ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 1992, p. 2656–2660
0066-4804/92/122656-05$02.00/0

Vol. 36, No. 12

Efficacy and Safety of Amphotericin B Colloidal Dispersion Compared with Those of Amphotericin B Deoxycholate Suspension for Treatment of Disseminated Murine Cryptococcosis

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Received 12 June 1992/Accepted 21 September 1992

The efficacy and safety of amphotericin B colloidal dispersion (ABCD) were compared with those of amphotericin B deoxycholate suspension (ABDS) (Fungizone) in a murine model of disseminated cryptococcosis. Mice were treated intravenously with either ABDS at 0.2, 0.8, or 3.2 mg/kg of body weight per dose or ABCD at 0.8, 3.2, 6.4, 12.8, or 19.2 mg/kg per dose three times per week for 2 weeks. Excluding mice treated with ABDS at 3.2 mg/kg, which was acutely lethal in 100% of mice, and ABCD at 19.2 mg/kg, which also resulted in two early deaths, the survival of ABCD- and ABDS-treated groups was prolonged over survival of controls ($P \leq 0.05$). Survival of ABCD (3.2 mg/kg)-treated mice was improved over that of ABDS (0.2 mg/kg)-treated mice ($P < 0.05$); however, comparisons of mice given all other dosages of ABCD with mice given sublethal dosages of ABDS did not demonstrate differences in survival. Comparative fungal burdens in organs showed a decrease in liver ($P < 0.05$) and spleen ($P < 0.05$) burdens for ABCD with the 19.2-mg/kg therapy versus those with ABDS with the 0.8-mg/kg therapy and liver burdens for ABCD with the 12.8-mg/kg therapy versus ABDS with the 0.8-mg/kg therapy ($P < 0.05$). There was no difference in organ burdens between therapy with ABCD at 0.8 mg/kg and ABDS at 0.8 mg/kg. These data show that the efficacy of ABCD is equal to that of ABDS on a milligram-per-kilogram basis for murine disseminated cryptococcosis. Because of its decreased toxicity, greater efficacy with ABCD could be achieved through doses fourfold higher than the 100% lethal dose for ABDS. Thus, ABCD shows promise as an effective but less toxic alternative to ABDS for the treatment of disseminated cryptococcosis.

Cryptococcal meningitis has become the most common life-threatening fungal infection in patients with AIDS (5, 28). Optimal therapy for this infection has not yet been established. Current treatment regimens include amphotericin B (with or without flucytosine) or fluconazole. Amphotericin B is associated with considerable toxicity, including nephrotoxicity which is often dose limiting (7). Fluconazole may be less efficacious than aggressive amphotericin B therapy for initial treatment (17).

In an effort to improve the therapeutic index of amphotericin B, new carrier systems and formulations have been devised. Among the most promising are lipid-based carriers, which are less toxic than the micellar amphotericin B deoxycholate suspension (ABDS; Fungizone; Bristol-Myers Squibb, Princeton, N.J.) and allow for higher doses with an improved therapeutic index (8, 11, 16, 20).

Lopez-Berestein and coworkers (18, 19, 21) demonstrated in open-label human trials the clinical efficacy and improved tolerance of a liposomal carrier system incorporating amphotericin B into large, multilamellar vesicles of dimyristoyl phosphatidylcholine (DMPC) and dimyristoyl phosphatidylglycerol (DMPG) (7:3 [mol/mol]) for systemic fungal infections. Szoka et al. (27) studied the incorporation of sterols such as ergosterol or cholesterol into small unilamellar liposomes and demonstrated their improved safety over that of large sterol-containing liposomes of the same composition as well as pure DMPC- and DMPG-containing phospholipid liposomes in a murine toxicity model. That study (27) also demonstrated the decreased toxicity of a pure cholesterol sulfate carrier used in combination with amphotericin B in a 1:1 molar ratio in comparison with DMPC- and DMPG-containing liposomes. Thus, the size and composition of lipid-based carriers appear to influence toxicity.

Amphotericin B colloidal dispersion (ABCD; Amphocil; Liposome Technology, Inc., Menlo Park, Calif.) is a novel lipid-based complex consisting of amphotericin B and cholesteryl sulfate in a 1:1 molar ratio. ABCD forms discoidal complexes of lipid and amphotericin B of about 150 nm in diameter. It is filter sterilizable and can be lyophilized to obtain a shelf-life of at least 1 year (11, 22). Tissue distribution studies of ABCD in rats (6) showed a three- to sevenfold lower concentrations of ABCD in the kidneys (the major target organ of toxicity) and three- to fourfold longer half-lives relative to those of ABDS at equal dosages. A single-dose pharmacokinetic study in healthy human volunteers, however, showed that ABCD has pharmacokinetics similar to those of ABDS (25).

