly susceptible to A. nidulans infection. The $p47^{phox-/-}$ and wild-type mice were challenged with conidia of wild-type *A. nidulans* (RYC13) by intranasal inhalation. No wild-type mice became ill with an inoculum of 10^5 spores per animal (Fig. 5 *A*). In contrast, pulmonary disease and death occurred in all $p47^{phox-/-}$ mice.

The gross appearance and histology of the lungs from wild-type mice challenged with RYC13 and killed 4 d after inoculation appeared normal and no fungal structures were detected in GMS-stained sections. In contrast, there were multiple foci of hyphal invasion associated with neutrophilic infiltrates in the lungs of $p47^{phox-/-}$ mice by 4 d after respiratory challenge (Fig. 6, *A* and *B*). Extensive *Aspergillus* pneumonia occurred in all mice that died of infection. The majority of the pulmonary parenchyma was effaced by pyogranulomatous inflammation. GMS staining revealed invasive hyphae scattered throughout the lung fields.

Experimental infection of CGD mice with catalase-deficient A. nidulans. To investigate the relative susceptibility of the $p47^{phox-/-}$ mice to various catalase-deficient mutants, mice were challenged intranasally with 10^5 conidia of wild-type or catalase-deficient (*catA*, *catB*, and *catA/catB*) strains. Fig. 5 *B* shows that the majority of $p47^{phox-/-}$ mice inoculated with either single or double catalase mutants died of infection. The time course of mortality after inoculation was similar among $p47^{phox-/-}$ mice infected with wild-type (RYC13) and the three catalase-deficient strains. Histopathology of $p47^{phox-/-}$ mice infected with wild-type and different catalase deficient *A. nidulans* was similar. All mice showed extensive pulmonary inflammation and hyphal invasion (Fig. 6, *C* and *D*). Even at a relatively low inoculum (4×10^3 spores per mouse), invasive hyphae and extensive granulomatous inflammation were present in all lung sections from mice infected with catalase-positive and catalase-deficient strains when examined on days 14 to 15 after inoculation. Typical granulomata were composed of a central focus of neutrophils surrounded by lymphocytes and macrophages. Even at 9 wk after inoculation, invasive hyphae and extensive pyogranulomatous inflammation with variable fibrosis were present in all lung sections. Taken together, these data demonstrate that genetically engineered catalase-deficient *A. nidulans* remains equally virulent compared with the wild-type strain in $p47^{phox-/-}$ mice.

Catalase-deficient strains are also virulent in immunosuppressed mice. To test whether catalases are required for the pathogenicity of *A. nidulans* in immunosuppressed mice with intact NADPH oxidase, cortisone-treated BALB/c mice were inoculated intranasally with 10^5 conidia of wild-type and cata-

