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Combined Effect of Amphotericin B and Rifampin on *Candida* Species

JOHN E. EDWARDS, JR.,[1]* JOAN MORRISON,[1] DAVID K. HENDERSON,[1] AND JOHN Z. MONTGOMERIE[2]

Department of Medicine, Harbor-UCLA Medical Center, Torrance, California 90502,[1] University of California-Los Angeles School of Medicine, Los Angeles, California 90024,[1] and Department of Medicine, Rancho Los Amigos Hospital, Downey, California 90241,[2] and University of Southern California School of Medicine, Los Angeles, California 90025[2]

Synergism with the combination of rifampin and amphotericin B in vitro has been demonstrated with a limited number of *Candida* strains. To extend these studies (particularly at clinically achievable levels of rifampin), we evaluated the in vitro combined activity of serial twofold dilutions of amphotericin B (0.1 to 6.0 μg/ml) against 11 concentrations of rifampin (0.2 to 200 μg/ml) for 40 strains of *Candida*: 20 *Candida albicans*, 7 *Candida parapsilosis*, 8 *Candida tropicalis*, 2 *Candida stellatoidea*, 2 *Candida guilliermondii*, and 1 *Candida krusei* by using a modified checkerboard microtitration technique. An additive or synergistic effect was seen for 45% of strains with 6.25 μg of rifampin per ml added to amphotericin B. Whereas the minimal inhibitory concentration to amphotericin B alone was 0.4 μg/ml or less for 50% of the strains, the percentage increased to 90 with the addition of 6.25 μg of rifampin per ml. A similar effect was seen with killing: 25% of the strains were killed by 0.4 μg or less of amphotericin B alone per ml, and 75% of the strains were killed with the addition of 6.25 μg of rifampin per ml. In vivo studies are needed for confirmation of the usefulness of combined amphotericin B and rifampin therapy.

Despite an interval of 21 years since its introduction, amphotericin B remains the most efficacious agent in the treatment of systemic mycoses (4). Numerous attempts to reduce the toxicity of amphotericin B have met with limited success. Currently, the most satisfactory ways to limit the undesirable side effects of the drug are to reduce the total quantity administered or to give amphotericin B over a prolonged time period (every-other-day dosage). One method to reduce the amount without compromising efficacy is to combine the drug with a compound which increases killing of organisms by combined action without adding toxicity. Tetracycline, 5-fluorocytosine, rifampin, actinomycin D, and mycophenolic acid glucuronide have all shown a propensity to increase the potency of amphotericin against various fungi and yeasts (8, 9).

Although *Candida* species are generally susceptible to relatively small quantities of amphotericin B, *Candida* endophthalmitis, endocarditis, meningitis, osteomyelitis, peritonitis, pancreatitis, arthritis, and systemic candidiasis are difficult to treat. Reports of all these *Candida* infections have increased impressively. This study was designed to determine the potential for rifampin to increase the potency of amphotericin B against multiple clinical isolates of *Candida* in vitro at clinically achievable levels of both drugs and to evaluate the microtiter system for testing synergism with these antifungal components.

MATERIALS AND METHODS

Forty strains of *Candida* were tested. Twenty-eight strains were isolated from the laboratory of Harbor General Hospital; the remaining strains were from the American Type Culture Collection (Rockville, Md.). These were the same strains used in a previous study of the combined activity of 5-fluorocytosine and amphotericin B and were identified and maintained as previously described (11).

