Synergistic Action of Amphotericin B and Rifampin against Rhizopus Species

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A 16-year-old diabetic patient developed Rhizopus pneumonia and was initially treated with amphotericin B for 7 days. Because of clinical deterioration of the patient, rifampin was added empirically. The patient improved clinically, and lung tissue removed surgically 8 weeks later showed no fungal elements by histopathological studies or by culture. An in vitro study of amphotericin B alone and in combination with rifampin against the isolate from the patient and 11 additional isolates of Rhizopus spp. was designed. The activity of amphotericin B in the presence of rifampin (10 or 5 μg/ml) increased fourfold against 9 of 10 clinical and 1 of 2 environmental isolates. Amphotericin B activity in the presence of 2 μg of rifampin per ml increased fourfold against 6 of 10 clinical isolates and increased twofold against an additional 3 clinical isolates. Amphotericin B in the presence of 1 μg of rifampin per ml inhibited 9 of 10 isolates at a concentration of one-half the MIC of amphotericin B alone. These findings were confirmed by dose-response curves calculated from fungal dry weight determinations of Rhizopus spp. incubated in serial dilutions of amphotericin B combined with rifampin. These observations demonstrate in vitro, and possibly in vivo, synergy between amphotericin B and rifampin against Rhizopus spp.

Invasive infections caused by Rhizopus spp. occur primarily in patients with underlying diseases such as leukemia, lymphoma, and diabetes mellitus and are associated with high morbidity and mortality (14). Successful management of these patients depends on how early the diagnosis is made, prolonged antifungal therapy, and aggressive surgical debridement. Various rates of survival have been reported (13). Amphotericin B is the drug of choice for these infections; however, the prolonged therapy with high dosage that is usually required may be associated with severe toxicity (13).

On the basis of previous reports (1, 3, 4, 8–10) of in vitro synergy between amphotericin B and rifampin against various fungi, we used this combination to treat a 16-year-old diabetic patient with Rhizopus pneumonia. Further study was undertaken to analyze the in vitro activity of amphotericin B alone and in combination with rifampin against the isolate from this patient and 11 additional isolates of Rhizopus spp.

CASE REPORT

A 16-year-old female in diabetic ketoacidosis was hospitalized on 16 March 1984. A chest radiograph taken on admission showed a right-lung upper-lobe consolidation with an early cavitary lesion. A bronchoscopy performed several days later yielded Rhizopus oryzae from a fungal culture. A bronchial biopsy performed at the same time showed tissue invasion of the mucosa and cartilage by fungal elements that were consistent with Rhizopus infection. The patient was treated parenterally with amphotericin B for 7 days with no clinical improvement (total dosage, 4.6 mg/kg). Because of clinical deterioration of the patient, rifampin was added empirically to the amphotericin B therapy. Serum fungistatic titers increased from <1:2 when the patient was on amphotericin B alone (1 mg/kg per day) to 1:4 while the patient received the combination. She improved clinically, and lung tissue, removed surgically 8 weeks later because the right lung was destroyed and nonfunctional, showed no fungal elements by histopathological studies or by culture. Approximately 3 years after this fungal infection, the patient expired from an unrelated illness (diabetic nephropathy). Tissue specimens from multiple organs (lung, spleen, and liver) taken at autopsy failed to demonstrate any infective process by histopathological studies or by fungal cultures.

MATERIALS AND METHODS

Rhizopus isolates. Eight isolates of R. oryzae and two isolates of R. arrhizus were obtained from patients with invasive disease (six with pulmonary mucormycosis and four with deep wounds). Two additional isolates (R. nigricans and R. stolonifer) were environmental species.

Serum fungistatic titers. The method used to assess the fungistatic power of serum from the patient was adapted from the method described by Schoenknecht et al. (19). Serial dilutions of serum were made in yeast nitrogen base without glucose, to which 1% serum was added. Agar slants were then made. An inoculum of 10⁴ or 10⁵ Rhizopus spores per ml was added to each slant. Slants were incubated at 30°C in ambient air and were read for growth at 24 and 48 h.

