Effect of Amphotericin B and Micafungin Combination on Survival, Histopathology, and Fungal Burden in Experimental Aspergillosis in the p47phox−/− Mouse Model of Chronic Granulomatous Disease

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Chronic granulomatous disease (CGD) is an inherited disorder of the NADPH oxidase characterized by recurrent life-threatening bacterial and fungal infections. We characterized the effects of single and combination antifungal therapy on survival, histopathology, and laboratory markers of fungal burden in experimental aspergillosis in the p47phox−/− knockout mouse model of CGD. CGD mice were highly susceptible to intratracheal Aspergillus fumigatus challenge, whereas wild-type mice were resistant. CGD mice were challenged intratracheally with a lethal inoculum (1.25 × 104 CFU/mouse) of A. fumigatus and received one of the following regimens daily from day 0 to 4 after challenge (n = 19 to 20 per treatment group): (i) vehicle, (ii) amphotericin B (intraperitoneal; 1 mg/kg of body weight), (iii) micafungin (intravenous; 10 mg/kg), or (iv) amphotericin B plus micafungin. The rank order of therapeutic efficacy based on prolonged survival, from highest to lowest, was as follows: amphotericin B plus micafungin, amphotericin B alone, micafungin alone, and the vehicle. Lung histology showed pyogranulomatous lesions and invasive hyphae, but without hyphal angiinvasion or coagulative necrosis. Treatment with micafungin alone or combined with amphotericin B produced swelling of invasive hyphae that was not present in mice treated with the vehicle or amphotericin B alone. Assessment of lung fungal burden by quantitative PCR showed no significant difference between treatment groups. Serum galactomannan levels were at background despite documentation of invasive aspergillosis by histology. Our findings showed the superior efficacy of the amphotericin B and micafungin combination compared to either agent alone after A. fumigatus challenge and also demonstrated unique features of CGD mice as a model for experimental aspergillosis.

Invasive aspergillosis is a major cause of morbidity and mortality in highly immunocompromised persons. Chronic granulomatous disease (CGD) is an inherited disorder of the NADPH oxidase complex in which phagocytes are defective in generating the reactive oxidant superoxide anion and its metabolites, hydrogen peroxide, hydroxyl anion, and hypohalous acid. Activation of preformed granular proteases is likely to be principally responsible for NADPH oxidase-mediated destruction of pathogens (25, 31). As a result of the defect in this key host defense pathway, CGD patients suffer from recurrent life-threatening bacterial and fungal infections. CGD patients are susceptible to a broad spectrum of opportunistic filamentous fungi, but Aspergillus infection is by far the most common. Invasive aspergillosis is the most important cause of mortality in CGD (10, 26–28). Genetically engineered CGD mice (X-linked and p47phox−/−) recapitulate the human disease and are highly susceptible to Aspergillus infection (5, 9, 24).

p47phox−/− mice were used in experimental pulmonary aspergillosis to evaluate single and combination antifungal regimens on the basis of survival, histopathology, and fungal burden in lungs. The following four treatment groups were evaluated: (i) vehicle, (ii) amphotericin B, (iii) micafungin, and (iv) amphotericin B plus micafungin. The combination of amphotericin B and micafungin was more effective than either agent alone in prolonging survival after Aspergillus fumigatus challenge. Our study also demonstrated unique features of the CGD mouse model that are distinct from other immunocompromised animal models of experimental aspergillosis.

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MATERIALS AND METHODS

Mice. Mice with a targeted disruption of the p47phox+ gene have a defective NADPH oxidase, rendering phagocytes incapable of generating measurable superoxide (12). CGD mice were derived from C57BL/6 and 129 intercrosses, and mice used were backcrossed either 5 (N5) or 14 (N14) generations in the C57BL/6 background. In all experiments, treatment groups were matched with respect to age (16 to 24 weeks) and generation of backcrossing. Mice were bred...
and maintained under specific-pathogen-free conditions at the animal care facility at Roswell Park Cancer Institute, Buffalo, NY. All procedures performed on animals in this study were approved by the Animal Care and Use Committee of the Roswell Park Cancer Institute and complied with all state, federal, and NHH regulations.

Preparation of conidia. A clinical strain of *A. fumigatus* previously used for experimental aspergillosis in mice (19) was provided to us by Vivanath Kourup (Department of Immunology, Medical College of Wisconsin). Conidia were plated on Sabouraud brain heart infusion (BHI) slants with chloramphenicol and gentamicin (Becton Dickinson, MD), incubated for 7 to 10 days at room temperature, and harvested by washing the slant with 10 ml of 0.01% Tween 20 in normal saline. The conidial suspension was then passed through a 100-μm filter (Falcon), counted on a hemacytometer, and diluted to the desired concentration. By use of approved CLSI (formerly NCCLS) methods for antifungal susceptibility testing of filamentous fungi (MSA®) (20), the 24- and 48-h MICs of amphotericin B for the isolate were 0.5 μg/ml and the MICs of micafungin were ≤0.03 μg/ml.

