ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1974, p. 473-478

Vol. 5, No. 5
Printed in U.S.A.

Combined Amphotericin B-Tetracycline Therapy for Experimental Coccidioidomycosis

M. HUPPERT, SUNG H. SUN, AND KATHERINE R. VUKOVICH
Veterans Administration Hospital, Long Beach, California 90801

Received for publication 15 February 1974

Although amphotericin B is the principal antibiotic for treating systemic mycoses, its clinical use is restricted, primarily because of the toxicity associated with the required prolonged therapy. Other investigators have reported results from in vitro experiments demonstrating that amphotericin B can potentiate antifungal activity of other antibiotics which are ineffective when used alone. In the present study, amphotericin B was used in combination with tetracycline for treating experimental coccidioidomycosis in mice. The results show that the combination of antibiotics is effective with a dosage of amphotericin B reduced 2.5 to 4 times of that required for effective chemotherapy with amphotericin B alone.

The choice of chemotherapy for patients with systemic mycoses is limited almost exclusively to the polyene antibiotic, amphotericin B, although 5-fluorocytosine has been used with some success for yeast infections. Unfortunately, treatment with amphotericin B is accompanied by immediate and delayed toxic effects which may become more severe and even irreversible with prolonged use (3). The antibiotic must be administered by slow, intravenous infusion with the dosage increasing gradually from 0.1 to 1.0 mg/kg/day. Patients frequently experience nausea and fever during the infusion. Therapy must be continued for extended periods and is associated with hypokalemia and changes in blood urea levels and creatinine clearance. Repetitions of therapy are usually required, and kidney damage may become permanent when the total accumulated dose exceeds 3 to 4 g. Obviously, current treatment for systemic mycoses has severe limitations.

Medoff, Kobayashi, Kwan and their colleagues (5, 6, 9, 10, 12) have published results of in vitro experiments indicating that combined drug therapy might be more effective than amphotericin B alone. Their hypothesis was based on the known mechanism of action of several antibiotics. The polyenes increase the permeability of the fungus cell membrane. This damage might permit entry of other drugs which were excluded by normal cells. Therefore, the combined effect of a polyene with a second drug which blocks intracellular synthetic mechanisms might be synergistic. The results of their in vitro experiments supported this hypothesis.

The experiments reported here were designed to test this hypothesis in an in vivo situation. Mice infected with *Coccidioides immitis* were treated with amphotericin B alone or in combination with tetracycline. The results were not dramatic, but did show that addition of tetracycline to the therapy achieved effective treatment with possibly one-fourth the dosage of amphotericin B required for the same result when the polyene was used alone.

MATERIALS AND METHODS

Animals. Male mice (dba strain) were obtained from two commercial sources: Leonell C. Strong Research Foundation, Inc., San Diego, Calif., and Laboratory Supply, Indianapolis, Ind. Animals from different sources were not mixed, but were used in repetitions of experiments. There were no significant differences related to the source of animals, and they have been considered as a uniform supply under Results. On arrival, mice were weighed and distributed at random, five per cage, and held for a minimum of 2 weeks before use in experiments. Weighings were repeated at weekly intervals. Food and water were available ad libitum at all times.

Chemicals. Amphotericin B (Fungizone intravenous, amphotericin B for injection, U.S.P.) was obtained from E. R. Squibb & Sons, and the tetracycline was from Milan Pharmaceuticals, Inc. Dilutions of the former were made with sterile 5% aqueous glucose solution (wt/vol) and of the latter with distilled water.

Culture and infection inoculum. A single strain of *C. immitis* (C-34) was used. Cultures were grown on 1% glucose agar enriched with 0.5% yeast extract (1) at room temperature for 1 to 2 months, and arthrospore suspensions were harvested in minimal amounts of sterile saline by the spinning bar technique de-

scribed earlier (4). Visual arthrospore counts were made by hemocytometer, and the suspensions were adjusted to contain the desired number of spores per milliliter. Viable counts were obtained by dilution, plating, and averaging the number of colonies on all countable duplicate plates.