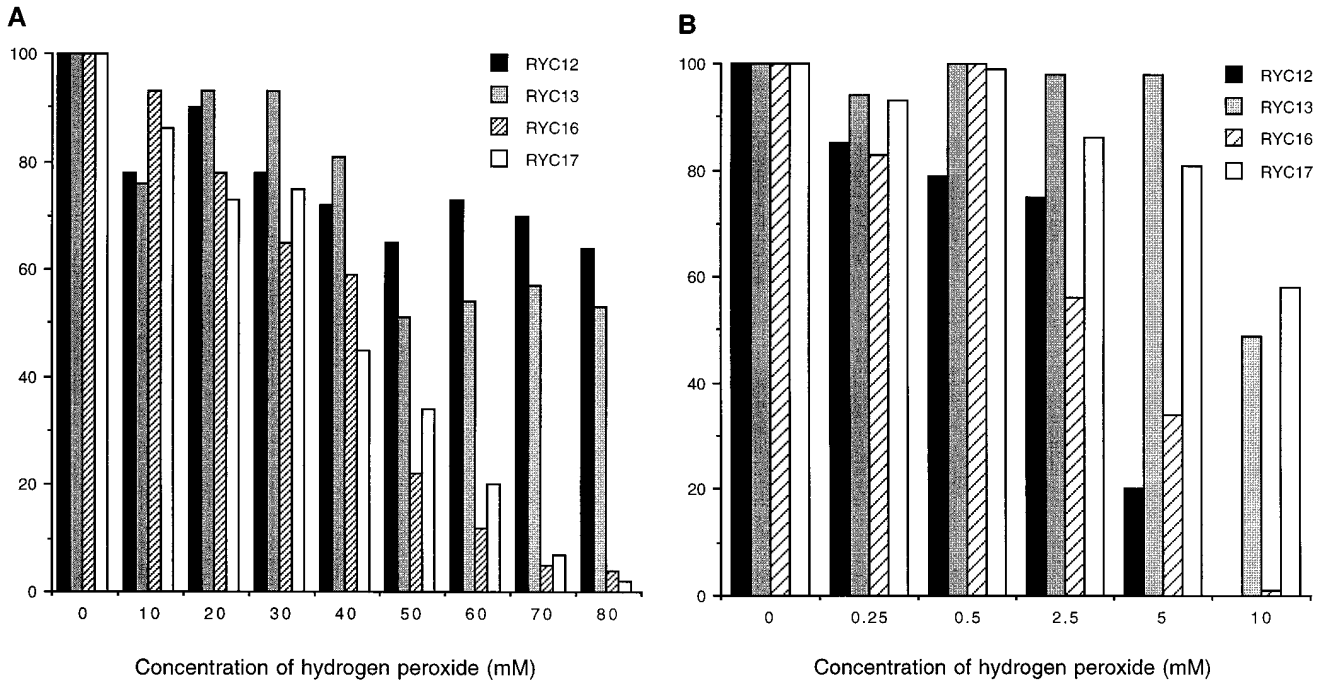

Figure 4. Hydrogen peroxide sensitivity test. (*A*) Conidia from wild-type (RYC13), *catA* (RYC17), *catB* (RYC12), and *catA*/*catB* (RYC16) mutants were treated with various concentrations of H_2O_2 at room temperature for 30 min, diluted 100 times, and plated. The number of colonies was recorded and expressed as a percentage of the control plates. The results shown are mean values of three plate counts for each treatment. (*B*) Conidia were inoculated on minimal-nitrate media and grown for 30 h at 37°C to form branching hyphae. The plates were overlaid with 10 ml of various concentrations of H_2O_2 for 10 min, washed twice with water, drained, and incubated for an additional 24 h. The number of sporulating colonies was recorded and expressed as a percentage of the control plates. The results shown are mean values of three plate counts for each concentration of H_2O_2.

lase-deficient strains and mortality was monitored. Fig. 7 shows that the majority of immunosuppressed mice inoculated with either single or double catalase mutants died of infection. The time course of mortality after inoculation was similar among the mice infected with wild-type (RYC13) and the three catalase-deficient strains. Thus, similar to the CGD model catalase-deficient *A. nidulans* remains highly virulent in immunosuppressed mice containing an intact $p47^{phox}$ gene.

