Experimental Pulmonary Aspergillosis Due to Aspergillus terreus: Pathogenesis and Treatment of an Emerging Fungal Pathogen Resistant to Amphotericin B

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Aspergillus terreus is an uncommon but emerging fungal pathogen, which causes lethal infections that are often refractory to amphotericin B (AmB). In comparison to Aspergillus fumigatus, A. terreus was resistant to the in vitro fungicidal effects of safely achievable concentrations of AmB. These in vitro findings correlated directly with resistance of A. terreus to AmB in experimental invasive pulmonary aspergillosis. Residual fungal pulmonary burden and galactomannan antigenemia demonstrated persistent infection, despite therapy with deoxycholate AmB or liposomal AmB. By comparison, posaconazole and itraconazole resolved GM antigenemia, reduced residual fungal burden, and improved survival. There were no differences in phagocytic host response to A. terreus versus A. fumigatus; however, the rate of conidial germination of A. terreus was slower. The strain of A. terreus with the highest minimum inhibitory and minimum lethal concentration of AmB also had the lowest membrane ergosterol content. The hyphae of A. terreus in vivo displayed distinctive aleurioconidia, which may be a practical microscopic feature for rapid preliminary diagnosis.

Invasive pulmonary aspergillosis is an important cause of morbidity and mortality in immunocompromised patients [1–4] and is the leading cause of infectious pneumonic mortality in blood and marrow transplant recipients [5]. Aspergillus fumigatus accounts for the majority of these cases. However, there have been increasing reports of pneumonia and disseminated infections due to Aspergillus terreus [6–21]. These reports underscore the observation that invasive pulmonary aspergillosis caused by A. terreus is often refractory to standard treatment with amphotericin B (AmB).

However, little is known about the pathogenesis and treatment of infections due to A. terreus. Whether its refractoriness to AmB is caused by the intrinsic resistance of the organism or the profound immunosuppression is not well understood. Using persistently neutropenic rabbit models of invasive pulmonary aspergillosis due to A. terreus and A. fumigatus, we comparatively examined the pathogenesis and treatment of these diseases. The antifungal activity of conventional and liposomal AmB, as well as that of two antifungal triazoles, posaconazole (SCH56592) and itraconazole, were investigated in these animal models to examine alternative treatment strategies for A. terreus infections. The possible mechanisms of resistance of A. terreus were further investigated by microbicidal time-kill assays, colorimetric MTT assays of hyphal damage, and sterol composition analysis of the fungal cell membrane by gas-liquid chromatography (GLC). Finally, the monocye-macrophage host response to A. terreus compared with A. fumigatus was examined using peripheral blood monocytes (MNCs) and rabbit pulmonary alveolar...
Materials and methods

Organisms
Organisms were identified by standard mycological methods [31]. A. terreus (National Institutes of Health [NIH] isolate 2624, Fun-gus Testing Laboratory isolates 95–644, and 96–1290) and A. fumigatus (NIH isolate 4215, Fungus Testing Laboratory isolates 97–2350, and 97–2025) were obtained from lung, sputum, and bronchoalveolar lavage specimens of patients with pulmonary aspergillosis. Conidia were prepared from frozen isolates that were subcultured onto potato dextrose agar (PDA) slants; these slants were incubated for 24 h at 37°C and then kept at room temperature for 5 days. Conidia were harvested under a laminar air flow hood with a solution of 0.025% Tween 20 (Fisher Scientific) in normal saline, transferred to a 50-mL conical tube, washed, and counted in a hemacytometer.

In Vitro Antifungal Studies
Minimum inhibitory concentrations (MICs) and minimum lethal concentrations (MLCs). MICs were determined by broth macrodilution methods described by the National Committee for Clinical Laboratory Standards (NCCLS) [22]. A conidial suspension was adjusted with a spectrophotometer to 68%–71% transmission at λ = 530 nm and diluted 100-fold to yield an inoculum of 1–5 × 10⁵ cfu/mL. Deoxycholate AmB (D-AmB; Bristol Myers-Squibb) was tested in Antibiotic Medium 3 (AM3) buffered to a pH of 7.0 (NIH Media Department, Bethesda, MD), and the antifungal triazoles were tested in RPMI 1640 medium supplemented with 0.3 g/L L-glutamine, 0.165 mol MOPS (buffered to a pH of 7.0), and phenol red (BioWhittaker). A 100-μL quantity of concentrated antifungal compound was diluted 10-fold into 900 μL of inoculum suspension and incubated at 35°C for 24 and 48 h. Final concentration ranges for AmB, itraconazole (Janssen Pharmaceutica), and posaconazole (SCH56592; Schering-Plough) were 0.03–16.0 μg/mL. The azoles were provided in reagent grade powders. The MIC was defined as the lowest concentration of antifungal compound that rendered no growth. The MLC was determined as the lowest concentration of antifungal compound that rendered no growth. The MLC was determined by culturing 100 μL of broth from the next 4 tubes above the MIC exhibiting no growth, onto Sabouraud glucose agar (SGA) plates, and incubating at 35°C. A growth of ≤3 colonies was considered to be fungicidal. The MLC was defined as the lowest concentration of antifungal compound with growth of ≤3 colonies. MICs and MLCs were run in duplicate and repeated on 3 different days. Determinations of MICs and MLCs were performed in 7 experiments.

Time-kill assay. The time-kill assay was performed using 3 concentrations of D-AmB (1, 4, and 20 μg/mL) and an untreated growth control. An inoculum of 1 × 10⁵ conidia/mL of each of the 3 isolates of A. terreus and 3 isolates of A. fumigatus was incubated in 10-mL volumes of AM3 alone or AM3 plus antifungal compound in polypropylene tubes at 37°C. Samples were taken from each tube at 0, 2, 6, and 24 h. These samples were cultured on SGA and incubated at 37°C for 24 h, and then placed at room temperature for an additional 24 h. Colonies were counted, and the calculated colony-forming unit per milliliter was plotted for each time point.

