Novel Inhalational Murine Model of Invasive Pulmonary Aspergillosis

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Received 4 December 2003/Returned for modification 31 December 2003/Accepted 15 January 2004

We developed a novel model of invasive aspergillosis (IA) that recapitulates human disease. Mice were immunosuppressed with cyclophosphamide and cortisone acetate and then infected in an aerosol chamber. This procedure reproducibly delivered 1×10^3 to 3×10^3 conidia to the lungs. Lethal pulmonary IA developed over 2 weeks and was prevented by amphotericin B.

The last 2 decades have seen a rise in the incidence of invasive infections due to *Aspergillus* species, particularly *A. fumigatus* (4, 8). Despite the development of new antifungal agents, mortality from invasive aspergillosis (IA) remains at least 50% (1, 2, 6). As a result, there is an urgent need for further study of this disease and the development of new clinical strategies to reduce mortality associated with IA. A simple, reproducible animal model that recapitulates the pathogenesis of human IA is a critical tool for developing new methods to prevent, diagnose, and treat this disease (7).

IA is initiated by the inhalation of small numbers of *A. fumigatus* conidia which, by virtue of their small size, evade the mucociliary barrier of the bronchial airways and are deposited in the pulmonary alveoli (7). In the immunosuppressed host, conidia germinate to produce invasive hyphal forms, which propagate by extension and invade local pulmonary tissues. Angioinvasion is a prominent histopathological and clinical feature of IA and is likely the mechanism for dissemination of hyphal fragments to other organs in a subset of patients.

We have developed a murine model of invasive pulmonary aspergillosis that recapitulates many of the key factors of human IA. By using an inhalation chamber, a relatively small number of conidia are delivered by small-particle aerosol to immunocompromised mice. This delivery system is reproducible both among mice within an experiment and between experiments. This route of inoculation results in a lethal infection in 60 to 75% of animals within 16 days. Additionally, we show that amphotericin B (AMB) is protective in this model.

Strains and media. *A. fumigatus* strain AF293 (a generous gift from P. Magee) was used for all experiments in this study. To prepare the inoculum, *A. fumigatus* was grown on Sabouraud dextrose agar plates for 2 weeks at 37°C. Conidia were collected by flooding the plates with sterile phosphate-buffered saline containing 0.2% (vol/vol) Tween 80. The conidia were concentrated by centrifugation and counted using a hemacytometer.

Inhalation apparatus. To infect animals via inhalation, we adapted the aerosol chamber described by Pal and Horwitz

(10) for use with *A. fumigatus* conidia (Fig. 1). Mice were introduced via a hinged doorway to a Plexiglas exposure chamber (South Bay Plastics, Torrance, Calif.). The inoculum was introduced by aerosolizing a conidial suspension with a small-particle nebulizer (Hudson Micro Mist; Hudson RCI, Temecula, Calif.) driven by compressed air at 100 lb/in². The nebulizer was connected to a channel that ran along the top of the chamber and vented the aerosol from the middle of the chamber ceiling. A standard exposure time of 1 h was used for all experiments to allow time for complete aerosolization and uniform exposure of the mice. The entire apparatus was contained within a laminar flow hood in a negative pressure room. Multiple immunosuppressive regimens and inocula were evaluated as outlined below. In all experiments, mice were infected 2 days after the initiation of immunosuppression and received daily ceftazidime (Western Medical Supply, Arcadia, Calif.) at 5 mg in a 0.2-ml volume per mouse subcutaneously for 10 days to protect against bacterial infection.

Mice. Female BALB/c mice (National Cancer Institute, Bethesda, Md.), 18 to 22 g, were used for these experiments. Mice were housed five per microisolator cage, with irradiated food and nonsterile water available ad libitum. All procedures involving mice were approved by the institutional animal use and care committee, according to the National Institutes of Health guidelines for animal housing and care.

Titration of infectious inoculum. As an initial step in the evaluation of the aerosol chamber, mice were exposed to aerosols generated from conidial suspensions of various concentrations. Immediately after exposure, mice were sacrificed, and the lungs were homogenized and quantitatively cultured to determine the number of conidia delivered to the lungs. Nebulizing 12 ml of a suspension containing 1×10^9 conidia per ml delivered a reproducible number of conidia per mouse within and between experiments (median, 2.4×10^3 CFU/mouse; interquartile range [IQR], 2.2×10^3 to 2.9×10^3 [results from three independent experiments of three animals each]). Lower-concentration conidial suspensions resulted in both lower conidial delivery and more mouse-to-mouse variability in inoculum (data not shown). Therefore, 12 ml of a suspension containing 1×10^9 conidia/ml was used for all subsequent experiments.