Intratracheal infections. CGD mice were anesthetized with intraperitoneal (i.p.) injections of Avertin (380 mg/kg of body weight). Mice were restrained, hair was plucked from the throat, and the area was cleansed with alcohol. A medial cut was made in the skin above the trachea, followed by a medial cut in the tracheal sheath. An Abbocath (Fisher Scientific, Atlanta, GA) cannula was inserted into the trachea just above the bifurcation, and 25 μl of the conidial suspension followed by 25 μl of air was injected. Mice were given 1 ml of sterile phosphate-buffered saline i.p. for rehydration, placed on a heating pad, and monitored for recovery.

Preparation of organs. Commercial amphotericin B desoxycholate (McKesson, West Sacramento, CA; 50 mg per vial) was reconstituted in 10 ml of 5% dextrose water and diluted to a concentration of 0.2 mg/ml. Micafungin (Fujisawa, Deerfield, IL; 50 mg per vial) was reconstituted in 10 ml of 0.9% saline and diluted to a concentration of 1.2 mg/ml.

Treatment groups. CGD mice (n = 19 to 20 per treatment group pooled from three separate studies) were challenged with a lethal inoculum of *A. fumigatus* (1.25 × 10^7 CFU/mouse) and received one of the following regimens daily from day 0 to 4 after challenge: (i) intravenous (i.v.) vehicle plus i.p. vehicle, (ii) i.v. gentamicin (10 mg/kg; 166 μg/ml) and the MICs of micafungin were determined by the software, then interpolated from a 6-point standard curve of CT values prepared by spiking naive uninfected mouse lungs with 1 × 10^2 to 1 × 10^7 *A. fumigatus* 293 conidia. Results were reported as *A. fumigatus* DNA conidial equivalents. Lungs from sham-infected mice were used as a specificity control in each experiment.

Serum galactomannan levels. Serum samples were collected from CGD mice 5 days after intratracheal *A. fumigatus* challenge (2.5 × 10^7 CFU/mouse) and frozen at −20°C. Batched serum samples were thawed, and serum galactomannan levels were assessed using the Platelia Aspergillus enzyme immunoassay (Bio-Rad Laboratories, Redmond, WA) as previously described (21, 23).

Statistics. Kaplan-Meier curves were generated to assess time to euthanasia (GraphPad Prism 4.0) and analyzed using the log rank method (lifetest software; SAS, Cary, NC). A P value of <0.05 was considered significant. In experiments involving quantitation of fungal burden (qPCR and serum galactomannan), pairwise comparisons between treatment groups were assessed by the Mann-Whitney test.

**RESULTS**

Amphotericin B and micafungin combination prolongs survival after *A. fumigatus* challenge. Mice receiving the micafungin-plus-amphotericin B combination had significantly longer survival than those receiving amphotericin B alone (P = 0.014 by the log rank test) or micafungin alone (P < 0.0001) (Fig. 1). Mice receiving amphotericin B alone had improved survival compared with those receiving micafungin alone (P = 0.0019) or the vehicle (P < 0.0027). Micafungin alone produced longer survival than the vehicle (P = 0.003 by the log rank test). No morbidity or invasive disease occurred in wild-type mice challenged with an inoculum 100-fold higher than that used for CGD mice (data not shown).

Histopathology. Early (day-5) lung histology following challenge with a lethal inoculum of *A. fumigatus* (1.25 × 10^6 CFU/mouse) was characterized by multiple focal neutrophilic infiltrates and hyphal parenchymal invasion. In mice challenged with a sublethal inoculum (1.25 × 10^5 CFU/mouse), lung histology at day 14 showed discrete well-defined pyogranulomatous lesions in which neutrophils were the predominant cell type in the center and were surrounded by histiocytes and lymphocytes (Fig. 2). Based on our predefined semiquantitative score, the extent of lung involvement was in the group 2 category (26% to 50%) for most of the mice and did not differ between treatment groups. Focal areas of necrosis and giant cells were occasionally observed within these lesions that did not differ among the treatment groups. Angioinvasion and coagulative necrosis, which are typically observed in invasive aspergillosis in neutrophic hosts, were not observed in CGD mice.

At both the early (day-5) and later (day-14) time points, a treatment effect in mice receiving micafungin occurred. There was a striking swelling of invasive hyphae, particularly at the tips, in mice treated with micafungin alone or in combination with amphotericin B (Fig. 3). This morphology was not observed in mice treated with the vehicle or amphotericin B alone.

