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# In Vitro Susceptibilities of *Naegleria fowleri* Strain HB-1 to Selected Antimicrobial Agents, Singly and in Combination

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The overall prognosis of primary amoebic meningoencephalitis remains poor. The results of this study support previous finding that amphotericin B is the most efficacious drug against the *Naegleria* species in in vitro testing. In additon, the methyl ester of amphotericin B, a new derivative, also appears to be an effective agent. Of the drug combinations studied, amphotericin B plus minocycline and amphotericin B plus tetracycline showed synergy. The clinical significance of these findings remains to be determined.

A strain of *Naegleria fowleri* (HB-1) was tested in vitro for susceptibility to amphotericin B (AMB), the methyl ester of AMB, miconazole, minocycline, and tetracycline, both singly and in combination with one another. The mean minimal inhibitory concentrations (MICs), defined as an absence of cytopathic effect by amoebae on monkey kidney cell (MKC) monolayers, were, respectively: AMB, 0.024 μg/ml; methyl ester of AMB, 0.103 μg/ml; minocycline, 2.8 μg/ml; miconazole, 6.25 μg/ml; and tetracycline, 10.0 μg/ml. Synergism, defined as at least a fourfold reduction in the MIC of each drug when used in combination as opposed to singly, was demonstrated in one of two runs each for AMB plus minocycline and AMB plus tetracycline. Because AMB and its methyl ester were the most active drugs in vitro, they are candidates for trial use in the treatment of primary amoebic meningoencephalitis (PAM). Administration of AMB in combination with minocycline or tetracycline also deserves consideration.

PAM, caused by free-living amoebae of the genus *Naegleria*, usually results in death; of the approximately 80 documented cases, there have been only three survivors (1, 2, 6). Many different antimicrobial agents (and treatment regimens) have been used to treat PAM, but the most promising appears to be the antifungal polyene, AMB. Both in vitro amoebicidal activity (5, 8, 9, 17) and successful treatment of the three reported survivors with AMB indicate its potential usefulness in treating PAM. Recently, the methyl ester of AMB has received trial in the treatment of fungal infections. AMB methyl ester is less toxic than AMB and is soluble in water. Moreover, Visvesvara et al. (G. Visvesvara, G. Healy, and D. Jones, J. Protozool. **23:** 26A, 1975) have reported that the methyl ester is active against *Naegleria* spp. Accordingly, further investigations of AMB methyl ester seem warranted.

Many of the human patients (including at least one of the survivors) received other drugs in combination with AMB. Although no definite therapeutic value of tetracycline has been demonstrated in PAM patients, Thong et al. (18) have apparently obtained synergism between tetracycline and AMB in mice infected experimentally with *N. fowleri*.

In this report a tissue culture technique for assay of anti-*Naegleria* activity is described. MICs were determined for several drugs, and the potential for synergy was measured.

## MATERIALS AND METHODS

**Drugs tested.** AMB (Fungizone; E. R. Squibb & Sons, New Brunswick, N.J.) was obtained as stock solution (5 mg/ml). The methyl ester of AMB was obtained from E. R. Squibb & Sons as the aspartate salt. A stock solution of 2 mg/ml, methyl ester base equivalent, was prepared. Miconazole (Janssen Pharmaceuticals, lot 73J04/14) was used as the stock solution (10 mg/ml). Minocycline (Minocin, Lederle, lot 472-160) was used as the stock solution (10 mg/ml). Tetracycline hydrochloride (Tetracyn, Pfizer) was used as the stock solution (50 mg/ml).

**Preparation of MKC monolayers.** MKC monolayers were established in slant culture by seeding 1 ml of Leibowitz L-15 medium (10% [vol/vol] fetal bovine serum added) with approximately 50,000 trypsinized cells in screw-cap test tubes (16 by 125 mm). Monolayers were also grown in 250-ml flasks by adding approximately 250,000 trypsinized cells to 20 ml of L-15 + 10% fetal bovine serum. Layers were allowed to grow for 1 week and were checked for confluency before use.

**Production of amoebae.** The strain of *N. fowleri*

(HB-1)used has been well characterized regarding cytopathogenicity in tissue culture and animal virulence (21, 22).

Amoebae used for drug testing were obtained from MKC flask cultures at the end of the logarithmic phase of growth (when most of the MKC layer was destroyed). The flasks were gently shaken to loosen debris and necrotic cells, and the culture maintenance medium (L-15 with 2% [vol/vol] fetal bovine serum added) was poured off and replaced with fresh L-15. The amoebae were gently scraped from the bottom of the flask, enumerated by duplicate hemacytometer counts, and diluted in L-15 to give a suspension of approximately 1,000 trophozoites per 0.025 ml of medium. This volume was then used to inoculate the appropriate tubes in each test.

