Activity of Amphotericin B, 5-Fluorocytosine, and Rifampin Against Six Clinical Isolates of *Aspergillus*

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Amphotericin B in combination with 5-fluorocytosine was synergistic against three clinical isolates of *Aspergillus fumigatus* and one of three clinical isolates of *A. flavus*. Amphotericin B in combination with rifampin was synergistic against all six clinical isolates of *Aspergillus* tested. The levels of 5-fluorocytosine and rifampin required for synergism were higher than clinically achievable concentrations when measurements of synergism were based on visual turbidity; but when the effects of the drugs were measured by inhibition of ribonucleic acid synthesis or dry-weight increase, much lower concentrations were effective.

The increasing frequency of disseminated *Aspergillus* infections (7), particularly in the compromised host, has stimulated us to reevaluate present therapeutic measures for this infection and to try to develop newer forms of therapy. As a first approach to this problem, we have attempted to define the variables and to standardize susceptibility testing of *Aspergillus* to amphotericin B (AmB); and we have also tested the responsiveness of this organism to 5-fluorocytosine (5FC) and rifampin (5). In this report, we show that, with some clinical isolates of *Aspergillus*, AmB in combination with either 5FC or rifampin is synergistic and leads to an enhanced antifungal effect. In this regard, aspergilli behave in a similar fashion to several other fungi we have tested (6, 8, 9).

MATERIALS AND METHODS

Chemicals. AmB, in the form of Fungizone, was purchased from E. R. Squibb & Sons Inc., Princeton, N.J. It was dissolved in sterile water before use. Rifampin was obtained from Dow Chemical Co., Zionsville, Ind. Eight milligrams of rifampin powder was dissolved in 0.5 ml of absolute ethanol plus 7.5 ml of distilled water. 5FC powder was obtained from Hoffmann-La Roche, Nutley, N.J., and was dissolved in distilled water. [5-³H]uridine (specific activity, 8 Ci/mmol) was purchased from Schwarz/Mann, Orangeburg, N.J.

Organisms. The three isolates of *Aspergillus fumigatus* and of *Aspergillus flavus* used were isolated by the mycology laboratory, Barnes Hospital, St. Louis, Mo., from human clinical specimens. The organisms were maintained on Sabouraud agar at room temperature and transferred weekly.

Susceptibility tests. All of the susceptibility tests were done in duplicate at least three times, and there was no significant variation within each experimental method. Conidiospores were harvested from 7-day-old cultures by flooding the surface

growth with phosphate-buffered saline and shaking the tubes. The conidiospores dislodged from the mycelium were then agitated with glass beads to break down aggregates and yield a uniformly dispersed suspension of single spores. The spores were then separated from the beads and resuspended in 50-ml Erlenmeyer flasks in 2× Salvin liquid medium at a concentration of 2×10^8 spores per ml. We used Salvin medium in these experiments because of our findings in the accompanying paper (5). One-milliliter samples from each of the flasks were pipetted into capped tubes, the drugs were added at an equivalent volume to each of the flasks or tubes, and the cultures were incubated at 37 C for 24 h. At the end of the 24-h incubation, the tubes were read visually for turbidity and graded 0 to 4+. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of drug or drug combination that caused complete growth inhibition or no detectable visual turbidity. Subcultures of the clear tubes were done to determine the minimal fungicidal concentrations. Synergism was judged to be present when complete growth inhibition occurred with combinations of drugs in concentrations less than half of their respective MICs. This conformed to our previous criterion for synergy (6, 8).

After the turbidity readings, 0.5 μCi of [³H]uridine per ml was added to each tube and the cultures were reincubated at 37 C for 1 h. An equal volume of 10% trichloroacetic acid was added to each tube, and the tubes were cooled in ice for 30 min and then filtered on 2.4-cm glass-fiber filters (Reeve-Angel, Clifton, N.J.). The filters were dried under a heat lamp, placed in counting vials, and counted in Bray solution on a liquid scintillation counter.

The cultures in the Erlenmeyer flasks (18 ml) were cooled, and 2 ml of 50% trichloroacetic acid was added. They were filtered on preweighed glass filters, dried in an oven, and weighed in preweighed vials on a Mettler H20T balance. After the weights were noted, 2 ml of 1 N NaOH was added to each vial and the vials were incubated at 37 C for 1 h. The supernatants from this hydrolysis were recovered by

centrifugation, and the amounts of ribonucleic acid (RNA) in them were determined by a modification of the Schmidt-Thannhauser procedure (3).

RESULTS

The MICs of the three drugs against all six isolates of *Aspergillus* are shown in Table 1. The three isolates of *A. fumigatus* were more susceptible to AmB than were the three isolates of *A. flavus*. The MICs of 5FC and rifampin for all of the isolates were high and well above clinically achievable levels.