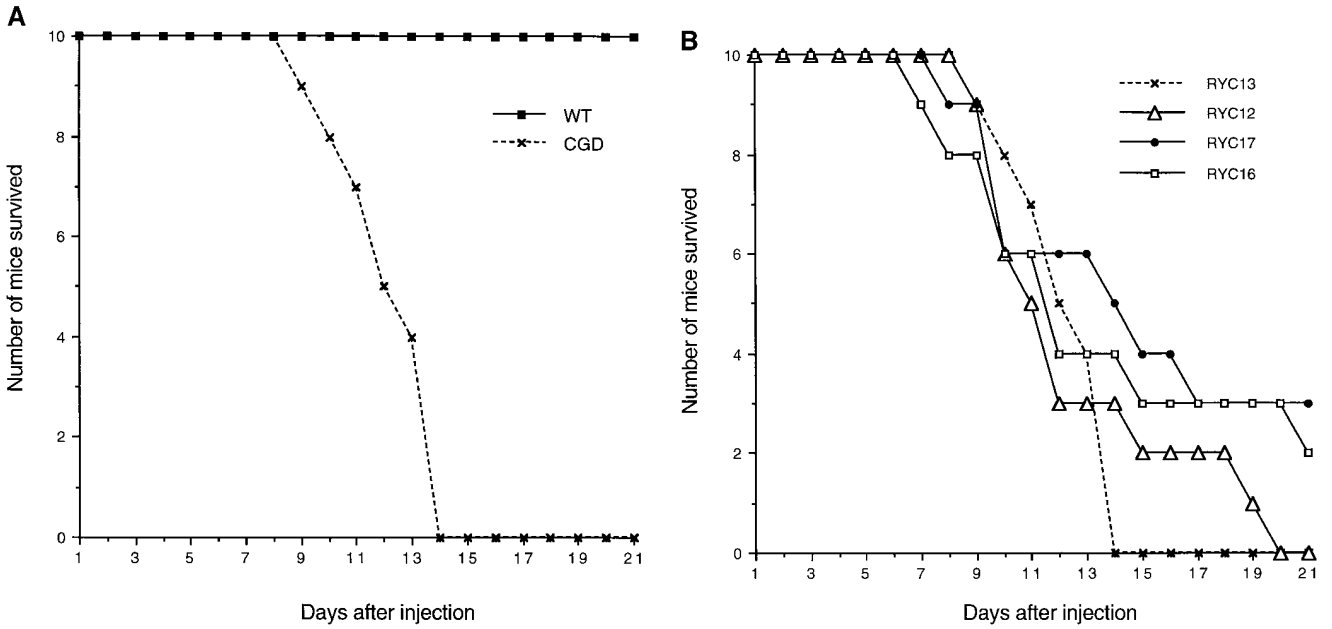

Figure 5. Virulence test of *A. nidulans* in mouse model. (*A*) $p47^{phox-/-}$ (*CGD*, $n = 10$) and wild-type (*WT*, $n = 10$) mice were challenged with 10^5 conidia of wild-type *A. nidulans* (RYC13). (*B*) $p47^{phox-/-}$ mice were inoculated with 10^5 conidia of wild-type (RYC13), *catA* (RYC17), *catB* (RYC12), and *catA*/*catB* (RYC16) mutants. Time course to mortality was similar in wild-type and catalase-deficient strains. Log-rank: $P = 0.4$.