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FIG. 1. Inhalation infection apparatus. Inset panel depicts the compressed air-driven nebulizer connected to a channel across the top of the chamber. This channel leads to an opening located in the center of the chamber ceiling. Nebulized conidia enter the chamber through this orifice and are dispersed throughout the chamber. A door at the right end of the chamber permits access.

Immunosuppression. Mice were rendered susceptible to infection with *A. fumigatus* by immunosuppression with a combination of cyclophosphamide and cortisone acetate. Cyclophosphamide (Western Medical Supply) was reconstituted in sterile distilled water and administered via intraperitoneal injection. Cortisone acetate (Sigma-Aldrich, St. Louis, Mo.) was prepared as a suspension in sterile phosphate-buffered saline with 0.02% Tween 80 and given by subcutaneous injection. To determine the duration of leukopenia resulting from immunosuppression, serial tail vein phlebotomy (10- μ l sample) was performed on groups of five mice, and leukocytes were enumerated by using the Unopette system (Fisher Scientific, Hampton, N.H.). A single dose of cyclophosphamide (200 mg/kg of body weight) and cortisone acetate (250 mg/kg) resulted in profound leukopenia for 6 days. However, mice treated with this combination did not consistently develop a lethal infection, even when exposed to the highest aerosol concentrations (data not shown). Increasing the dose of cyclophosphamide to 250 mg/kg increased the duration of leukopenia by only 1 day (data not shown). Therefore, mice were given an initial dose of cyclophosphamide and cortisone acetate (250

mg/kg for both drugs) and then a second dose of cyclophosphamide (200 mg/kg) combined with cortisone acetate (250 mg/kg) 5 days later (Fig. 2). This regimen extended the duration of leukopenia to 9 to 10 days, providing 7 to 8 days of leukopenia after infection and permitting the development of lethal infection (see below). With this combination, uninfected mice had lost more than 20% of their initial body weight and were too ill to receive further immunosuppression. This regimen was therefore used for all subsequent experiments.

Course of infection. Using two doses of immunosuppression and aerosolizing 12 ml of a suspension containing 1×10^9 conidia/ml resulted in lethal infection with mortality ranging from 60 to 75% (Fig. 3). Mortality occurred late in infection, usually beginning five or more days after inoculation. Of note, smaller mice (18 to 20 g) displayed increased susceptibility to infection, with mortality as high as 90%. In a separate experiment, infected mice were sacrificed at select time points, and the lungs were removed for histopathological examination by periodic acid-Schiff (PAS) staining. Early on in infection, the fungal burden was too low to be detected using PAS staining. By day 7, large numbers of isolated foci of hyphae could be

FIG. 2. Duration of leukopenia induced by two doses of cyclophosphamide and cortisone acetate. Data are shown as mean leukocyte counts $\pm$ standard deviations. Five mice were sampled at each time point. The X denotes the days of administration of cyclophosphamide and cortisone acetate. WBC, white blood cells.

FIG. 3. Survival of mice infected by inhalation and treated with two doses of immunosuppression. Results are the combination of three independent experiments (total n of 27 for each group). *, P of <0.001 by log-rank test compared with values for uninfected mice. ◆, uninfected mice; ■, infected mice.

identified throughout both lungs, with prominent tissue destruction and vascular invasion evident (Fig. 4A and B). After leukocyte recovery, these foci of infection became the target of dramatic leukocyte infiltration, forming large fungal abscesses

(Fig. 4C and D). These infiltrates were composed predominantly of neutrophils, although some mononuclear cells were observed. Angioinvasion was also present, often associated with thrombosis, and in some mice, areas of pulmonary infarction were seen. In long-term survivors, destruction and fragmentation of hyphal elements within abscesses occurred, although intact hyphae were visible up to 16 days after infection (data not shown).