MTT assay for hyphal damage. Hyphal damage was assessed by a colorimetric assay using MTT, a yellow salt that is cleaved by dehydrogenases in eukaryotic cells to its purple formazan derivative (MTT-formazan), as described elsewhere [23, 24]. In brief, conidia of A. fumigatus or A. terreus were incubated in yeast-nitrogen base broth with 2% glucose in flat-bottom 24-well microtiter plates at 35°C for 16–24 h to promote germination. The plates were removed from the incubator and were examined for uniform hyphal growth. Medium was aspirated, and the organisms then were exposed to AmB in AM3 at 0.25, 0.5, 1.0, 2.0, or 4.0 μg/mL for 2, 6, or 24 h at 37°C. At the respective time points, the drug was aspirated, and the organisms were washed 3 times with sterile water. Subsequently, 1 mL of RPMI 1640 medium without phenol red containing 0.5 mg/mL of MTT was added to each well, and the plates were incubated at 37°C for an additional 3-h period. The wells then were aspirated dry, and 200 μL of isopropanol was used to extract the dye from the viable hyphae. Aliquots of 150 μL were transferred to a 96-well microtiter plate, and absorbance was measured on a Titerhek Multiscan microplate spectrophotometer (Titertek Multiscan MMC/340) at the dual wavelength 570/690 nm. A blank well (containing only isopropyl alcohol and control wells (containing only hyphae and media alone) were included on each plate. The percentage of hyphal damage was determined as follows: percentage of hyphal damage = [(OD of control wells – OD of test wells)/OD of control wells] × 100, where control wells contained hyphae only.

Membrane Sterol Analysis
Sterol extraction. Sterols were extracted by the method of Ghannoum et al. [25]. In brief, 1.5 g of KOH in 2 mL of distilled water and 8 mL of ethanol were added to 0.2 g (wet weight) of hyphae. The mixture was refluxed for 3 h under nitrogen. The refluxed mixture was filtered, diluted with an equal amount of water, and extracted 4 times with heptane. The extract was dried with anhydrous Na₂SO₄, and the solvent was removed by using a rotovaporizer. The purified sterols were
weighed and dissolved in chloroform (10 mg/mL) and stored under nitrogen at −20°C until being analyzed.

**Sterol analysis.** Sterols were analyzed by GLC. Silylated sterols were prepared for GLC studies by a modification of the method of Vandenheuvel and Court [26, 27]. In brief, 2 mg of extracted sterols was reacted with 0.1 mL of hexamethyldisilazane (Sigma Chemical) and 0.1 mL of 10% (vol/vol) trimethylchlorosilane (Sigma) in chloroform. The reaction mixture was gently mixed, covered with aluminum foil, and stored at room temperature for 4 h. Next, the excess solvent was evaporated under nitrogen, and the samples were redissolved in 50 μL of hexane. One-microliter samples were analyzed by GLC with an OV-1 column (3% on 100/120 gaschrome Q) in a Vista 6000 (Varian) gas chromatograph. The samples were eluted with helium as the carrier gas (30 mL/min). The column temperature was 230°C, whereas the injection temperature was set at 250°C, and the flame ionization temperature was set at 300°C. Sterols were identified by a comparison of their retention times with authentic standards or relative to the retention time of ergosterol. GLC peaks were quantified by use of a Varian Vista 402 integrator. Unless otherwise specified, all chemicals used were reagent grade purity and were purchased from Fisher Scientific. Differences in sterol content between hyphae of A. terreus and A. fumigatus were expressed as the percentage of variability.

**Host Defense Studies**

**Cell separation.** Human monocytes (MNCs) were isolated from blood samples obtained from healthy adult volunteers by elutriation at the Transfusion Medicine Department of the Clinical Center (NIH). MNC pellets were >95% CD14+ and nonspecific esterase stain. To allow for differentiation of MNCs to MNC-derived macrophages, cells were resuspended to a concentration of 1 × 10^6/mL in RPMI 1640 medium containing 25% pooled human serum (BioWhittaker), 100 μ/mL penicillin, and 100 μg/mL streptomycin (CM-hu), and then incubated with MTT for 3 h. Dye was extracted, and supernatants were read spectrophotometrically, as described above.

**Phagocytosis of conidia by PAMs.** One million PAMs suspended in 200-μL aliquots of CM were placed on 18-mm sterile round glass coverslips in 12-well plates (Costar) and were incubated at 37°C with 5% CO_2 for 1 h. The coverslips were washed once with prewarmed MHS, and 1 mL of medium containing 10^6 A. fumigatus or A. terreus conidia was added. After incubation for 60 min, coverslips were washed, and cells were fixed and stained by periodic acid–Schiff (PAS) stain. Phagocytic activity was assessed by light microscopy in triplicate coverslips, as described elsewhere [16]. Percentage of phagocytosis was the percentage of PAMs that had >1 conidia phagocyted or attached among 100 cells counted. Phagocytic index was the average number of conidia that had been ingested or attached to each phagocytosing cell.

**Conidiodial assay.** A standard colony-forming unit assay was used to assess MDM conidiodial activity. MDMs were mixed with 10^6 conidia at an E:T ratio of 1:1 or 5:1 in a final volume of 1 mL of CM. Control tubes containing conidia only were included in each experiment. Mixtures were rotated at 37°C for 4 h, followed by complete lysis of MDMs with sterile H_2O. Serial dilutions were prepared, and aliquots were plated in duplicate on SGA plates and incubated at 37°C for 24 h and at room temperature for an additional 24 h. Each set of conditions was tested in duplicate for each experiment, including control tubes. Colonies were counted, and conidiodial activity was calculated by use of the following formula: percentage of killing = (1 − X/C) × 100, where X is the number of colony-forming units with MDMs at 4 h, and C is the number of colony-forming units of conidia only at 4 h.