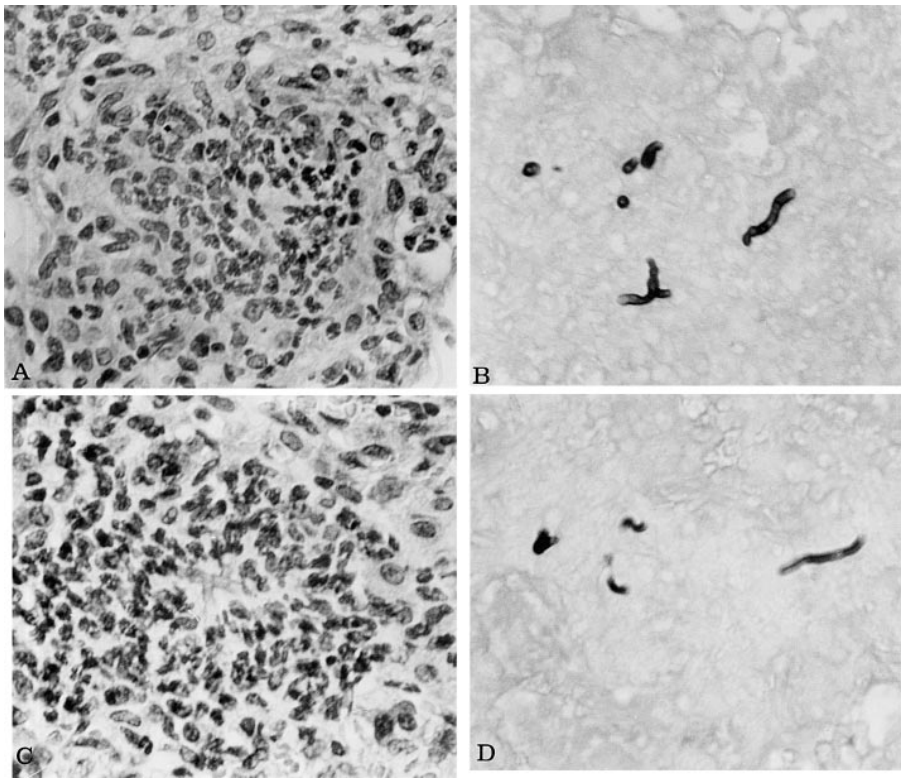

Figure 6. Pulmonary histopathology. Histopathology of p47$^{phox-/-}$ mice infected with wild-type and catalase-deficient *A. nidulans* was similar. Lung sections from p47$^{phox-/-}$ mice infected with the wild-type strain 4 d after challenge (10^5 conidia per mouse) showed several focal lesions composed of neutrophils and macrophages (*A*, H&E, ×450; *B*, GMS, ×450). (*C* and *D*) Lung sections from a p47$^{phox-/-}$ mouse similarly infected with *catA*/*catB* strain (*C*, H&E, ×450; *D*, GMS, ×450).

Neutrophil-induced damage of hyphae. The tetrazolium dye MTT has been useful for quantitative assessment of leukocyte-mediated damage to fungal hyphae (29–31). In our initial experiments, the MTT assay did not provide sufficient sensitivity to demonstrate wild-type neutrophil-mediated hyphal damage.

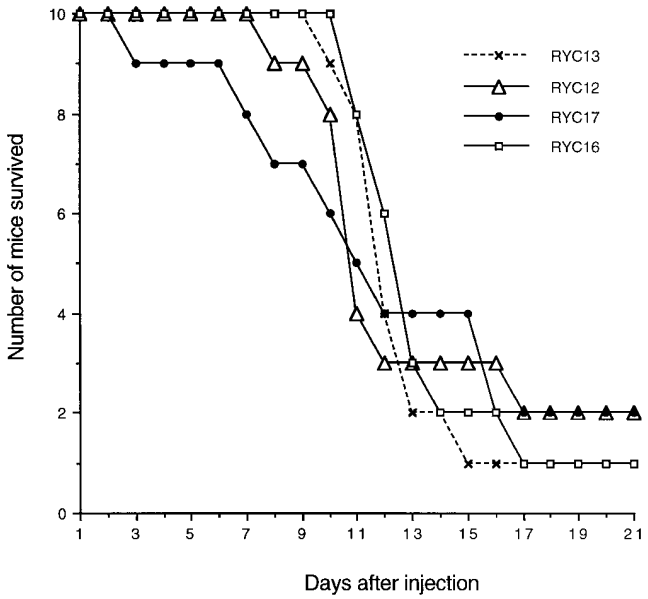

Figure 7. Virulence test of *A. nidulans* in immunosuppressed BALB/c (p47$^{phox+/+}$) mice. Cortisone-treated mice were inoculated with 10^5 conidia of wild-type (RYC13), *catA* (RYC17), *catB* (RYC12), and *catA*/*catB* (RYC16) mutants. Time to mortality was similar in wild-type and catalase-deficient strains. Log-rank: $P = 0.98$.

This lack of sensitivity may relate to the fact that it was necessary to modify the MTT assay protocol by coating assay plates with an agarose layer to prevent loss of hyphae during washing (see Methods). The XTT assay is based on the same principal as the MTT method, but provides greater sensitivity for evaluating hyphal damage (18). Using the XTT assay, wild-type neutrophil-mediated hyphal damage occurred in all *A. nidulans* strains; hyphal metabolic activity was reduced consistently by $\sim$ 30% compared with hyphae incubated without neutrophils (Fig. 8). In contrast, CGD neutrophils failed to cause hyphal damage regardless of the catalase status of the *A. nidulans* strain.