Efficacy of AMB. To investigate the utility of this model in the evaluation of antifungal therapies, we evaluated the efficacy of AMB in the treatment of inhalational IA. Mice were immunosuppressed and infected with *A. fumigatus* as described above. The day after infection, mice received either AMB (solubilized AMB; Sigma-Aldrich) reconstituted in 5% glucose (D5W) at a dose of 3 mg/kg or an equivalent volume of D5W intraperitoneally. Therapy was continued until the predicted resolution of neutropenia 8 days after infection. To determine the pulmonary fungal burden, mice were immunosuppressed and infected with *A. fumigatus* as described above. Six days after infection, mice were sacrificed; the lungs were removed aseptically and homogenized in 5 ml of sterile saline. Quanti-

FIG. 4. PAS-stained lung sections of mice infected with *A. fumigatus* by inhalation. (A) Lung tissue 7 days after inoculation showing a focal lesion of IA with invasion of a neighboring blood vessel indicated by the arrow ($\times 100$). (B) Higher magnification ($\times 400$) demonstrating complete penetration of the blood vessel wall by hyphae (arrow). (C) Lung tissue 10 days after inoculation (after leukocyte recovery) showing the formation of a parenchymal fungal abscess ($\times 100$). (D) Higher magnification ($\times 400$) demonstrating abundant hyphae surrounded by leukocytes.

FIG. 5. Survival of mice infected with *A. fumigatus* and treated with AMB, combined results of two experiments, total n of 20 per group. *, P of <0.001 by log-rank test compared with values for mice receiving 5% dextrose agents. ◆, AMB at 3 mg/kg/day; ■, vehicle (5% dextrose).

tatively cultured AMB treatment markedly reduced mortality compared with that for infected mice receiving D5W alone (Fig. 5). This protection was associated with a modest, but significant, reduction in fungal burden determined after 7 days of infection (median $\log_{10}$ CFU per gram, 3.3; IQR of 2.9 to 3.8 in control mice versus 2.7 and IQR of 2.6 to 3.0 in AMB-treated mice [$P < 0.05$ by Wilcoxon rank-sum]).

We have developed a simple, relatively inexpensive murine model of IA that uses a combination of high-dose immunosuppression and small-particle inhalation to produce infection by delivering a relatively low inoculum of conidia directly to the alveoli. This methodology allows for infection of up to 40 animals at a time in a single chamber, with an inoculum that is consistent between experiments and among mice in any given experiment. Lethal, invasive pulmonary infection results in the majority of mice, producing a histopathological picture very similar to human IA.

The murine model described here is unique in that infection is initiated with a relatively small infectious inoculum, and consequently, the duration of infection is significantly longer than in models of IA which use a higher inoculum (10^4 to 10^9 depending on immunosuppressive regimen and strain of *A. fumigatus*) to initiate infection and result in a more precipitous course of disease (3, 5, 7).

The initiation of infection with an inoculum that approximates that of human infection has important implications for the testing of novel therapeutics and diagnostic strategies. Inoculum size is particularly relevant when testing an antifungal compound for prophylaxis against IA. Since such an agent needs only to be active against the relatively small number of conidia being inhaled on a daily basis, it may perform adequately as a prophylactic agent and yet fail to demonstrate efficacy when tested in models that utilize higher infectious inocula. Furthermore, in the inhalational model described here, mice survived for 5 to 15 days after infection. Not only does this subacute course parallel the development of human IA, but it also provides a reasonable window for the study of antifungal agents as well as diagnostic modalities designed to detect early disease.

In addition to these therapeutic considerations, the use of a low-dose inhalational model should more faithfully recapitulate the human host response to IA. Since the immune response to a wide range of pathogens has been shown to be dependent on inoculum (9, 11), approximating an inoculum similar to that found in human IA provides an appropriate model for studying the pathogenesis of IA.

This inhalational model of IA provides a simple method to study this important fungal infection. It is inexpensive, with an initial chamber construction cost of approximately \$200 and the nebulizers costing only \$1 to \$2 each. This model provides an alternative to existing intranasal models of infection and may offer new insights into the pathogenesis, diagnosis, and treatment of IA.

We are grateful to M. Horwitz for assistance with the design of the inhalation chamber, S. French for assistance with histopathological studies, B. Spellberg for helpful comments, and F. Monroy for technical assistance.

This project was supported in part with federal funds from the National Institute of Allergy and Infectious Diseases, under contract no. N01-AI-30041 and by a grant from Hollis-Eden Pharmaceuticals Inc. D.C.S. is supported by a Burroughs Wellcome Fund Career Award in the Biomedical Sciences and a Clinician Scientist award from the Canadian Institutes of Health. A.S.I. and S.G.F. are supported by Burroughs Wellcome Fund New Investigator Awards in Molecular Pathogenic Mycology.

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