**Conidial Germination Assay**

The rate of germination by Aspergillus species may be a potential virulence factor in initiating early pulmonary invasion. Conidia of A. terreus or A. fumigatus in a concentration of 10^6 suspended
in 1-mL aliquots of CM were placed in 12-well Costar plates (Costar) containing 18-mm sterile round glass coverslips. Plates were incubated for 1 h at 37°C with 5% CO₂. After 1 wash with prewarmed MHS, wells were refilled with 1-mL aliquots of fresh prewarmed CM and incubated for an additional 7 or 23 h. After this incubation, wells were washed 2 additional times with prewarmed MHS. Coverslips were removed, fixed in methanol, and stained with PAS stain. Percentage of germination was assessed by light microscopy and calculated as the number of conidia containing germings or hyphal elements greater than twice the length of the conidia among 100 organisms counted.

**Experimental Pulmonary Aspergillosis**

**Animals.** Female New Zealand White rabbits (Hazleton) weighing 2.0–3.5 kg were used in all experiments. Rabbits were individually housed and maintained according to NIH guidelines for animal care and in fulfillment of American Association for Accreditation of Laboratory Animal Care (AAALAC) criteria [29]. A total of 72 rabbits were used for all experiments. Vascular access was established in each rabbit by the surgical placement of a silastic tunneled central venous catheter [30].

**Inoculation.** Invasive pulmonary aspergillosis was established, as described elsewhere [32]. Suspensions of *A. terreus* and *A. fumigatus* conidia were prepared as described above. The concentration was adjusted to give each rabbit a predetermined endotracheal inoculum of 1 × 10⁴ conidia of *A. terreus* or *A. fumigatus* in a volume of 250–50 μL. The concentrations of the inocula were confirmed by culturing serial dilutions of each preparation onto SGA plates and by calculating colony-forming units per milliliter. Inoculation was performed on day 2 of the experiments under general anesthesia with 0.8–1.0 mL of a 2:1 mixture (vol/vol) of ketamine (100 mg/mL; Fort Dodge Labs) and xylazine (20 mg/mL; Mobay) administered intravenously.

**Immunosuppression, induction, and maintenance of neutropenia.** Induction, maintenance, and supportive care of neutropenia were performed as described elsewhere [32]. In brief, cytarabine (Ara-C; Cytosar-U; Upjohn) was initiated 1 day before endotracheal inoculation of rabbits. Profound and persistent granulocytopenia (<100/μL) was achieved by an initial course of 525 mg/m² of Ara-C for 5 consecutive days. A maintenance dose of 484 mg/m² of Ara-C was administered for 4 additional days, on days 8, 9, 13, and 14 of the experiment. Concomitant thrombocytopenia ranged from 30,000 to 50,000/μL. Methylprednisolone (5 mg/kg of body weight; Abbott Laboratories) was administered on days 1 and 2 of the experiment, to inhibit macrophage activity against conidia and to facilitate establishment of infection. Absolute neutrophil counts (ANCs) were determined from the product of percentage of neutrophils and total white blood cell (WBC) count.

**Treatment groups and antifungal compounds.** Rabbits received D-AmB (0.5 mg/kg/day), liposomal AmB (5 mg/kg/day; L-AmB; AmBisome, Fujisawa Healthcare), itraconazole (6 mg/kg/day and 20 mg/kg/day), posaconazole (6 mg/kg/day and 20 mg/kg/day), or no treatment (untreated controls). Antifungal therapy was initiated 24 h after endotracheal inoculation. Therapy was continued throughout the course of the experiments for a maximum 12 days in surviving rabbits.

**Polyenes.—** D-AmB was reconstituted with sterile distilled water, maintained at 4°C, and diluted 1:5 (vol/vol) with 5% dextrose in water immediately before use. Reconstituted D-AmB was slowly administered intravenously (0.1 mL every 10 s).

L-AmB in small, unilamellar vesicles was provided as a lyophilized powder, maintained at 4°C, reconstituted with distilled water to a concentration of 2 mg/mL, and then filtered. The solution then was diluted to a final concentration of 0.5 μg/mL with 5% dextrose in water. L-AmB and D-AmB preparations were sheltered from light and administered intravenously via central silastic venous catheter during a minimum of 5 min.

**Triazoles.—** Two antifungal triazoles were studied: posaconazole and itraconazole. Posaconazole stock solution (30 mg/mL) was prepared by dissolving the antifungal powder in a solution of distilled water and Tween 80 (Fisher Scientific). Itraconazole was administered in β-hydroxypropyl cyclodextrin (10 mg/mL). As described elsewhere [33], posaconazole and itraconazole achieved similar plasma concentrations across the dosage range from 6 to 20 mg/kg/day when administered orally to rabbits.

**Outcome variables.** The following outcome variables were used to assess differences in experimental invasive pulmonary aspergillosis due to *A. terreus* versus *A. fumigatus*: clearance of residual fungal burden in tissue (cfu/g), serial serum galactomannan levels, survival, pulmonary infarct score, lung weight, and computed tomography (CT) scan score. Pulmonary infarct score, lung weight, and CT scan score are measures of organism-mediated pulmonary injury.

**Quantitative tissue cultures.—** Lung tissue in each individual rabbit was sampled and cultured by excision of a representative region of the lung. Each specimen was weighed individually, placed in sterile bags (Tekmar), and homogenized with sterile saline for 15 s per tissue sample (Stomacher 80; Tekmar) [34]. Lung homogenate dilutions of 10⁻¹ and 10⁻² were prepared in sterile saline, and aliquots of 100 μL from homogenates and their dilutions were plated onto SGA. Plates were incubated at 37°C for the first 24 h and then at room temperature for another 24 h. Colony-forming units of *A. terreus* or *A. fumigatus* were counted and recorded for each lobe and the colony-forming units per gram were calculated. A finding of 1 colony of *A. terreus* or *A. fumigatus* was considered to be positive.