Infection. Mice were anesthetized by intraperitoneal (i.p.) injection of sodium pentobarbital (54 mg/kg) and then were infected intranasally (i.n.) by inhalation of a measured drop (0.025 ml) of the spore suspension suspended from the tip of a 1-ml pipette (graduated in 0.01 ml). The volume of the drop was controlled with a tuberculin syringe attached by a short piece of rubber tubing to the lower half of the 1-ml pipette. The pipette was filled with 0.4 ml of the suspension and rinsed three times with the mixed suspension before refilling.

Design of experiments. After the animals had stabilized to their new environment and were gaining weight, the population was made more homogeneous by including only those mice weighing within ±2 standard deviations of the average for the entire population. The average weights and standard deviations in three separate experiments were: 24.9 ± 3.1, 21.5 ± 2.2, and 21.2 ± 1.7. Twenty anesthetized mice were sham infected with 0.025 ml of saline (i.n.). Ten were caged for treatment with the highest dose of amphotericin B (i.p.), and water was administered per os (p.o.) by stomach tube. The second 10 mice were caged for treatment with the highest dose of amphotericin B (i.p.) and with tetracycline (p.o.). None of these treatment controls exhibited any ill effects, and all gained weight throughout the experiment. The remaining anesthetized mice were infected (i.n.) by inhalation of 0.025 ml of the spore suspension. These were distributed at random in groups of five per cage. The assignment of cages to specific treatments was randomized again with two cages for each treatment.

Therapy was begun at 5 days postinfection, a time at which infected mice showed evidence of being sick; i.e., weight loss, lethargy, ruffled fur. Amphotericin B was injected i.p. (0.5 ml) on a daily schedule with different groups receiving dosages ranging from 0.006 to 0.4 mg/kg in fourfold increments. The groups treated with only amphotericin B were given 0.2 ml of water p.o. on the same schedule. A second set of mice receiving amphotericin B were given tetracycline (8 mg/kg) in place of the water. Treatments were continued for 5 days. Deaths were recorded on a daily basis and weights on a weekly interval for 40 days when survivors were sacrificed. Confirmation of infection was obtained by demonstration of endosporulating spherules in excised lung.

The significance of differences among treatments was evaluated by nonparametric tests in terms of survival times, total survivors, and 50% end points. Gehan's generalized Wilcoxon test (2) was used for survival time, Fisher's exact probability test for total survivors (13), and a logarithmic-probit graph plot for 50% end points (11).

RESULTS

Preliminary. Certain conditions were set for the experimental animal model of coccidioidomycosis. The mice should exhibit clinical evidence of illness by the 5th day postinfection, at which time chemotherapy would be initiated. No mice should die before the 7th day, and the most rapid rate of death should occur between 10 to 15 days, with the day of 50% survival in the same interval. The rapidly rising curve of cumulative mortality would serve as the comparative base for measuring effects of chemotherapy. A substantial majority of untreated animals should succumb prior to 30 days, at which time the developing acquired resistance (7 and 8) might influence evaluation of chemotherapeutic effects.

A preliminary experiment was performed to determine the number of arthrospores administered by inhalation which would produce a course of disease satisfying the established conditions. Groups of mice were infected via the i.n. route with graded doses of arthrospores. The results in Table 1 show that 10^3 spores by the i.n. route produced a suitable pattern of infection. In three separate experiments, the suspension of spores was adjusted to 4×10^4 spores/ml by visible count, and the comparable viable counts were in the 3.5 to 5.7 $\times$ 10^4/ml range. Therefore, the infecting dose of arthrospores was 0.9 to 1.4 $\times$ 10^3 spores contained in 0.025 ml.

Combined drug therapy. The experiments involving treatment of infected animals with amphotericin B alone or in combination with tetracycline were performed at two separate times with strains of mice from two different commercial sources. There were no significant differences in the patterns of response among these two strains of mice, and the following results are from the combined data. The reported results, therefore, are based on 20 mice per treatment group. In addition, there were no differences in terms of survival times or total number of survivors among the groups of infected animals treated with the 5% glucose solution (i.p.) in combination with either water or tetracycline (p.o.), and the results for these 40 mice have been combined as a single "control" group.