Discussion

A. nidulans strains containing *catA*, *catB,* or *catA*/*catB* mutation were constructed and the in vitro growth rate was similar between wild-type and different catalase mutants. Deletion of one or both catalase genes greatly affected H_2O_2 sensitivity in a stage-dependent fashion. The *catA* gene deletion (RYC17) was associated with enhanced H_2O_2 sensitivity in the conidial stage and the *catB* deletion (RYC12) caused enhanced H_2O_2 sensitivity in growing hyphae. The *catA*/*catB* double deletion strain (RYC16) demonstrated increased sensitivity to H_2O_2 in both the conidial and hyphal stages. Interestingly, *A. fumigatus* also possesses at least two catalases (32). However, disruption of one of the genes encoding catalase of *A. fumigatus* did not affect H_2O_2 sensitivity in this fungus (33).

Surprisingly, deletion of one or both catalase genes did not affect virulence in p47$^{phox-/-}$ or cortisone immunosuppressed mice with an intact p47^{phox} gene. At an inoculum of 10^5 spores in p47$^{phox-/-}$ mice, evidence of extensive multifocal fungal pneumonia was present by day 4 after intranasal inoculation.

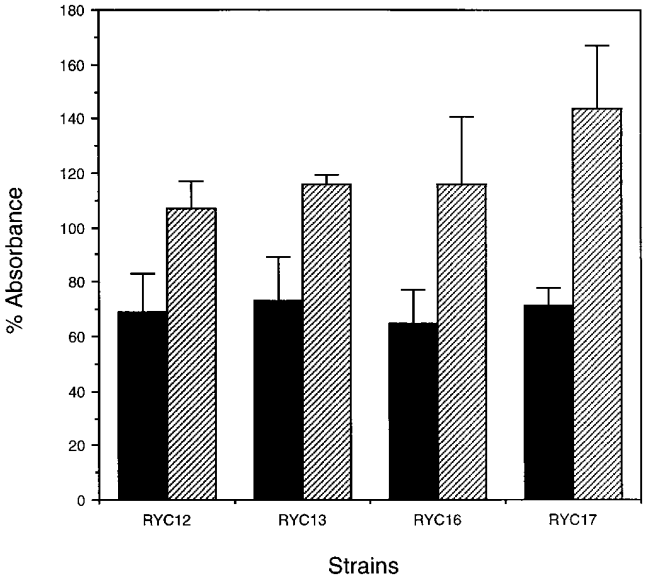

Figure 8. Neutrophil-mediated hyphal damage assayed by the XTT test. Percent absorbance is a measure of hyphal metabolism after incubation with wild-type (*solid bars*) or p47$^{phox-/-}$ (*hatched bars*) neutrophils compared with hyphae incubated without neutrophils (see Methods). Data are derived from three to four separate experiments for each *A. nidulans* strain. The absorbance difference between wild-type and p47$^{phox-/-}$ in all strains of *Aspergillus* is statistical significantly (t test; $P < 0.01$). Data are means±standard deviation. Strains: RYC13 (wild-type); RYC17 (*catA*); RYC12 (*catB*); RYC16 (*catA/catB*).

The majority of p47$^{phox-/-}$ mice died of infection, and necropsy showed mycotic pneumonia involving greater than half of the pulmonary parenchyma (based on microscopic examination of representative sections) regardless of the catalase status of the fungal strain. In contrast, no histopathological changes were observed in wild-type mice at 4 d after inoculation and no deaths occurred in this group. The ability of *A. nidulans* to cause fatal infection in p47$^{phox-/-}$ mice but not in wild-type mice confirms the critical role of the host respiratory burst response in this model, and is consistent with studies of *A. fumigatus* experimental infection in the X-linked mouse model of CGD (10–12).

These in vivo findings are consistent with in vitro XTT studies in which wild-type neutrophils were able to damage *A. nidulans* hyphae as evidenced by reduction in hyphal metabolic activity, whereas CGD neutrophils failed to elicit hyphal damage. This observation is similar to the MTT assay using normal and CGD human neutrophils in *A. fumigatus* (30). Moreover, the catalase status of the *A. nidulans* strains did not affect the antihyphal activity of either wild-type or CGD neutrophils.