**Lung weights and pulmonary lesion scores.—** The entire heart-lung block was carefully resected at autopsy. The heart then was dissected away from the lungs, leaving an intact tracheobronchial tree and lung preparation. The lungs were weighed.
and inspected by at least 2 observers who were unaware of the treatment group and recorded hemorrhagic infarct lesions (if any) in each individual lobe. Positive lobes were added together, and the mean value of all positive lobes was calculated for each treatment group. Hemorrhagic infarcts were dark red consolidated lesions that corresponded histologically to coagulative necrosis and intra-alveolar hemorrhage.

CT.—CT of the lungs was performed during all experiments to monitor the effects of antifungal treatment on infection-mediated tissue injury during life [36]. In brief, rabbits were sedated with ketamine and xylazine and then placed prone, head first, on the scanning couch. CT was performed by use of the Imatron C-100XL ultrafast electron beam CT scanner (Oyster Point), as described elsewhere [36]. Ultrafast CT (UFCT) scans were performed by use of the high resolution, table-incremented, volume-acquisition mode. Slices, 3-mm thick, were made every 4 s. The pulmonary lesion score in each lobe was initially zero. Each lobe was evaluated and scored independently. A score of +0.5, +1, 0, −1, or −0.5 was assigned to the previous score, if the lobe demonstrated worsening, stabilization, or improvement. CT was performed on days 1–8 and day 10 of treatment. The mean CT pulmonary lesion score for that day represents the mean of all lobes of all rabbits in each group.

Survival.—Duration of survival in days after inoculation was recorded for each rabbit. All surviving rabbits were killed by pentobarbital anesthesia on the thirteenth day after inoculation.

Histopathologic analysis.—Pulmonary lesions were excised and fixed in 10% neutral buffered formalin. Paraffin-embedded tissue sections were stained with PAS and Grocott-Gomori methenamine silver (GMS) stains. Tissues were microscopically examined for pulmonary injury and structural changes in Aspergillus hyphae.

Serum galactomannan levels. Serum from each rabbit infected with Aspergillus terreus and A. fumigatus was separated from blood collected every other day and stored at −20°C until used. Serum galactomannan was determined by the Platelia Aspergillus EIA (Sanofi Diagnostic Pasteur/BioRad) 1-stage immunoenzymatic sandwich microplate assay method. The assay was performed on days 1–8 and 10 of treatment. The mean value of all positive lobes was calculated for each treatment group and recorded hemorrhagic infarct lesions (if present) in the lungs of all rabbits in each group.

RESULTS

In Vitro Antifungal Studies

MICs and MLCs. The MICs and MLCs of AmB against A. terreus isolates were higher than those of A. fumigatus isolates. The median MIC for A. terreus isolates was 2 μg/mL (range, 2–4 μg/mL), whereas the median MIC for A. fumigatus isolates was 0.5 μg/mL (range, 0.25–0.50 μg/mL). Similarly, the median MLC was 8 μg/mL (range, 8–16 μg/mL) for A. terreus isolates and 1 μg/mL (range, 0.5–1.0 μg/mL) for A. fumigatus isolates. By comparison, the MICs and MLCs of itraconazole and posaconazole were similar for both A. fumigatus and A. terreus. The median MICs for itraconazole against A. terreus isolates were 0.5–1.0 μg/mL (range, 0.25–2.0 μg/mL), whereas the median MICs for posaconazole were 0.125–2.0 μg/mL (range, 0.06–1.0 μg/mL), respectively, and the median MLCs for posaconazole were 0.06–0.50 μg/mL, respectively. The median MICs for itraconazole against A. terreus and A. fumigatus were 0.125 μg/mL (range, 0.03–0.125 μg/mL) and 0.25 μg/mL (range, 0.06–0.50 μg/mL), respectively, and the median MICs for posaconazole were 0.06–0.50 μg/mL, respectively. The median MICs for itraconazole against A. terreus and A. fumigatus were 0.50 μg/mL (range, 0.125–2.0 μg/mL) and 1.0 (0.50–2.0 μg/mL), respectively, and the median MLCs for posaconazole were 0.125 μg/mL (range, 0.06–0.50 μg/mL) and 0.25 μg/mL (range, 0.06–1.0 μg/mL), respectively.

Statistical Analysis

Comparisons between groups of continuous variables were performed by use of analysis of variance (ANOVA) with Bonferroni’s correction for repeated measures or by use of the Mann-Whitney U test, as appropriate. Comparisons between each treatment group and untreated controls also were performed by ANOVA with Bonferroni’s correction for repeated measures. Differences in survival were analyzed by log-rank test and were plotted by Kaplan-Meier curves. All P values were 2-sided, and P < .05 was considered to be significant. Values are expressed as mean ± SEM for most of the results. Values are expressed as median and range when presenting results of susceptibility testing and sterol analysis.

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Figure 1. Time-kill assay of Aspergillus terreus versus Aspergillus fumigatus. A. terreus is resistant to the fungicidal activity of amphotericin B (AmB) (n = 4 experiments).

Time-kill assay. Compared with A. fumigatus, A. terreus was resistant to the fungicidal effects of AmB (figure 1). The antifungal activity of AmB against A. terreus and A. fumigatus was similar for the first 2 h of exposure. After more prolonged exposure of ≥6 h, the differences in the efficacy of AmB against A. terreus and A. fumigatus were evident. A. terreus isolates did not demonstrate concentration-dependent susceptibility. Moreover, at 24 h, there was <1 log kill of A. terreus at all concentrations of AmB tested. By comparison, A. fumigatus demonstrated concentration-dependent antifungal activity of AmB with an ∼10^5-fold reduction in viable colony-forming units by 24 h at AmB concentrations ≥4 μg/mL.