TABLE 1. *Determination of infective dose of arthrospores for meeting conditions set for experimental animal model of coccidioidomycosis*

Concn of spores	Earliest day of death	Days to 50% survival	(%) Survival on day 30
10^2	15	25.0	30
10^3	11	13.0	22
10^4	7	8.5	0
10^5	7	7.0	0

The effect of these several treatments on infected mice is illustrated in Fig. 1 and 2 in terms of cumulative percentage of mortality over a 40-day postinfection period. When various doses of amphotericin B were used in combination with water (Fig. 1), apparently only amphotericin B at 0.400 mg/kg provided some degree of effective therapy compared with the control group. In contrast, when the amphotericin B treatments were supplemented with tetracycline (Fig. 2), the combination appeared to be effective (compared with the control group) at an amphotericin B dose of 0.100 mg/kg as well as at 0.400 mg/kg. These impressions were supported by statistical analyses for significance of the differences in survival times (Table 2) and in total number of survivors (Table 3) when comparing the response for each treatment group to that for the control group.

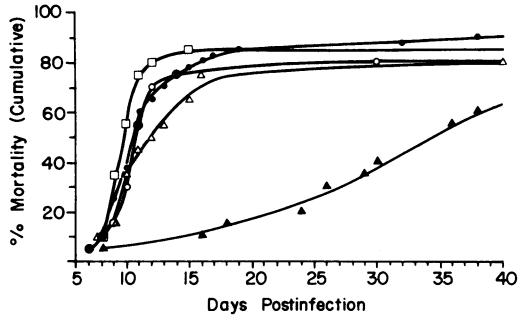

FIG. 1. *Combined drug therapy for coccidioidomycosis in mice by using amphotericin B (i.p.) and water (p.o.), single treatment daily for 5 days: ●, controls, 5% aqueous glucose (i.p.) and water (p.o.); ○, amphotericin B, 0.006 mg/kg; □, amphotericin B, 0.025 mg/kg; △, amphotericin B, 0.100 mg/kg; ▲, amphotericin B, 0.400 mg/kg.*

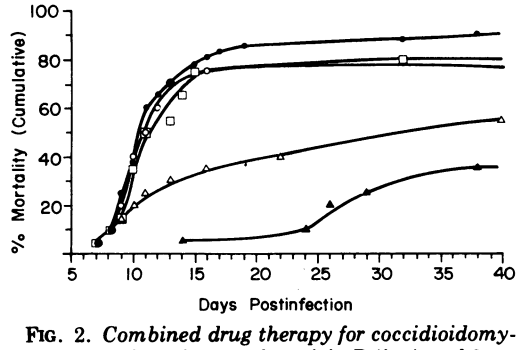

FIG. 2. *Combined drug therapy for coccidioidomycosis in mice by using amphotericin B (i.p.) and 8 mg of tetracycline per kg per day (p.o.), single treatment daily for 5 days; ●, controls, 5% aqueous glucose (i.p.) and tetracycline (p.o.); ○, amphotericin B, 0.006 mg/kg; □, amphotericin B, 0.025 mg/kg; △, amphotericin B, 0.100 mg/kg; ▲, amphotericin B, 0.400 mg/kg.*

TABLE 2. *Comparison of survival times among mice infected (i.n.) with C. immitis: treatment groups versus controls*

Amphotericin B (mg/kg/day)	Treatment per os[a]	Significance of survival times		
		Day 20	Day 30	Day 40
0.006	Water	NS[b]	NS	NS
0.025	Water	NS	NS	NS
0.100	Water	NS	NS	NS
0.400	Water	<0.001	<0.001	<0.001
0.006	Tetracycline	NS	NS	NS
0.025	Tetracycline	NS	NS	NS
0.100	Tetracycline	0.02	0.02	0.02
0.400	Tetracycline	<0.001	<0.001	<0.001

[a] Water or tetracycline (8 mg per kg per day), single treatment daily for 5 days.
[b] NS, Not significant; $P > 0.10$ in all comparisons.