Assessment of fungal burden. Fungal burden was assessed by quantitative fungal PCR of whole lungs and serum galactomannan levels after *A. fumigatus* challenge. In mice receiving a
sublethal inoculum (1.25 × 10³ CFU/mouse), followed by anti-
fungal therapy (days 7 to 11) and lung harvest on day 14, there was no difference in lung fungal DNA burden between treatment groups in two separate experiments. When a lethal inoculum was used (1.25 × 10⁴ CFU/mouse), followed by antifungal therapy (days 0 to 4) and lung harvest on day 5, there was significant variability between two independent experiments, but no consistent treatment effect was observed in pairwise comparisons (data not shown). Eight to 10 mice per treatment group were used in each experiment.

In experiments involving the galactomannan assay, mice were challenged with *A. fumigatus* (2.5 × 10³ CFU/mouse), received antifungal therapy daily from days 0 to 4 (as described above), and were terminally bled on day 5. Serum galactomannan levels were at background in all treatment groups (n = 5 mice per treatment group) and were similar to those of sham-infected mice, despite histologic evidence of invasive aspergillosis (Fig. 4).

**DISCUSSION**

We characterized the effect of single and combination anti-
fungal therapy on survival, histopathology, and laboratory markers of fungal burden in experimental aspergillosis in the p47^phox^−/− mouse model of CGD. The combination of amphotericin B and micafungin was more effective than either agent alone in prolonging survival. The rank order of therapeutic efficacy, from highest to lowest, was as follows: amphotericin B plus micafungin, amphotericin B alone, micafungin alone, and the vehicle (Fig. 1). The sample size (n = 19 to 20 per treatment group), the low variation within each group, and the magnitude of intergroup differences all contributed to identi-

![FIG. 1. CGD mice were challenged intratracheally with *A. fumigatus* conidia (1.25 × 10⁶ CFU/mouse). Mice (n = 19 to 20 per treatment group pooled from three experiments) received antifungal therapy daily from day 0 to 4 after challenge. Mice receiving combination micafungin plus amphotericin B had significantly longer survival than those receiving amphotericin B alone (P = 0.014 by the log rank test) or micafungin alone (P < 0.0001).](image)

![FIG. 2. Histopathology of invasive aspergillosis in CGD mice. Mice were administered a sublethal intratracheal *A. fumigatus* challenge (1.25 × 10³ CFU/mouse), followed by antifungal therapy (see Materials and Methods) and lung harvest on day 14. (A) A representative lung section shows a well-circumscribed pyogranulomatous lesion (H&E staining; magnification, ×100). (B) Higher-power magnification demonstrates a predominance of neutrophils in the center of the lesion (H&E staining; magnification, ×400), surrounded by histiocytes and lymphocytes. No difference in inflammation was observed between antifungal regimens and the vehicle. Coagulative necrosis, a characteristic feature of pulmonary aspergillosis during neutropenia, was not observed in CGD mice.](image)
fying statistically significant intergroup differences. Changes in hyphal structure that we observed in CGD mice treated with micafungin were previously reported in experimental pulmonary aspergillosis in animals treated with an echinocandin (22, 35) and likely result from the echinocandin abrogating the structural integrity of the hyphal cell wall.

Our study demonstrating prolonged survival in CGD mice receiving the amphotericin B and micafungin combination following *Aspergillus* challenge is consistent with other models of experimental aspergillosis showing enhanced efficacy of combination regimens pairing an echinocandin with either an azole or an amphotericin B formulation (13, 15, 23). Other studies showed no significant enhanced benefit of combination antifungal regimens in experimental aspergillosis (8, 11, 29). Indeed, the lack of consistent results between studies is not surprising considering the variability in the type of animal

FIG. 3. Micafungin alone and in combination with amphotericin B caused distinctive swelling of hyphae in lungs of CGD mice. Mice were administered a sublethal intratracheal *A. fumigatus* challenge (1.25 × 10⁵ CFU/mouse), followed by antifungal therapy (see Materials and Methods) on days 7 to 11 and lung harvest on day 14. Representative lung sections from mice treated with vehicle (A), amphotericin B (B), or micafungin (C) are shown (silver staining; magnification, ×400).

FIG. 4. Serum galactomannan levels following *A. fumigatus* challenge and correlation with histopathology. CGD mice (n = 5 per treatment group) challenged with intratracheal *A. fumigatus* (2.5 × 10⁵ CFU/mouse) received antifungal therapy from day 0 to 4 and were terminally bled on day 5 after challenge. (A) Serum galactomannan levels were at background and were similar to those in sham-infected mice despite the demonstration of invasive aspergillosis. Amb-D, amphotericin B. (B and C) Representative lung sections at day 5 after *A. fumigatus* challenge show neutrophilic infiltration (H&E staining; magnification, ×200) (B) and hyphal invasion (silver staining; magnification, ×200) (C).
model tested, the immunosuppression used, the route of fungal challenge, and the timing and dosing of antifungal regimens. In the clinic, combination antifungal therapy as salvage therapy for invasive aspergillosis has produced promising results (14, 16, 18). A well-designed randomized study will be required to demonstrate the value of combination regimens as primary therapy for invasive aspergillosis.