MTT hyphal damage assay. The MTT hyphal assay demonstrated that A. terreus was resistant to persistent AmB-induced hyphal damage (figure 2). Within the first 6 h, the magnitude of AmB-induced hyphal damage was initially similar for A. terreus and A. fumigatus. This level of hyphal damage due to AmB persisted throughout 24 h in A. fumigatus isolates. By comparison, A. terreus demonstrated recovery from AmB-induced hyphal damage over the same time period from 6 h to 24 h, which is consistent with polyene resistance of this organism. For example, by 24 h, hyphal damage was ≤50% in A. terreus isolates exposed to ≤1 μg/mL of AmB, whereas hyphal damage was >85% in A. fumigatus isolates exposed to ≤1 μg/mL of AmB (P < .01).

Membrane sterol composition analysis. Ergosterol levels were more variable in isolates of A. terreus than in A. fumigatus (table 1). One isolate (95–644) of A. terreus demonstrated substantially reduced ergosterol levels (20.3%), with correspondingly increased levels of zymosterol (17.1%) and squalene (17.5%). This isolate also had the highest MLC of AmB (16 μg/mL) among the isolates of A. terreus. However, there were no significant differences between the groups of isolates of A. terreus and A. fumigatus among the overall contents of sterols.

Experimental Invasive Pulmonary Aspergillosis

Resistance to AmB. Consistent with the aforementioned in vitro data, D-AmB had no effect in clearing fungi from lung tissues in rabbits infected with A. terreus (figure 3A). However,
Table 1. Membrane sterol analysis of *Aspergillus terreus* and *Aspergillus fumigatus*.

<table>
<thead>
<tr>
<th>Organism, sterol or lipid</th>
<th>Percentage of content, median (range)</th>
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<tr>
<td><em>A. terreus</em></td>
<td></td>
</tr>
<tr>
<td>Ergosterol</td>
<td>56.1 (20.3–75.4)</td>
</tr>
<tr>
<td>Zymosterol</td>
<td>8.1 (3.5–17.1)</td>
</tr>
<tr>
<td>Squalene</td>
<td>4.5 (2.5–17.5)</td>
</tr>
<tr>
<td><em>A. fumigatus</em></td>
<td></td>
</tr>
<tr>
<td>Ergosterol</td>
<td>60.0 (49.7–64.1)</td>
</tr>
<tr>
<td>Zymosterol</td>
<td>7.0 (6.9–11.2)</td>
</tr>
<tr>
<td>Squalene</td>
<td>10.7 (7.1–16.1)</td>
</tr>
</tbody>
</table>

**NOTE.** Three isolates each of *A. terreus* and of *A. fumigatus* were analyzed.

D-AmB achieved a significant reduction of residual fungal burden in lungs of rabbits infected with *A. fumigatus* (*P* < .001). There were no significant inter-isolate differences in any of the outcome variables.

Serum galactomannan concentration-time curves paralleled these microbiological differences (figure 3B). There were no significant differences in the pattern of galactomannan concentration-time curves between treated and nontreated rabbits with invasive aspergillosis due to *A. terreus*. Although, there was a marked reduction of galactomannan antigenemia in D-AmB–treated animals infected with *A. fumigatus* (*P* < .0001).

Compatible with these microbiologic and antigenic findings, lung tissues from D-AmB–treated rabbits infected with *A. terreus* were histologically indistinguishable from those of untreated controls, as evidenced by vascular invasion and hemorrhagic infarction (figure 4). By comparison, lung tissue from treated rabbits infected with *A. fumigatus* demonstrated complete histologic clearance of organisms.

A notable histopathological feature distinguishing *A. terreus* was the presence of aleurioconidia formation along the lateral walls of hyphal elements invading lung tissue (figure 4E). These structures are morphologically compatible with those described in culture for *A. terreus*. Such structures were not observed in the hyphae of animals infected with *A. fumigatus*.

We then further sought to ascertain whether a higher dosage of AmB in the form of L-AmB would be more effective against *A. terreus* in vivo (figure 5). L-AmB (5 mg/kg/day) had no significant effect on residual fungal burden or survival, compared to untreated controls. However, there was a reduction in organism-mediated pulmonary injury in L-AmB–treated animals (*P* < .05).

**Treatment with antifungal triazoles.** Posaconazole and itraconazole were active in treatment of invasive pulmonary aspergillosis due to *A. terreus* (figure 5). Posaconazole significantly reduced fungal tissue burden (log cfu/g), as well as organism-mediated pulmonary injury (measured by lung weights and pulmonary infarct scores), in persistently neutropenic animals infected with *A. terreus*. Itraconazole also significantly reduced pulmonary injury, but the trend toward reduction in residual fungal tissue burden did not reach statistical significance. Although there was a reduction in organism-mediated pulmonary injury (measured by reduced pulmonary infarct scores and lung weights) in animals treated with L-AmB, there was no significant reduction of fungal tissue burden.

Only posaconazole and itraconazole doses of 20 mg/kg/day significantly improved survival over controls (figure 5D). Posaconazole and itraconazole at the lower dosage of 6 mg/kg/day improved survival, compared to that in controls and L-AmB–treated animals; however, survival was lower than that achieved at 20 mg/kg/day dosages and not significantly different from that of controls. Survival in L-AmB–treated animals also was not significantly different from that of controls.