Susceptibility tests. Amphotericin B (E. R. Squibb & Sons, Princeton, N.J.) was reconstituted in distilled water according to the instructions of the manufacturer. Rifampin (Merrell Dow Pharmaceutical Inc., Cincinnati, Ohio) was reconstituted in 10% dimethyl sulfoxide in phosphate-buffered saline (pH 7.0). Both drugs were further diluted in antibiotic
medium 3 (Difco Laboratories, Detroit, Mich.) to the desired concentrations (final concentration of dimethyl sulfoxide in rifampin, 0.1%). To prepare the agar slants, SeaKem HE agarose (FMC Corp., Marine Colloids Div., Rockland, Maine) was added to antibiotic medium 3. Susceptibility testing was performed by (i) an agar tube dilution technique as described by McGinnis (15) and (ii) a method for the calculation of dose-response curves based on fungal-dry-weight determinations. Appropriate controls and control isolates with known amphotericin B MICs were included. Each isolate was tested against serial twofold dilutions of amphotericin B alone (range, 1 to 0.015 µg/ml) and in combination with 10, 5, 2, or 1 µg of rifampin per ml in a checkerboard fashion. Each isolate was also tested against rifampin in concentrations from 1 to 50 µg/ml.

To prepare the inocula, isolates were grown on cornmeal agar at 30°C to enhance sporulation. Spores were obtained by swabbing the surface of the colonies, immersing the swabs in normal saline, and examining a wet mount to ensure suspension of >95% spores. Two isolates required filtering through a glass-wool column to obtain spore-rich suspensions. The inoculum size for the agar dilution tests was 5 × 10⁴ CFU/ml (actual range, 2.3 × 10⁴ to 8 × 10⁴ CFU/ml) calculated by spore count with a hemocytometer. Tubes were incubated at 30°C and observed for macroscopic and microscopic (×100 magnification) fungal growth at 24 and 48 h. For this method, the MIC was defined as the lowest antifungal concentration inhibiting microscopic fungal growth on the agar slant.

The inoculum size for the fungal-dry-weight determinations was 5 × 10⁶ to 8 × 10⁶ CFU/ml (final inoculum in broth, 5 × 10⁵ CFU/ml). Flasks (500 ml, containing 200 ml of broth), each containing a different concentration of drugs, were incubated at 30°C on a shaker for aeration and observed for macroscopic growth at 24 and 48 h. At 48 h, the contents of all flasks were filtered through sterile cheese cloth and placed in preweighed vials. These were placed in a freeze-dryer (Freezer Dryer 18; Labco Corp., Kansas City, Mo.) for 24 h. The fungal dry weight was then determined by weighing the dry fungal mass in an analytical balance (Mettler Instrument Corp., Highstown, N.J.). With the contents of the control flask considered equal to 100% growth, the fungal dry weight was calculated for all other amphotericin B- and rifampin-containing flasks and expressed as a percentage of control growth. Dose-response curves were then constructed for each isolate (10).

We also examined the effect of amphotericin B alone and in combination with rifampin on dose-response curves for Rhizopus spores germinated by incubation for 4 h at 37°C in flasks without antimicrobial agents. After germination, the flasks were incubated at 30°C for 48 h.

**Criteria for synergy.** In the tube dilution method, synergy was defined as at least a fourfold reduction in the MIC of amphotericin B and rifampin when tested in combination compared with the MIC of each drug when administered alone. Synergism in dose-response curves was defined as at least a 50% reduction in fungal dry weight in flasks containing a drug combination compared with the fungal weight in flasks containing the same concentration of amphotericin B alone (11).

**RESULTS**

**Tube dilution method.** The MICs of amphotericin B alone and in combination with clinically achievable concentrations of rifampin are shown in Table 1. Synergy between amphotericin B and rifampin was noted in 9 of 10 clinical and in 1 of 2 environmental isolates. The activity of amphotericin B increased by fourfold or more in 9 of 10 clinical isolates when either 10 or 5 µg of rifampin per ml was added. In the presence of 1 µg of rifampin per ml, the MIC of amphotericin was reduced twofold for 5 of 10 clinical isolates.

Rifampin alone was inactive against all isolates tested (MIC > 50 µg/ml). However, when subinhibitory concentrations of amphotericin B were used (1/4 MIC), rifampin MICs decreased to 2 µg/ml in 6 of 10 clinical isolates and to 5 µg/ml in an additional 3. No antagonism was noted with these drug combinations against any of the isolates tested.