Susceptibility testing method. The susceptibility of *Candida* species to amphotericin B and rifampin was tested alone and in combination by a modification of the checkerboard microtitration technique described by Harwick et al. (7). The organisms were incubated overnight at 37°C in yeast nitrogen broth (YNB) supplemented with 0.15% L-asparagine and 1% glucose and adjusted to pH 7.0 with 8 N sodium hydroxide. After incubation, the organisms (in the yeast phase) were counted with a hemocytometer and adjusted with medium to a concentration ranging from 1×10^5 to 7×10^5 organisms per ml. Serial twofold dilutions of amphotericin B (0.1 to 6.0 μg/ml) were

tested with 11 concentrations of rifampin (0.19 to 200 μg/ml). Stock solutions of amphotericin B were prepared from U.S.P. reference standard amphotericin B 0672-G (United States Pharmacopeial Convention, Bethesda, Md.) in dimethylsulfoxide (J. T. Baker Chemical Co., Phillipsburg, N.J.), and rifampin (Calbiochem Lot 501463, La Jolla, Calif.) was also dissolved in dimethylsulfoxide. Both drugs were stored in the dark at −20°C.

Analysis of results. The minimum inhibitory concentration (MIC) was defined as the concentration in the first well with no visible turbidity (read with a Microtiter Reading Device, Cooke Engineering Company, Alexandria, Va.) after incubation for 48 h at 37°C. The minimal cidal concentration (MCC) was the concentration in the first well from which two or fewer organisms were recovered by subculturing 50 μl of the solution dropped onto Sabouraud dextrose agar (BBL Microbiology Systems, Cockeysville, Md.) with a microdiluter (Cooke Laboratory Products, Alexandria, Va.). Synergistic activity was defined arbitrarily as a reduction in MIC (or MCC) of two or more twofold dilutions of one antibiotic in the presence of the other. The entire experiment was repeated on different days (in duplicate) starting from the beginning. For instance, organisms and media were prepared fresh from the first step as outlined above and new dilutions were made from the stock drug solutions.

RESULTS

Rifampin alone was totally inactive against all of the *Candida* strains, even at the concentration of 200 μg/ml. All species were inhibited by amphotericin B at concentrations ranging from 0.2 to 6.25 μg/ml (only one isolate had an MIC of 6.25 μg/ml). Figure 1A shows the effect of concentrations of rifampin on the susceptibility of *Candida* strains to amphotericin B. With a concentration of 6.25 μg of rifampin per ml (approximately the average peak serum level), the percentage of strains which were inhibited (MIC) by 0.4 μg of amphotericin B per ml was 90% compared with only 50% in the absence of rifampin. Similar results were seen in the MCC where only 25% of strains were killed with 0.4 μg of amphotericin B per ml alone, and 75% were killed with the addition of 6.25 μg of rifampin per ml.

The tendency for rifampin to increase the percentage of strains inhibited and killed by a given concentration of amphotericin B continued until a concentration of 200 μg of rifampin per ml was reached, where relative antagonism of the two drugs was noted as illustrated by the curve shift to the right.

Figure 2A shows the percentage of strains which had a fourfold or more reduction in the MIC and MCC to amphotericin B at the various concentrations of rifampin. For instance, a concentration of 25 μg of rifampin per ml reduced the MIC of amphotericin B for 55% of strains by fourfold or more. Relative antagonism was again noted at 200 μg/ml. When these data are viewed from the opposite perspective, i.e., percentage of strains which had a fourfold or more reduction in MIC and MCC to rifampin at various concentrations of amphotericin B (Fig. 2B), the 50% value was found at low concentrations of amphotericin B (0.1 to 0.2 μg/ml). This observation was consistent with the fact that no organisms