![Figure 3. Lack of efficacy of deoxycholate amphotericin B (AmB) in reducing the residual fungal burden of *Aspergillus terreus* in persistently neutropenic rabbits with invasive pulmonary aspergillosis.](https://academic.oup.com/jid/article-abstract/188/2/305/906413)
Figure 4. Comparative histopathologic features of experimental pulmonary aspergillosis due to *Aspergillus terreus* and *Aspergillus fumigatus* (Gomori methenamine silver stain). A, Untreated controls infected with *A. terreus* causing angioinvasion of small pulmonary artery with formation of an intralumenal thrombus (long arrow). Branching septate are invading the vascular wall and infiltrating adjacent infarcted pulmonary parenchyma (short arrows) (original magnification, ×200; bar, 50 μm). B, Untreated controls infected with *A. fumigatus* forming branching septate hyphae also cause similar patterns of angioinvasion, thrombosis, and pulmonary infarction (original magnification, ×200; bar, 50 μm). C, Rabbits infected with *A. terreus* and treated with deoxycholate amphotericin B (AmB) demonstrate no change in extent or patterns of invasive aspergillosis, compared with that of untreated controls (original magnification, ×200; bar, 50 μm). D, Rabbits infected with *A. fumigatus* and treated with deoxycholate AmB demonstrate virtually complete eradication of fungal elements in lung tissue. Only a few small fragments (long arrows and bracket) of *A. fumigatus* are visible in infarcted tissue change in association with pulmonary alveolar macrophages (short arrows) (original magnification, ×200; bar, 50 μm). E, In vivo formation of aleurioconidia along the lateral walls of hyphal elements of *A. terreus* (arrows) (original magnification, ×1000; bar, 10 μm). F, No in vivo production of aleurioconidia occurred along walls of the hyphal elements of *A. fumigatus* (arrows) (original magnification, ×1000; bar, 10 μm).
Reduced virulence of *A. terreus* versus *A. fumigatus*. To exclude the possibility that the differences in greater resistance to AmB of *A. terreus* versus *A. fumigatus* may have been due to potentially greater virulence of *A. terreus*, we studied several parameters of virulence of these 2 species in vivo. Although *A. terreus* was more resistant to AmB, it was less virulent in vivo in comparison to *A. fumigatus*. Untreated rabbits infected with *A. terreus* survived longer (*P < 0.001*) than did those infected with *A. fumigatus* (figure 6A). Mean pulmonary infarct scores and lung weights were lower in rabbits infected with *A. terreus* (figure 6B and 6C).

Further consistent with the observation of reduced organism-mediated tissue injury, pulmonary CT scan scores and rates of development of pulmonary infiltrates were lower in animals infected by *A. terreus* versus *A. fumigatus* (figure 6D and figure 7). Although vascular invasion, hemorrhagic infarction, and coagulative necrosis were histologically indistinguishable between untreated control rabbits infected with *A. terreus* or *A. fumigatus*, these lesions were more widely distributed radiologically in the lungs of rabbits infected with *A. fumigatus*.

The lower virulence of *A. terreus* versus *A. fumigatus* also is reflected in the rates of change of the galactomannan antigenemia concentration-time curves of the untreated controls (figure 3B). The galactomannan antigenemia concentration-time curve of untreated *A. terreus*–infected animals rose more slowly than that of the rapidly increasing antigen curve of *A. fumigatus*–infected rabbits.

**Host-Defense Studies**

We also sought to determine whether potential differences in phagocytic host defense may contribute to in vivo resistance of *A. terreus* or to its reduced virulence in comparison to *A. fumigatus*. There were no differences in host response to *A. terreus* versus *A. fumigatus*, as measured by peripheral blood monocyte-mediated hyphal damage, conidiocidal activity by monocyte-derived macrophages, and phagocytosis of conidia by pulmonary alveolar macrophages to account for these differences in resistance and virulence.

**Monocyte-mediated hyphal damage.** There were no significant differences detected in the capacity of MNCs to damage hyphae of *A. terreus* versus *A. fumigatus* at any of the E:T ratios.
Figure 6. Reduced virulence of Aspergillus terreus vs. Aspergillus fumigatus (*; §; and ¶). Untreated rabbits infected with A. terreus survive longer (A) and demonstrate reduced pulmonary infarct scores (B), lower lung weights (C), as well as reduced pulmonary computed tomography score, compared with those animals infected with A. fumigatus (D). UC, untreated control.

This study demonstrated that A. terreus is resistant in vitro and in vivo to the fungicidal effects of AmB. By comparison, A. fumigatus was susceptible in vitro and in vivo to AmB. Although A. terreus was less virulent than A. fumigatus in vivo, this organism was unable to be eradicated from pulmonary tissue of rabbits with persistent neutropenia. A. terreus in rabbits with persistent neutropenia produced pulmonary infarctions, angiinvasion, and thrombosis of pulmonary arteries and veins. The resistance of A. terreus to the fungicidal effects of AmB also correlated with galactomannan antigenemia, which continued to increase in plasma, despite continued AmB antifungal therapy. Compared with the lack of efficacy of AmB, the antifungal triazoles, posaconazole and itraconazole, exerted significantly more antifungal activity in pulmonary aspergillosis due to A. terreus.

A review of the literature reveals an increasing number of reported cases of infection caused by A. terreus [6–21]. This increase may be related to an increasing number of immunocompromised patients at risk for the development of invasive fungal infections. Such patients include those undergoing bone marrow transplantation, solid organ transplantation, and cy-
Figure 7. Thoracic computed tomography scans of persistently neutropenic rabbits with invasive pulmonary aspergillosis due to *Aspergillus terreus* (lower panels) and *Aspergillus fumigatus* (upper panels) obtained on the same days (day 1, 4, or 6) after endotracheal inoculation demonstrate more pulmonary infiltrates associated with *A. fumigatus* infection than with *A. terreus* infection at a given time point. On day 1, pulmonary infiltrates are more severe in the right middle lobe of the animal infected with *A. fumigatus*, with that in the animal infected with *A. terreus*. By day 4, the entire right middle lobe of the rabbit infected with *A. fumigatus* is consolidated, in contrast to the similar region of lung in the *A. terreus*-infected animal, which remains partially aerated and less extensive. On day 6, the infiltrates in the *A. fumigatus*-infected lung have extended posteriorly well into the right lower lobe, whereas the infiltrates in the *A. terreus*-infected lung remain confined to the right middle lobe.