**MIC.** The MIC was defined as the concentration of drug required to prevent cytopathic effect on MKC cultures to which an inoculum of approximately 1,000 amoebae had been added. The concentration of drug at which the cell layer remained intact 48 to 72 h after exposure to both drug and amoebae was called the MIC. Control cell cultures to which only amoebae were added were 90 to 95% destroyed in 48 to 72 h.

From the stock solutions of drugs, serial dilutions were made in Leibowitz L-15 medium. The final volume of the drug solution in each tissue culture tube (16 by 125 mm) was 1 ml. The ranges of drug concentrations, which encompassed all clinically attainable concentrations and were obtained by serial twofold dilutions of the highest concentration, were as follows: AMB, 5.00 to 0.0024 μg/ml; AMB methyl ester, 20.00 to 0.0024 μg/ml; and miconazole, minocycline, and tetracycline, 10.0 to 0.0781 μg/ml each.

Each drug was tested in at least four separate runs. Appropriate cell layer, drug, and amoeba controls were done concurrently with each run.

**Synergy studies.** The method used was a modification of the procedure employed in the microbiology laboratory of the University of California–Davis Medical Center, Sacramento, for bacteriological synergy studies.

Drug A was tested against drug B using a checkerboard arrangement of sterile screw-cap tubes (16 by 125 mm) containing MKC and L-15 medium. Stock solutions of antimicrobial agents at concentrations four times those used in the MIC determinations were employed as follows: 0.25 ml of drug A at each of the 4× concentrations was micropipetted (MLA 250 microtiter pipette) into the appropriate tube in the first vertical row of tubes containing 0.75 ml of L-15 medium. Drug B was similarly pipetted into the first horizontal row of tubes containing 0.75 ml of L-15 medium. The final volume was 1 ml. The ranges of concentrations for the drug pairs were 0.15 to 0.024 μg of AMB or AMB methyl ester per ml with 10.0 to 0.0781 μg of miconazole, minocycline, or tetracycline per ml.

In the remaining tubes of the checkerboard, 0.25 ml of drug A at each of the above concentrations and 0.25 ml of drug B at each of its concentrations were micropipetted into appropriate tubes containing 0.50 ml of L-15 for a final volume of 1.0 ml. In this manner all possible concentrations of drug A and drug B in combination were made over the selected range of concentrations listed above for all the drugs. Each combination was tested at least in duplicate.

Approximately 1,000 amoebae in 1 drop of liquid (0.025 ml) were added to each tube. The cytopathic effect on the cell monolayers was assessed at 48 to 72 h after inoculation. Cell layer, drug, and amoeba controls were set up concurrently with each run. Synergy was defined as a fourfold decrease in the MIC of each drug when used in combination, compared to the use of each drug alone.

## RESULTS

The MIC data are summarized in Table 1.

The synergy studies are summarized in Table 2. Although there were differences in the results of the duplicate assays, the data summarized in Table 2 provide strong evidence of synergy when AMB was combined with minocycline or tetracycline. There was no indication of synergy when AMB was combined with miconazole or when the methyl ester of AMB was combined with either minocycline, tetracycline, or miconazole.

## DISCUSSION

AMB (5, 8, 9, 17), tetracycline (19), rifampin (19), clotrimazole (9, 12), and miconazole (9, 21) have all shown activity against *Naegleria* spp. The majority of the assays of these reagents have been carried out either on agar plates or in axenic media, both with and without bacteria as an added nutrient. Chang (7) first utilized an MKC culture system for testing the susceptibility of *Naegleria* spp. to AMB, chloroquine phosphate, and emetine hydrochloride. After inoculating cultures with unstated numbers of amoebae, Chang (7) found some antiamoebic activity against *N. fowleri* (including the HB-1 strain) at 1-μg/ml levels of AMB; 2 μg/ml was necessary to prevent amoebal growth. In our system we have found that a mean concentration of 0.024 μg of AMB per ml will kill at least $10^3$ amoebae.

TABLE 1. *MIC determinations*

| Drug | No. of determinations | Arithmetic mean MIC (μg/ml) | Range (μg/ml) |
|---|---|---|---|
| AMB | 8 | 0.024 | 0.009–0.039 |
| AMB methyl ester | 8 | 0.103 | 0.078–0.156 |
| Minocycline | 4 | 2.800 | 1.250–5.000 |
| Miconazole | 4 | 6.250 | 5.000–10.000 |
| Tetracycline | 4 | 10.000 | 10.000 |

TABLE 2. *Synergy studies*

| Drugs | Run 1 | | Run 2 | |
|---|---|---|---|---|
| | MIC alone (μg/ml) | MIC in combination (μg/ml) | MIC alone (μg/ml) | MIC in combination (μg/ml) |
| AMB + Miconazole | 0.019<br>5.000 | 0.009<br>0.156 | 0.019<br>5.000 | 0.009<br>2.500 |
| AMB + Minocycline | 0.019<br>2.500 | 0.005<br>1.250 | 0.009<br>5.000 | 0.0024*[a]<br>1.250* |
| AMB + Tetracycline | 0.019<br>10.000 | 0.009<br>2.500 | 0.038<br>10.000 | 0.009*<br>0.625* |
| AMB methyl ester + Miconazole | 0.078<br>5.000 | 0.019<br>2.500 | 0.156<br>10.000 | 0.078<br>1.250 |
| AMB methyl ester + Minocycline | 0.156<br>2.500 | 0.078<br>1.250 | 0.156<br>1.250 | 0.156<br>1.250 |
| AMB methyl ester + Tetracycline | 0.078<br>10.000 | 0.078<br>10.000 | 0.078<br>10.000 | 0.039<br>1.250 |

[a] *, Synergistic combination.