Although a number of lipid-based carrier systems have been developed, it remains to be seen what the optimal formulation and treatment regimen(s) for systemic fungal infections will be. In the present study we compared the

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efficacy and safety of ABCD with those of ABDS in a murine model of acute disseminated cryptococcal infection.

MATERIALS AND METHODS

Preparation of inoculum. *Cryptococcus neoformans* 9-759 was kindly provided by Christine Morrison (Centers for Disease Control, Atlanta, Ga.). The lyophilized culture was reconstituted and streaked onto slants of Sabouraud dextrose agar (Difco, Detroit, Mich.) and was incubated at 35°C. Organisms were passaged through murine brain and then stored under water at 25°C. Prior to challenge, organisms were streaked onto Sabouraud dextrose agar plates and incubated at 35°C. Colonies from these plates were subcultured twice in a defined broth (12, 14) and were incubated at 35°C to produce cultures in the logarithmic phase of growth, centrifuged, washed twice, and resuspended in saline. Organisms were enumerated by counting with a hemacytometer and further diluted in saline to produce an inoculum stock of 5×10^6 yeasts per ml. Viability was assessed by plate counts on Sabouraud dextrose agar and defibrinated sheep blood agar plates (Becton-Dickinson, Irvine, Calif.) after incubation at 35°C for 2 to 4 days.

Inoculation of mice. Ten-week-old specific-pathogen-free female CD-1 mice (Charles River Laboratories, Portage, Mich.) were infected via the lateral tail vein with 1.25×10^6 (0.25 ml) yeasts of *C. neoformans*. This inoculum was used in preliminary experiments to establish an infection resulting in an approximate 1-month 90% lethal dose survival curve, with no deaths occurring before 5 days postinfection. In the present experiment, mice were grouped according to their drug-dose combination, with 10 mice per group. Sterilized food and acidified water were provided ad libitum.

Therapy regimens. ABCD was supplied as a lyophilized cake and was reconstituted with sterile water to a concentration of 5 mg/ml. Dilutions for administration to mice were made in sterile 5% glucose (Kendall McGaw Laboratories, Inc., Irvine, Calif.). ABDS was reconstituted to 5 mg/ml according to the manufacturer's instructions and was diluted in sterile 5% glucose.

Dosages were selected to test the comparative efficacy and therapeutic index of ABDS versus those of ABCD. Drug regimens consisted of ABCD at 0.8, 3.2, 6.4, 12.8, or 19.2 mg/kg of body weight per dose and ABDS at 0.2, 0.8, and 3.2 mg/kg per dose. Control groups included one group that received ABCD buffer (equivalent to the concentration in a 19.2-mg/kg dose of ABCD), another group that received ABDS diluent, and a third group that was not treated. Treatment was initiated 4 days after the establishment of infection. All treated groups were dosed three times per week (Monday, Wednesday, and Friday) for 2 weeks.

Deaths were recorded through 49 days postinfection. At the end of the study, all surviving mice were killed by CO_2 narcosis, and necropsies were performed immediately. The brain, lungs, liver, kidneys, and spleen were removed aseptically and weighed. Organs were subsequently homogenized with a Tissuemizer (Tekmar Co., Cincinnati, Ohio) in 5 ml of saline, and the homogenates were diluted and plated onto Sabouraud dextrose agar plates with chloramphenicol. After incubation at 37°C for 3 days, the quantitative CFU per organ was determined and was expressed as the number ($\log_{10}$) of CFU per organ. Organs with no detectable CFU ($\log_{10}$ value, 0) were considered completely cleared of infection, although the theoretical lower limit of detection of the assay method is approximately 5 to 7 CFU per organ.

In vitro studies. Determination of MICs and minimum fungicidal concentrations for the challenge isolate was performed by previously described methods (13).