TABLE 3. *Comparison of total number of survivors among mice infected (i.n.) with C. immitis: treatment groups versus controls*

Amphotericin B (mg/kg/day)	Treatment per os[a]	Significance of total survivors		
		Day 20	Day 30	Day 40
0.006	Water	NS[b]	NS	NS
0.025	Water	NS	NS	NS
0.100	Water	NS	NS	NS
0.400	Water	<0.001	<0.001	0.006
0.006	Tetracycline	NS	NS	NS
0.025	Tetracycline	NS	NS	NS
0.100	Tetracycline	<0.001	<0.001	0.004
0.400	Tetracycline	<0.001	<0.001	<0.001

[a] Water or tetracycline (8 mg per kg per day), single treatment daily for 5 days.
[b] NS, Not significant; $P > 0.10$ in all comparisons.

Similar analyses were performed for the water and the tetracycline supplements to treatment for comparable dosage groups of amphotericin B. Addition of tetracycline to therapy with 0.100 mg of amphotericin B per kg was significantly more therapeutic than the polyene with water at this dose in terms of prolonged survival time (Table 4) and an increase in total survivors (Table 5) for the time period studied. The effect of the tetracycline supplement was not apparent with mice treated with 0.400 mg of amphotericin B per kg, because the latter was therapeutically effective even in combination with the water supplement. By 40 days postinfection, however, it was apparent that treatment with the 0.400 mg of amphotericin B-tetracycline per kg combination was better than with the polyene-water combination, although the statistical probability was not significant at the usually acceptable level of $P = 0.05$. Additional support for the superiority of treatment with the amphotericin B-tetracycline combina-

TABLE 4. *Significance of survival times among mice infected (i.n.) with C. immitis: amphotericin B and water versus amphotericin B and tetracycline*

Amphotericin B + water[a] (mg/kg/day)	Amphotericin B + tetracycline[a]		
	Day 20	Day 30	Day 40
0.006	NS[b]	NS	NS
0.025	NS	NS	NS
0.100	0.02	0.03	0.03
0.400	NS	NS	0.08
All groups	0.05	0.05	0.04

[a] Water or tetracycline (8 mg per kg per day), single treatment daily for 5 days.
[b] NS, Not significant; $P > 0.10$ in all comparisons.

TABLE 5. *Significance of total number of survivors among mice infected (i.n.) with C. immitis: amphotericin B and water versus amphotericin B and tetracycline*

Amphotericin B + water[a] (mg/kg/day)	Amphotericin B + tetracycline[a]		
	Day 20	Day 30	Day 40
0.006	NS[b]	NS	NS
0.025	NS	NS	NS
0.100	0.02	0.03	0.09
0.400	NS	NS	0.09
All groups	0.10	0.10	NS[c]

[a] Water or tetracycline (8 mg per kg per day), single treatment daily for 5 days.
[b] NS, Not significant; $P > 0.10$ in all cases.
[c] $P = 0.12$.

tion is evident in the comparison between all groups on one or the other combination, especially in terms of survival time (Table 4).

A somewhat more precise evaluation can be obtained by determination of 50% end points. The time, in days, to survival of 50% of the mice in the various groups is presented in Table 6. Once again, an apparently effective chemotherapy is obtained with both higher levels of amphotericin B combined with tetracycline (27.0 days, $P = 0.06$; 45.1 days, $P < 0.001$). A comparable degree of therapeutic response occurs only with the highest dose of the polyene-water combination (34.8 days, $P = 0.009$). The dose of amphotericin B required to achieve survival of 50% of the infected mice (SD_{50}) is presented in Table 7. At each postinfection time interval, less amphotericin B is required when used in combination with tetracycline than with the water supplement. The ratio of the SD_{50} for the polyene-water combination relative to the SD_{50} for the polyene-tetracycline therapy varies from 3.19 to 2.50, indicating that this much more amphotericin B is required with the water supplement than when combined with tetracycline. These ratio values should be compared with the fourfold estimate derived from the data in Tables 2 to 6.