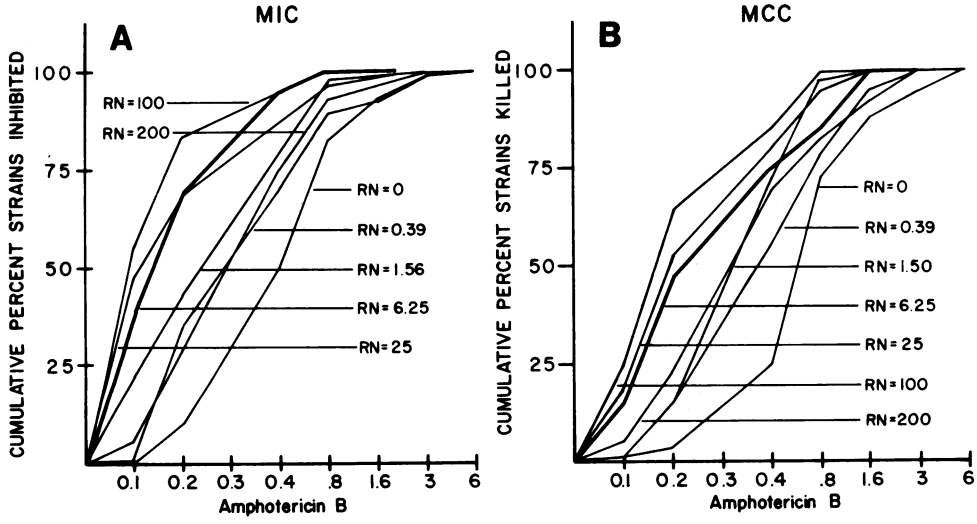

FIG. 1. *Cumulative percentage of Candida killed (left) and inhibited (right) by combinations of amphotericin B and rifampin. Concentrations of both drugs are given in micrograms per milliliter. The lower half is the data from the repeat experiment showing correlation with the data from the first experiment.*

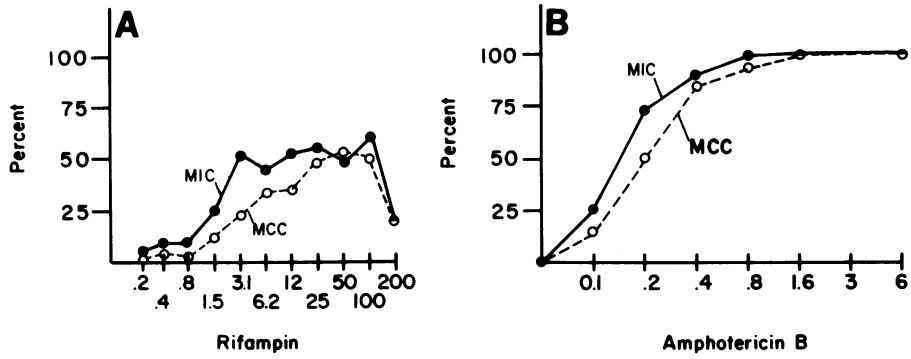

FIG. 2. *(A) Fourfold or greater reduction of rifampin MIC and MCC with various concentrations of amphotericin B. This graph demonstrates the marked change in rifampin effects when susceptibility to small, varying quantities of amphotericin B were added. (B) Percentage of strains showing a fourfold or greater reduction of amphotericin B MICs and MCCs at various doses of rifampin.*

were susceptible to rifampin alone and that most organisms were susceptible to small quantities of amphotericin B. Therefore, addition of small quantities of amphotericin B, to which the organism was highly susceptible, changed the MIC or MCC of rifampin alone extensively.

Table 1 illustrates the percentage of strains showing a fourfold or greater reduction in MIC and MCC of amphotericin B with a rifampin concentration level of 25 μg/ml or less (clinically achievable) according to the species types.

Reproducibility. Results of the duplicate experiments were compared by plotting the differences in selected corresponding values (every other point) along each curve as a frequency histogram. These histograms were essentially symmetric and consistent with a random (nonskewed) error distribution. The magnitude of the differences in points was nonsignificant within the confines of the variables (pipetting, dilutions, microtiter system, and inoculum size preparation), and the reproducibility of the experimental system was entirely satisfactory.

TABLE 1. *Percentage of strains showing fourfold or greater reduction in MIC and MCC to amphotericin B with 25 μg or less of rifampin per ml*

Species	No. tested	MIC	MCC
Candida albicans	20	45%	20%
Candida parapsilosis	7	86%	86%
Candida tropicalis	8	63%	63%
Candida stellatoidea	2	2[a]	2[a]
Candida guilliermondii	2	1[a]	1[a]
Candida krusei	1	0[a]	0[a]

[a] Number.