In light of the extensive patient experience with CGD, it was surprising that catalase-deficient *A. nidulans* retained virulence in our experimental model. A consistent clinical observation over the past 30 years or so has been that CGD patients are at increased risk from a diverse spectrum of catalase positive pathogens (1). Catalases are enzymes that convert the potentially damaging H_2O_2 to H_2O and O_2. In the setting of infection, catalase produced by the pathogen may protect it from harmful oxidants generated by host phagocytes. It is generally thought that CGD phagocytes, though incapable of generating a detectable respiratory burst response, may take advantage of the H_2O_2 produced by the pathogen and the reactive oxidants generated by the host as by-products of normal aerobic metabolism. Thus, in combination with nonoxidative host defense mechanisms, these low levels of reactive oxidants may enable the phagocyte in CGD to mount an effective host response. However, if the pathogen simultaneously produces catalase, then these low levels of hydrogen peroxide may be rapidly detoxified, and thus be unavailable to the host phagocyte. Therefore, it was reasonable to posit that catalase production by the pathogen may be necessary to establish disease in the CGD host. This model predicts that antihyphal activity of CGD neutrophils would be increased against catalase-deficient *A. nidulans* strains; this hypothesis is clearly refuted by the XTT assay.

Although the number of mice enrolled in mortality studies does not confer sufficient power to detect a small difference in pathogenicity associated with catalase production, the virulence of catalase-deficient *A. nidulans* in p47$^{phox-/-}$ mice was unequivocal. Thus, our data cause us to question the central role of pathogen-derived catalase as an important fungal virulence factor in CGD. It is possible that *A. nidulans* may produce an additional catalase(s) during pulmonary infection that was not detected in the in vitro catalase assays. However, this hypothesis is unlikely in view of the recent report by Kawasaki et al. (19) in which only catalase A and catalase B, but no other catalase, were detected in *A. nidulans* under a variety of experimental stress conditions: heat shock, osmotic stress, and incubation with reactive oxidants. Alternatively, *A. nidulans* may have evolved mechanisms, such as various peroxidases and free radical–scavenging substrates, for detoxifying reactive oxidants. There is ample precedent in various bacterial strains in which a series of protective detoxifying enzymes and "stress" proteins are induced during oxidant stress (34–36). Similar antioxidant mechanisms may occur in *A. nidulans* which enable genetically engineered catalase-deficient strains to retain virulence in the CGD model. This hypothesis is strengthened by the finding that *A. nidulans catA/catB* double mutants are able to grow in media in which low levels of H_2O_2 have been added, and in media containing H_2O_2-generating substrates (19). However, the net concentration of H_2O_2 produced was not determined and, therefore, it is unknown whether the presence or absence of catalase affected H_2O_2 production in the fungi.

We hypothesize that catalase may be one of several reactive oxidant scavengers that may contribute to virulence of pathogens in CGD. A certain threshold of H_2O_2 concentration, in combination with nonoxidative killing mechanisms, may be necessary for host defense against these pathogens. The normal phagocyte is able to generate sufficient reactive oxidants through the NADPH oxidase complex to overcome this threshold. In contrast, the phagocytes in CGD may not be able to accumulate a sufficient quantity of reactive oxidants to meet this threshold. In this situation, pathogen-derived catalase may further shift the balance toward survival of pathogens by reducing the level of H_2O_2 in its surrounding milieu. We speculate that other pathogen antioxidant systems may have a similar function, and may become dominant virulence factors when catalase genes are experimentally deleted.

Among bacteria, virtually all serious infections afflicting CGD patients are caused by catalase-positive pathogens. However, since all medically relevant fungi produce catalase,

the association between microbial catalase and virulence in CGD patients is less clear in fungi than in bacteria. Therefore, catalase may not be an important virulence factor among fungi in CGD patients, whereas its role in bacterial virulence has yet to be tested.

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