**Dose-response curves.** By the definition of synergism given above, three of six isolates tested by dose-response curves were synergistically inhibited by amphotericin B-rifampin (Fig. 1). Synergism is demonstrated in R. oryzae isolates 1, 8, and 7 (Fig. 1A, B, and C, respectively). Two other isolates showed synergism, but only at certain amphotericin B concentrations, i.e., one at 0.031 µg/ml (Fig. 1D, R. arrhizus) and the other at 0.015 µg/ml (Fig. 1E, R. oryzae isolate 4). This was observed at rifampin concentrations of 5 and 10 µg/ml.

The only environmental isolate (R. nigricans) tested demonstrated a dose-response curve suggestive of possible antagonism (Fig. 1F, isolate 11). Not depicted in the figure are the results of rifampin alone at various concentrations, which showed no antifungal activity.

The effect of the two compounds and their various combinations on the dose-response curves of germinated spores of isolates 7 and 8 is demonstrated in Fig. 1G and H. An enhancing effect of rifampin on the antifungal activity of amphotericin B was still noticeable.

**DISCUSSION**

Favorable activity of the amphotericin B-rifampin combination has been previously demonstrated in vitro against various fungal species including Candida spp. (1, 3), Histoplasma capsulatum (12), Coccidioides immitis (18),

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**TABLE 1. Antifungal activity of amphotericin B and rifampin against Rhizopus spp.**

<table>
<thead>
<tr>
<th>Rifampin concn (µg/ml)</th>
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<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
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*50% and 90%, MIC for 50 and 90% of isolates, respectively.

*Environmental isolates.*
**Cryptococcus neoformans** (15), and **Aspergillus** spp. (10, 11). In each study, including ours, rifampin alone had no effect on the fungal strains, whereas the addition of amphotericin B resulted in apparent synergy at clinically achievable drug concentrations. Medoff et al. (16) have studied the mechanism of enhancement and postulated that the avid binding of amphotericin B to the principal sterol in fungal cell walls, ergosterol, alters the permeability barrier of the surface membrane and allows increased penetration of rifampin into the cell. Once in the cell, rifampin has a direct inhibitory effect of DNA-dependent RNA polymerases similar to its effect in bacterial cells.

By establishing dose-response curves based on fungal dry weights, we were able to confirm our findings by the agar dilution method. Despite theoretical implications that antifungal susceptibility testing would be more reliable and have a better correlation with the infective state of the organism if the fungi were tested in a germinated state, based on the limited data from our study and those of others (2, 11), nongerminated or germinated spores yield similar results. Spores have been used without germination in the majority of reports on filamentous fungal susceptibility testing. This problem merits further study.

In contrast to the in vitro observations regarding amphotericin B-rifampin synergy, there are few in vivo studies that address this issue. In a murine candidiasis model, Graybill and Ahrens (6) found minimal, if any, increase in efficacy when the amphotericin B-rifampin combination was compared with amphotericin B alone. Similarly, Huppert et al. (8) found no improved activity with the combination, compared to amphotericin B alone, for the treatment of murine **C. immitis** infection, despite in vitro synergy. The authors postulated that the thick-walled spherule-endospore phase of **Coccidioides** spp. in tissue may be less permeable to rifampin, despite amphotericin activity, than the mycelial phase tested in vitro. In contrast, Kitahara et al. (9) demonstrated in vivo synergy of the above-described drug combination in the treatment of murine histoplasmosis and blastomycosis. There are few anecdotal case reports similar to ours, in which invasive fungal infections in humans were
successfully treated with the amphotericin B-rifampin combination (5, 7, 17). However, to date, no prospective comparative studies of the combination in invasive fungal infection of humans have been reported.

In vivo therapeutic studies of invasive Rhizopus infection are needed to extend our observations of synergy. Comparative clinical studies, however, are difficult to conduct, since infections caused by Rhizopus spp. and other fungi belonging to the order Mucorales are uncommon. Therefore, the use of patients as their own controls, by measuring the fungistatic activity of serum against their own isolates before and after rifampin is added to amphotericin B, may provide an indication of in vivo synergy in an individual patient. This may lead to an improved clinical outcome and possibly allow use of lower, less-toxic doses of amphotericin B.

It is possible that other members of the order Mucorales will demonstrate results similar to those observed with Rhizopus spp., but further studies are warranted.

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LITERATURE CITED