A limitation of our study is that we tested only one dose of amphotericin B (1 mg/kg/day) and micafungin (10 mg/kg/day). While these doses were selected based on previously published studies of experimental aspergillosis (33), the total daily dose and schedule of administration may not be optimal. Wiederhold et al. (35) showed that the echinocandin caspofungin demonstrated concentration-dependent pharmacodynamics in the treatment of murine pulmonary aspergillosis in mice rendered neutropenic and treated with corticosteroids. Because we did not assess a range of doses of study drugs, we cannot address the question of “in vivo synergy” between amphotericin B and micafungin. For example, amphotericin B at double the dose we used may produce the same (or a greater) survival advantage as the combination of amphotericin B and micafungin. Indeed, an analysis of in vivo synergy would require a prohibitively large number of mice, emphasizing the need for in vitro methods that effectively model the in vivo state.

Important differences exist between CGD mice and other experimental models of aspergillosis. In neutropenic animals, experimental pulmonary aspergillosis is characterized by hyphal angioinvasion, coagulative necrosis, and a paucity of inflammatory cells (2, 3). In contrast, Aspergillus infection causes robust pyogranulomatous responses in CGD mice with occasional foci of necrosis. Angioinvasion was not observed in CGD mice, nor is it a characteristic feature of invasive aspergillosis in CGD patients (1, 26). The presence or absence of ischemic necrosis will affect the local inflammatory response, tissue oxygen content, tissue pH, and drug delivery to the infected site, all of which may influence the efficacy of antifungal regimens (30, 34). Bignell et al. (4) recently reported that even Aspergillus nidulans mutants strain of low virulence caused mortality in experimental pulmonary aspergillosis in CGD (p47phox−/−) mice as a result of an excessive inflammatory response. This finding emphasizes the unique pathophysiological features of aspergillosis in CGD mice that must be considered in studies of pathogen virulence and antifungal agents.

Surprisingly, lung fungal burden assessed by qPCR did not differ among treatment groups despite significant differences in survival between treatment groups demonstrated in separate experiments. Using the same method to quantify lung fungal burden, Wiederhold et al. (35) demonstrated a treatment effect in a high-inoculum, hyperacute model of pulmonary aspergillosis in which survival and fungal burden were assessed 4 days after fungal challenge. Bowman et al. (7) demonstrated the utility of qPCR in monitoring the treatment effect of amphotericin B and caspofungin in murine disseminated aspergillosis. The lack of utility of qPCR in modeling a treatment effect in our studies may reflect factors specific to the CGD mouse model as well as variables related to the inoculum and the route of fungal challenge.

Serum galactomannan levels were at background in CGD mice, despite histological evidence of parenchymal invasion. The Platelia Aspergillus enzyme immunoassay (Bio-Rad Laboratories, Redmond, WA), a sensitive double sandwich enzyme-linked immunosorbent assay that detects the fungal cell wall constituent galactomannan, has been approved for the diagnosis of invasive aspergillosis. Response to therapy in patients (6, 17) and in experimental aspergillosis in neutropenic animals (17, 21) correlates with a decrease in galactomannan levels. While in neutropenic mice experimental pulmonary aspergillosis led to high levels of galactomannan in the lungs and extrapulmonary organs, galactomannan levels were close to background in corticosteroid-treated mice with pulmonary aspergillosis (2). In agreement with our findings for CGD mice, Walsh et al. (32) reported reduced expression of galactomannan antigenemia in patients with invasive aspergillosis and chronic granulomatous disease or Job’s syndrome. The lack of hyphal angioinvasion in aspergillosis in CGD may lead to reduced systemic release of galactomannan and lack of detection in serum. Thus, a negative serum galactomannan result should not be relied on to exclude invasive aspergillosis in CGD patients.

CGD mice are a valuable model for evaluation of antifungal therapeutics that is complementary to models of iatrogenic immunocompromised states. CGD mice are susceptible to Aspergillus challenge and do not require exogenous immunosuppressive agents. The low frequency of spontaneous infections in CGD mice reduces the likelihood of confounding causes of mortality in survival experiments, thus reducing intragroup variability in survival. When lower inocula of A. fumigatus conidia are used for challenge, survival, fungal burden, and pathology can be evaluated over prolonged periods, which more closely resembles the clinical situation. CGD mice may also be a promising model in which to evaluate immunotherapeutic strategies that augment NADPH oxidase-independent innate and antigen-specific host defense pathways.

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REFERENCES