toxic chemotherapy for hematologic malignancies. The empirical or prophylactic use of AmB in these high-risk patients may be another factor contributing to selection for polyene resistant strains of *A. terreus* over *A. fumigatus* in this high-risk population. Improved ability to recognize *A. terreus* in clinical microbiology laboratories may be another factor contributing to the increasing number of reported cases of *A. terreus*. However, because the distinctive microscopic and colonial morphology of *A. terreus* has been well described in standard textbooks of mycology and microbiology for the past 2 decades, improved recognition may play only a minor part in this increasing trend. Yet another factor contributing to the increase *A. terreus* infections may be environmental contamination. *A. terreus* thrives in soil, as well as in aqueous environments. The construction activity and the soil of potted plants have been implicated as potential reservoirs for nosocomial aspergillosis due to *A. terreus* [37, 38]. The organism recently has been reported within the water distribution system of major oncology centers [39]. *A. terreus* also was described recently as the most common species of the genus *Aspergillus* that is detectable in the bloodstream [40].

The natural history of pulmonary aspergillosis due to *A. terreus* in rabbits with persistent neutropenia is one of progressive hemorrhagic pneumonia due to angioinvasion and pulmonary infarction. The hyphal morphology and patterns of tissue invasion, including angioinvasion, hemorrhagic infarction, and arterial and venous thrombosis, are similar for both *A. terreus* and *A. fumigatus*. However, the rate of development of pulmonary infarction, as evidenced by serial chest CT scans, is slower than that of *A. fumigatus*, possibly accounting for the lower mortality in *A. terreus* infections. Because of the findings that *A. terreus* does not appear to be any more resistant to phagocytosis or phagocytic damage than *A. fumigatus* suggests that these 2 species differ instead in intrinsic virulence factors. These differences in
virulence between *A. terreus* and *A. fumigatus* may be related to differential rates of germination, as demonstrated here. Such differences in the onset of germination may be an important factor that dictates rates of early invasion, with that of *A. fumigatus* being greater than that of *A. terreus*. Other potential virulence factors yet to be explored in *A. terreus* are differential expression of proteinases, phospholipases, and rodlet proteins [41].

Because the phagocytic host response and capacity for conidial and hyphal damage appear to be similar for *A. terreus* and *A. fumigatus*, the refractoriness of eradication of *A. terreus* from tissue is more likely related to its intrinsic polyene resistance than to any differences in host defenses. Persistently neutrophic rabbits infected with *A. fumigatus* demonstrated a marked histologic reduction in hyphal elements and angioinvasion when treated with AmB. By comparison, there was virtually no effect of AmB in altering hyphal morphology, reducing the number of hyphal elements, or modifying the patterns of tissue invasion in persistently neutrophic rabbits infected with *A. terreus*. Culture results of multiple lung samples consistently demonstrated that these hyphal elements were viable and similar in concentration of the residual fungal burden of the untreated controls. These findings are consistent with the in vitro time-kill studies and MTT hyphal damage assays.

The progressive increase of galactomannan antigenemia in *A. terreus*-infected rabbits treated with AmB also is consistent with the persistence of viable hyphal burden in lung tissues. Reflecting the refractoriness of *A. terreus* to AmB, the antigen concentration-time curves of *A. terreus* infected rabbits were virtually identical between treated animals and untreated controls. Thus, the cumulative evidence of persistent hyphal invasion of lung tissue, progressive galactomannan antigenemia, and sustained residual fungal burden, despite administration of AmB, support the in vitro observations that *A. terreus* is resistant to the fungicidal effects of AmB. This resistance was observed in animals treated with either conventional D-AmB or L-AmB. These findings are also consistent with those of Dannaoui et al. [42], who found resistance of *A. terreus* to D-AmB in a murine model of disseminated aspergillosis.

These in vivo findings have a parallel in the recent report by Lass-Florl et al. [43], who described a direct relationship between in vitro resistance to amphotericin and therapeutic failure in treatment of invasive aspergillosis due to *A. terreus*. Among the *Aspergillus* species, *A. terreus* was the only species against which the MIC was consistently high (≥2 μg/mL). Twenty-two of 23 patients with aspergillosis caused by an *Aspergillus* species with a MIC <2 μg/mL survived, whereas none of 6 patients survived from aspergillosis with a MIC ≥2 μg/mL.

By exploring the possible mechanisms of polyene resistance of *A. terreus*, we investigated the possibility that relative depletion of ergosterol may be a contributory factor. A depletion of ergosterol may contribute substantially to diminished binding of AmB to the cytoplasmic cell membrane and hence more polyene resistance. The substituted nonergosterol cytoplasmic membrane sterols and lipids (e.g., zymosterol and squalene) may have further reduced affinity for AmB, resulting in diminished binding. The findings from thin-layer chromatography and GLC demonstrated no overall differences in ergosterol content in 3 isolates each of *A. terreus* and *A. fumigatus*. However, the 1 isolate of *A. terreus* that had diminished ergosterol content also was the isolate that had the highest MLC (16 μg/mL). Other mechanisms of polyene resistance are likely mediating polyene resistance in *A. terreus*.

Amphotericin B is considered to be a standard of first-line therapy for the treatment of invasive pulmonary aspergillosis in patients with neutropenia [44, 45]. This strategy, however, should be tempered by the diagnosis of a non–*fumigatus* *Aspergillus* species [46]. Clearly, if the diagnosis were one of *A. terreus*, an antifungal triazole would be predicted to have more efficacy than would AmB or its lipid formulations. Our experimental findings demonstrate that L-AmB, despite attainment of higher tissue and plasma concentrations, was inadequate to reduce the microbiologic burden of *A. terreus* in pulmonary infections.