DISCUSSION

Although at least 87 polyenes have been described adequately (3), only amphotericin B has been clinically useful for general treatment of systemic mycoses. Clinical experience, however, has demonstrated that practical considerations severely limit the potential of this drug. Administration by infusion is cumbersome and difficult, and the immediate responses among patients vary from discomfort to toxicity sufficiently severe to cause termination of therapy. The necessarily slow infusion requires several hours and must be repeated on a daily or alternate day schedule over a period of several weeks. Successful response to a single course of therapy frequently is not sustained, and repetitions of the therapeutic regimen are common. Although initial nephrotoxicity is usually reversible following cessation of therapy, prolonged or successive repetitions of chemotherapy can result in permanent changes. The need for improvement is obvious.

Because none of the currently available an-

TABLE 6. *Comparison of the days to 50% survival for mice infected with C. immitis*

Amphotericin B (mg/kg/day)	Days to 50% survival with amphotericin B plus	
	Water	Tetracycline
0	10.8	11.4
0.006	10.7	11.2
0.025	10.1	11.9
0.100	12.0	27.0
0.400	34.8	45.1[a]

[a] By extrapolation.

TABLE 7. *Dose of amphotericin B required for survival of 50% of mice infected with C. immitis*

Day	SD_{50}[a] (mg/kg/day) with amphotericin B plus		Ratio
	Water	Tetracycline	
20	0.102	0.032	3.19
30	0.145	0.053	2.74
40	0.220	0.088	2.50

[a] SD_{50}, Survival dose for 50% of mice. Standard errors of all values were ± 0.009 to 0.010.
[b] Ratio = SD_{50} (amphotericin B + water)/SD_{50} (amphotericin B + tetracycline).

tifungal agents is likely to replace amphotericin B as the drug of choice for most cases of systemic mycoses, one must consider alternatives in the use of this antibiotic. The demonstration in vitro that amphotericin B, by increasing cell membrane permeability, opens the way for other antibiotics which are not effective against the intact cell indicates a potential for combined drug therapy (5, 6, 9, 10, 12). In our opinion, the experimental animal model reported here is a valid one, with certain limitations, for testing this hypothesis in an in vivo situation. The route of infection corresponds to that in natural disease, and animals are clinically ill before therapy is instituted. On the other hand, the infecting dose of spores is probably far more massive than occurs in natural disease, the incubation period is shorter, the disease course is more fulminating, and mortality is considerably higher than in naturally acquired coccidioidomycosis. These factors, combined with the very short course of treatment, constitute a severe test for the efficacy of the chemotherapy. In this context, the evidence that a significant measure of effective therapy was achieved is very promising.

Several aspects of the combined drug therapy must be considered. The results certainly are not dramatic in that there were still a significant number of deaths among animals treated with the higher doses of antibiotics and in that active lesions containing endosporulating spherules were found in tissues of surviving mice after sacrifice and necropsy. It was necessary, therefore, to rely on statistical analyses for evaluating whether one should place any confidence in the apparent differences among the several treatments. All these evaluations—survival times, total number of survivors, and time to survival of 50% of the animals—justified the conclusion that the amphotericin B-tetracycline combination resulted in a more efficacious therapeutic regimen than amphotericin B alone. The question of how best to use this combination of antibiotics is another aspect requiring consideration. Should tetracycline be added to amphotericin B at the maximally tolerated dose or at a reduced dosage? The evidence that there were no significant differences among animals receiving 0.400 mg of amphotericin B per kg, with or without tetracycline, might suggest that the additive or synergistic effect of adding tetracycline is of no consequence when the dosage with amphotericin B alone is effective. Yet it is apparent that there were some differences, although not statistically significant, among these two groups on the highest dose of amphotericin B by 40 days postinfection (Tables 4 and 5). On the other hand, the evidence from all the statistical evaluations consistently indicates that the combination of antibiotics results in significantly effective therapy at an amphotericin B dosage which is not effective when used alone (Tables 2 to 6). Furthermore, the results in Table 7 indicate that a desirable therapeutic response can be achieved when the effective dosage of amphotericin B alone is reduced by at least 2.5 times, provided tetracycline is added to the regimen. This should be an important consideration because of the cumulative toxicity of prolonged treatment with amphotericin B.