The drug combinations with the lowest concentrations of each drug that resulted in complete growth inhibition, as determined by visual turbidity, are also shown in Table 1. When the concentrations of each drug in the combination were less than half the MIC of that drug, our criterion for synergy was met. Using this definition, AmB plus rifampin was synergistic for all six organisms, and AmB plus 5FC was synergistic for all three isolates of *A. fumigatus* and one of three isolates of *A. flavus* (Table 1). Although the concentrations of AmB in all of the effective combinations were clinically achievable, the concentrations of 5FC and rifampin necessary for an effect on turbidity were all at the upper limits or well beyond safe clinically achievable blood levels. None of the combinations shown were fungicidal for any of the organisms. We were able to achieve fungicidal effects with combinations of AmB and 5FC or rifampin at concentrations of AmB that were half the minimal fungicidal concentration of AmB alone, but this did not meet our criterion for synergism.

The effects of the drugs on RNA synthesis and dry weight are shown in Fig. 1 and 2. Here the drug combinations of AmB plus 5FC and AmB plus rifampin, at clinically achievable concentrations, significantly inhibited RNA synthesis and increases in dry weight. Moreover, if we define a significant effect on these parameters of growth as greater than a 50% inhibition when compared with controls, then

this effect was achieved with concentration of both drugs less than half of that required for the same effect when each drug was used alone. On this basis, the criterion for synergism was met for all three isolates of *A. fumigatus* (Fig. 1) and one of the three isolates of *A. flavus* (Fig. 2A), confirming the results we obtained by using visual turbidity.

DISCUSSION

Human infections with species of *Aspergillus* are extremely difficult to cure because patients who are infected with these organisms usually have serious underlying illness and severely compromised host defenses. Moreover, the antimicrobial therapy of *Aspergillus* infection is a problem because the only treatment available has been AmB, an extremely toxic drug, difficult to administer and with achievable serum levels below or close to the inhibitory concentrations for these organisms (8).

Recently, we have shown that AmB in combination with 5FC or rifampin is synergistic against several pathogenic fungi (6, 8, 9). We and others have also shown in vivo synergism in experimental fungal infections in animals (1, 10, 12; unpublished work). The synergistic action of these agents against aspergilli has not been systematically examined, although there has been one report of the treatment of a case of *Aspergillus* endocarditis with AmB and 5FC after in vitro susceptibility tests showed synergism against this organism (2).

Our results with six clinical isolates of aspergilli have shown that the combination of AmB and rifampin was synergistic against all of the six isolates tested, and that the combination of AmB and 5FC was synergistic against all three isolates of *A. fumigatus* and one of three isolates of *A. flavus* tested. The combination of AmB and 5FC appeared to be additive against the other two isolates of *A. flavus*. The response of *Aspergillus* to these drug combinations is therefore similar to what we have found for other pathogenic fungi of man (6, 8, 9).

TABLE 1. Minimum inhibitory concentrations of amphotericin B, 5-fluorocytosine, and rifampin alone and in combination

Organism	MIC ($\mu\text{g/ml}$) ^a	
	Single drug	Combination
<i>A. fumigatus</i> 1	A, 0.5; 5FC, 100; R, 1,000	0.1 A + 25 5FC; 0.1 A + 250 R
<i>A. fumigatus</i> 2	A, 0.5; 5FC, 200; R, 1,000	0.2 A + 25 5FC; 0.2 A + 250 R
<i>A. fumigatus</i> 3	A, 1.0; 5FC, 200; R, 1,000	0.15 A + 25 5FC; 0.15 A + 250 R
<i>A. flavus</i> 1	A, 2.5; 5FC, 200; R, 500	1.0 A + 50 5FC; 1.0 A + 100R
<i>A. flavus</i> 2	A, 4.0; 5FC, 250; R, 1,000	2.0 A + 50 5FC; 1.0 A + 250 R
<i>A. flavus</i> 3	A, 2.0; 5FC, 500; R, 1,000	1.5 A + 200 5FC; 0.5 A + 250 R

^a Abbreviations: A, amphotericin B; 5FC, 5-fluorocytosine; R, rifampin.

FIG. 1. Effects of amphotericin B alone and in combination with 5-fluorocytosine or rifampin on $[^3\text{H}]$ -uridine incorporation into RNA ($\square$), and determinations of RNA ($\triangle$) and dry weight ($\circ$). A, B, and C represent *A. fumigatus* 1, 2, and 3, respectively.

It is important to note that, when visual turbidity was used as a measure of susceptibility, the concentrations of 5FC and rifampin required to show synergism with AmB were at the level of, or well above, achievable serum levels for both drugs. However, when the effects of these agents were measured by the level of inhibition of RNA synthesis and dry-weight increase, then synergism was demonstrated at much lower concentrations of 5FC and rifampin. We will have to determine by in vivo stud-

ies which of the methods of measuring the effects of the drugs is a more valid indication of clinical effectiveness. If the effects on RNA synthesis and dry weight actually reflect true susceptibility, then these combinations will be useful additions to the therapeutic regimens for *Aspergillus* infection.

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FIG. 2. Same as Fig. 1. A, B, and C represent *A. flavus* 1, 2, and 3, respectively.

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