Testing anti-*Naegleria* drugs in a tissue culture system appears to have advantages over other in vitro methods. Most highly virulent isolates of *N. fowleri* that have been tested cause destruction of tissue culture monolayers through undefined cytotoxic mechanisms (7, 20, 22). For the HB-1 and Carter-66 strains of *N. fowleri* the ability to damage MKC cultures has been demonstrated to be associated with pathogenicity for experimental animal hosts (23). Our tissue culture assay system allows measurement of the effect of drugs on this product (cytotoxicity) of amoebic metabolic activity that is directly related to pathogenic potential. Using mammalian cells as the food source for the amoebae also provides a way to observe the possible effects of competitive binding or uptake of drug by nontarget cells on amoebicidal concentrations of the drug. For example, AMB acts on plasma membranes, combining with sterols and causing leakage of cellular components across the membrane (10). Binding of AMB to human tissues, rather than to amoebae, during treatment of cases of PAM could affect the outcome. Under the conditions of our tests in vitro, this would not appear to be a problem, since AMB was amoebicidal at low concentrations (mean, 0.024 μg/ml).

Antiamoebic effects of drugs that have been previously noted include inhibition of growth (5, 8, 9), inhibition of amoebic motility (5, 8, 9), and cytotoxic changes observed by light or electron microscopy (17). Definitions of the MIC of a drug have varied in previous studies (5, 8, 9). Minimal motility inhibitory concentration has been generally defined as light microscopic evidence of trophozoite immobilization (5, 8, 9). Comparative data on the activities of different drugs are limited. Such observations as are available indicate that AMB is more active than its methyl ester, and both are more active than clotrimazole or miconazole (5, 8, 12, 17; Visvesvara et al., J. Protozool. **23**:26A, 1975).

Our study using the tissue culture assay system confirms previous work showing that AMB is the most effective drug in vitro against *Naegleria* spp., with a mean MIC of 0.024 μg/ml. AMB methyl ester was slightly less active, with a mean MIC of 0.103 μg/ml. Minocycline, miconazole, and tetracycline were all less active, with mean MICs of 2.8, 6.25, and 10 μg/ml, respectively.

Several investigators have evaluated the efficacy of AMB in combination with other antimicrobial agents. Kwan et al. (13) showed a synergistic effect from combining AMB with mycophenolic acid glucoronide, tetracycline, and actinomycin D against the yeast *Saccharomyces cerevisiae*. Other studies by Medoff et al. (15) have shown synergistic effects against *Candida* spp. when using AMB in combination with

rifampin or 5-fluorocytosine. Lew et al. looked at potentiation of antifungal activity of four tetracycline analogs against *Candida albicans* by AMB and found synergistic activity with minocycline (14). Huppert et al. (11) describe a model in which AMB and tetracycline exhibited a synergistic effect in the treatment of experimental coccidioidomycosis in mice. Finally, Thong et al. (18) demonstrated the presence of synergism between tetracycline and AMB in mice infected with *Naegleria* spp.

No universally accepted definition of synergy exists. Huppert et al. (11) did not define synergy, but regarded as effective a combination of tetracycline with AMB if the desired therapeutic response was obtained when the original dose of AMB was reduced at least 2.5 times. Montgomerie et al. (16) defined synergy as being present when killing by two drugs given together was greater (by a difference in viable counts of the test organism of $>1$ $\log_{10}$) than that by the two drugs given separately. Medoff et al. (15) defined antifungal synergy as a decrease of 100-fold or more in colony count caused by the drugs in combination as compared with the count when the drugs are used singly. Beggs et al. (4) in their study of AMB and rifampin defined synergy as a greater rate and extent of killing with the drugs in combination than with either drug tested alone at a twofold increase in concentration.

For our studies we have adopted the definition described by Barry (3) that is used in evaluating antimicrobic synergy against bacteria. We considered synergy to be present in our tissue culture system if there was a fourfold decrease in the MIC of each drug when used in combination as compared to the MIC of each drug when used alone. Of the combinations tested only AMB plus tetracycline and AMB plus minocycline fulfilled this criterion in one of two tests. This result supports previously cited work by Thong et al. (18) in experiments with mice. No synergy was demonstrated for any of the combinations in which AMB methyl ester was used. There were no combinations that showed an antagonistic effect.

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