These aspects require further investigation. Extending the treatment period beyond 5 days and increasing the dosage of tetracycline by multiple daily administration are projected for further experiments. In addition, there are several other antibiotics (e.g., streptomycin and rifampin) which, in combination with amphotericin B, are synergistic against fungi in an in vitro environment (5, 6, 9, 10), and these are the subject of current studies with the experimental animal model of coccidioidomycosis.

ACKNOWLEDGMENTS

The authors express their gratitude to Georgia Workman and Betty Accomando for their invaluable assistance in tabulation of data for statistical analyses.

LITERATURE CITED

1. Friedman, L., D. Pappagianis, R. J. Berman, and C. E. Smith. 1953. Studies on *Coccidioides immitis:* morphology and sporulation capacity of forty-seven strains. J. Lab. Clin. Med. **42**:438–444.
2. Gehan, E. A. 1965. A generalized Wilcoxon test for comparing arbitrarily singly-censored samples. Biometrika **52**:203–223.
3. Hamilton-Miller, J. M. T. 1973. Chemistry and biology of the polyene macrolide antibiotics. Bacteriol. Rev. **37**:166–196; 407.
4. Huppert, M., S. H. Sun, and A. J. Gross. 1972. Evaluation of an experimental animal model for testing antifungal substances. Antimicrob. Ag. Chemother. **1**:367–372.
5. Kobayashi, G. S., G. Medoff, D. Schlessinger, C. N. Kwan, and W. E. Musser. 1972. Amphotericin B potentiation of rifampicin as an antifungal agent against the yeast phase of Histoplasma capsulatum. Science **177**:709–710.
6. Kwan, C. N., G. Medoff, G. S. Kobayashi, D. Schlessinger, and H. J. Raskas. 1972. Potentiation of antifungal effects of antibiotics by amphotericin B. Antimicrob. Ag. Chemother. **2**:61–65.
7. Levine, H. B., and Y. C. M. Kong. 1965. Immunity development in mice receiving killed *Coccidioides immitis* spherules: effect of removing residual vaccine. Sabouraudia **4**:164–170.
8. Levine, H. B., Y.-C. M. Kong, and C. E. Smith. 1965. Immunization of mice to *Coccidioides immitis:* dose, regimen and spherulation stage of killed spherule vaccines. J. Immunol. **94**:132–142.

9. Medoff, G., M. Comfort, and G. S. Kobayashi. 1971. Synergistic action of amphotericin B and 5-fluorocytosine against yeast-like organisms. Proc. Soc. Exp. Biol. Med. **138**:571–574.
10. Medoff, G., G. S. Kobayashi, C. N. Kwan, D. Schlessinger, and P. Venkov. 1972. Potentiation of rifampicin and 5-fluorocytosine as antifungal antibiotics by amphotericin B. Proc. Nat. Acad. Sci. U.S.A. **69**:196–199.
11. Miller, L. C., and M. L. Tainter. 1944. Estimation of the ED_{50} and its error by means of logarithmic-probit graph paper. Proc. Soc. Exp. Biol. Med. **57**:261–264.
12. Schwartz, S. N., G. Medoff, G. S. Kobayashi, C. N. Kwan, and D. Schlessinger. 1972. Antifungal properties of polymyxin B and its potentiation of tetracycline as an antifungal agent. Antimicrob. Ag. Chemother. **2**:36–40.
13. Siegel, S. 1956. Nonparametric statistics for the behavioral sciences. McGraw-Hill Book Co., Inc., New York.