DISCUSSION

The attainable peak serum levels with daily doses of amphotericin B usually used (0.37 to 1.4 mg/kg) have ranged from 0.5 to 3.5 μg/ml (11). The range for rifampin with a standard dose of 600 mg has been from 4 to 32 μg/ml with an average peak serum level of 7 μg/ml. We concluded from the results of these studies that concentrations of rifampin, within the range of attainable serum levels, increased the percentage of strains of *Candida* killed and inhibited by amphotericin B and augmented the potency of amphotericin B.

The definition of synergism for antibiotics is arbitrary and controversial; to facilitate data expression, we defined synergism as a drop in MIC or MCC by two or more twofold dilutions. Our method for detecting synergism underestimated a synergistic effect when the organism was exquisitely susceptible to one of the drugs. For instance, if a strain of *Candida* had an MIC of 0.19 μg/ml of amphotericin B, it was impossible to demonstrate a drop in MIC of two or more twofold dilutions because the lowest dilution tested (0.09 μg/ml) was only one twofold dilution lower. Therefore, synergism was not recorded in such an instance. Alternatively, if an organism was completely resistant to one drug, as was the case with rifampin, small concentrations of a second drug, to which the organism was highly susceptible, markedly lowered the MIC and a synergistic effect for amphotericin B was recorded in almost all instances.

Of interest was the relative antagonistic effect seen with concentration of rifampin of 200 μg/ml. The mechanism of the antagonism is not understood. Since this level of rifampin is considerably greater than levels one would find in practically all clinical situations, we did not explore the mechanism. However, the finding does

caution against using excessive doses of rifampin in the presence of amphotericin B and for monitoring the levels in clinical situations when the drugs are metabolized efficiently or where they might accumulate in a closed space (such as local instillation into a joint).

Our reproducibility data showed minor variation, usually one to two tubes, which occurred most consistently with changes in the inoculum size of the *Candida* organism within the range of 1.0×10^5 to 7.0×10^5 organisms per ml. This variation, related to inoculum size, is consistent with other observations indicating that fungal susceptibility testing of this type is dependent upon inoculum size (5).

Synergism of amphotericin B and rifampin for *Candida* has been demonstrated previously. Beggs et al. (2) added sublethal concentrations of rifampin and amphotericin B to a single strain of *Candida* and found a significant reduction in colony-forming units per milliliter for incubation times up to 8 h. They also examined the question of whether pretreatment of the organisms with amphotericin B increased susceptibility to rifampin alone and failed to demonstrate such an effect. In a subsequent study (3), using seven strains of *Candida*, they found class II synergism (defined as 1- to 4-log reduction in the number of colony-forming units per milliliter) in six of the strains when 25 μg of rifampin per ml was added to 0.2 μg of amphotericin B per ml. Levels less than 25 μg of rifampin per ml were not tested. Ansehn et al. (1) found a profound inhibitory effect on metabolism of a single *Candida albicans* isolate when rifampin (25 μg/ml) was combined with amphotericin B (0.1 μg/ml). A single isolate of *C. albicans* has been tested, using a microtiter system similar to the one used in this study, which exhibited synergism (6). Lou et al. (10) found the combination of amphotericin B (0.25 and 0.5 μg/ml) and rifampin (50 μg/ml) to be synergistic for a blood isolate of *C. albicans* from a patient with hematogenous *Candida* endophthalmitis. Therapy of the patient with amphotericin B (1 mg/kg per day) plus rifampin (15 mg/kg per day) resulted in dramatic clearing of the eye lesions.

With our studies, we substantiated these previous observations and extend the demonstration of rifampin potentiation of amphotericin B into ranges easily obtainable clinically. In vivo observations with animal models for disseminated candidiasis including endophthalmitis, endocarditis, arthritis, meningitis, and peritonitis are necessary to demonstrate the clinical usefulness of these observations. The mechanism for the relative antagonism seen at 200 μg of rifampin per ml justifies exploration. Mixing of the compounds or administration into closed compartments, such as the cerebral spinal fluid or joint spaces, may be contraindicated.

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