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**Figure 8.** Monocyte-mediated hyphal damage against *Aspergillus terreus* vs. *Aspergillus fumigatus*. There are no significant differences detected in the capacity of peripheral blood monocytes to damage hyphae of *A. terreus* vs. *A. fumigatus* at any of the effector-to-target (E:T) ratios studied. At the highest E:T ratio (50:1), the difference in percentage of hyphal damage was 66.2 ± 3.9 vs. 54.1 ± 1.5, respectively (*P* = .08).

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**Table 2.** Percentage of conidial germination of *Aspergillus terreus* and *Aspergillus fumigatus*.

<table>
<thead>
<tr>
<th>Time of incubation, h</th>
<th><em>A. terreus</em> (% ± SEM)</th>
<th><em>A. fumigatus</em> (% ± SEM)</th>
<th><em>P</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>1.36 ± 0.45</td>
<td>96.7 ± 0.40</td>
<td>&lt; .0001</td>
</tr>
<tr>
<td>24</td>
<td>97.0 ± 1.8</td>
<td>99.8 ± 0.30</td>
<td>.07</td>
</tr>
</tbody>
</table>

**NOTE.** Data are mean ± SEM of data from 5 experiments, unless otherwise indicated.
tissue. Thus, identification of *A. terreus* as a cause of invasive aspergillosis would logically warrant the use of an antifungal triazole in high dosages (e.g., ≥10 mg/kg/day of itraconazole). Our earlier work found that posaconazole achieved therapeutic plasma levels for treatment of experimental pulmonary aspergillosis due to *A. fumigatus* [33]. Such levels are ≥5 times the MIC and MLC of *A. terreus*.

The antifungal efficacy of antifungal triazoles against *A. terreus* is consistent with their known mechanism of activity of inhibition of ergosterol biosynthesis. This effect of antifungal triazoles in *Aspergillus* species is not merely fungistatic but may be fungicidal to the organism [47]. Azole-mediated inhibition of ergosterol biosynthesis appears to inflict a lethal effect on hyphal cells of *A. terreus*. The activity of antifungal triazoles against AmB-resistant *A. terreus* is reminiscent of that of *Pseudallescheria boydii*, which tends to be resistant to AmB but is susceptible to antifungal triazoles [48]. Previous work found that a combination antifungal therapy with AmB and antifungal triazoles was more efficacious than either agent alone against *P. boydii* [49]. Whether combination therapy with AmB and antifungal triazoles is more active than either agent alone against *A. terreus* remains to be further investigated.

Other emerging fungal pathogens, such as *Trichosporon* species, *Fusarium* species, and *Candida krusei* may be resistant in vitro and in vivo to increasing concentrations of AmB. *Trichosporon* species may be resistant to the fungicidal activity of AmB with correlation among high MLCs, time-kill assay results, and lack of in vivo and clinical response [50, 51]. Anaissie et al. [52, 53] previously demonstrated that *Fusarium* spp. may be resistant in vitro, in vivo, and in patients to AmB. Karyotakis et al. [54] also found that *C. krusei* may be resistant to elevated AmB concentrations in vitro and high dosages in vivo.

The in vitro susceptibility findings of *A. terreus* in this study are consistent with those of Sutton et al. [55]. *A. terreus* may be a useful tool for investigation of in vitro and in vivo correlations for antifungal susceptibility testing and for establishing breakpoints against filamentous fungi. There is a paucity of data correlating in vitro resistance to AmB, NCCLS standardized methodology, and in vivo outcome. Previous studies have endeavored to establish such in vitro/in vivo correlations for filamentous fungi and have demonstrated trends for resistance to AmB [56]. Considerably more work is required to establish clear interpretive breakpoints for filamentous fungi. As the preponderance of MICs for isolates of *A. fumigatus* fall within a narrow range of susceptibility to AmB, *A. terreus* may be a more versatile tool for further refinement of standardized methods, as well as for development of interpretive breakpoints for filamentous fungi.

Differentiation between *A. terreus* and *A. fumigatus* carries important therapeutic implications. Rapid identification of *A. terreus* would permit initiation of appropriate antifungal triazole therapy for this polyene-resistant pathogen. This study found in vivo formation of aleurioconidia along the lateral walls of hyphal elements of *A. terreus*. These aleurioconidia in tissue appear to be identical to the same structures produced in culture [31, 57]. Recognition of these aleurioconidia in clinical specimens, such as bronchoalveolar lavage, fine needle aspirates, or biopsies, may permit a preliminary identification of *A. terreus* and the initiation of an antifungal triazole. Illustrating this approach, Seligsohn et al. [58] reported morphologically distinct aleurioconidia on microscopic examination of open biopsy specimens from a case of *A. terreus* lumbar osteomyelitis and diskitis that was later confirmed by culture. Liu et al. [59] also have recommended that the identification of aleurioconidia and other structures may facilitate the early clinical recognition of *Fusarium, Paecilomyces*, and *Acremonium* species in tissues of infected patients. To our knowledge, *A. terreus* is the only medically important species of *Aspergillus* that produces aleurioconidia in vivo.

In summary, these studies demonstrate that *A. terreus* is resistant in vitro and in vivo to the fungicidal effects of AmB. This resistance occurs, despite its lower virulence, compared with that of *A. fumigatus*. Antifungal triazoles, such as itraconazole and posaconazole, are active in vitro and in vivo against *A. terreus* and should be considered for primary treatment of this emerging fungal pathogen resistant to AmB.

References

11. Goldberg SL, Geha DJ, Marshall WF, Inwards DJ, Hoagland HC. Suc-


29. Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Academy Press, 1996.


31. Sutton DA, Sanche SE, Revankar SG, Fothergill AW, Rinaldi MG. In vitro amphotericin B resistance in clinical isolates of Aspergillus terreus, with