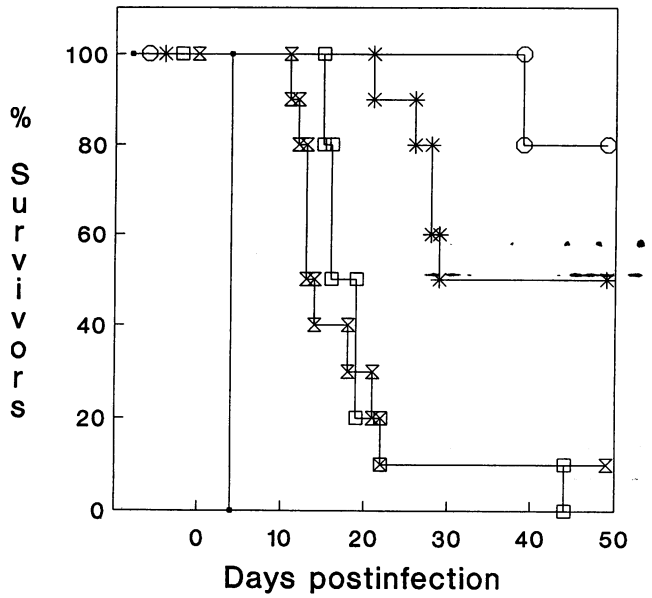

FIG. 1. Survival of CD-1 mice infected with *C. neoformans* and given intravenous ABDS, ABDS diluent, or no treatment. Ten mice treated with ABDS at 3.2 mg/kg died of acute toxicity. ■, ABDS at 3.2 mg/kg; ○, ABDS at 0.8 mg/kg; *, ABDS at 0.2 mg/kg; □, ABDS diluent; ⧖, no treatment.

Statistics. Differences in the cumulative mortalities and organ burdens of the various therapy groups were analyzed by nonparametric statistical tests, i.e., Wilcoxon rank sum test for mortality data and the Mann-Whitney U test for organ burdens (26). A Fisher's exact test was used for comparisons of numbers of sterilized organs and survivorship. Significance was assumed to be $P < 0.05$, although in some situations where multiple comparisons were possible, only those comparisons with lower P values (e.g., $P < 0.01$) could be regarded as important.

For analysis of organ burdens, datum points unavailable because of prior death of the animals from infection were assigned a value higher than the highest $\log_{10}$ CFU per organ for the same organ of living animals at the end of the experiment. This ensured that in nonparametric tests death caused by infection was assigned a worse outcome than survival with any organ burden. Early animal deaths during treatment apparently caused by toxicity were not included in the organ burden statistical analysis. This ensures that animals that died of infection contributed to the total organ burden of the group, although animals that died of toxicity did not.

RESULTS

In vitro studies. The MIC for the challenge isolate was 0.5 μg/ml for both preparations, and the minimum fungicidal concentrations were 1.0 μg/ml for ABDS and 2.0 μg/ml for ABCD.

Survival. Figure 1 shows the cumulative mortality of ABDS-treated mice and mice in control groups. Ninety percent mortality occurred in control groups (including untreated mice and mice receiving ABCD buffer or ABDS diluent) by 21 to 22 days. Only one control mouse (untreated) survived to the end of the experiment. One hundred

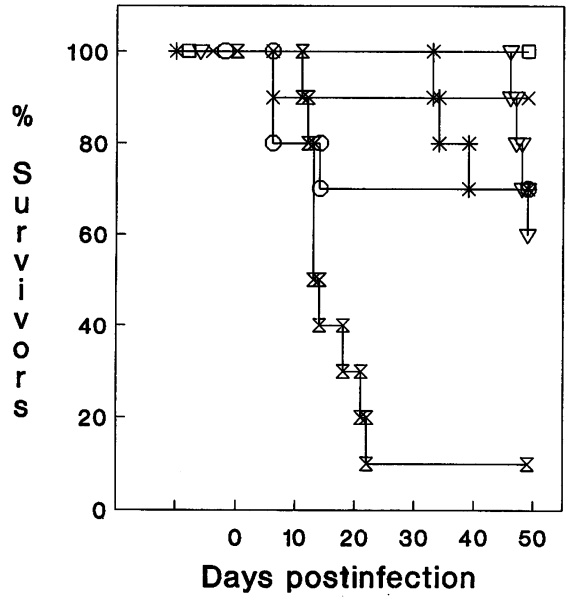

FIG. 2. Survival of CD-1 mice infected with *C. neoformans* and given intravenous amphotericin B colloidal dispersion (ABCD) or no treatment. *, ABCD at 0.8 mg/kg; □, ABCD at 3.2 mg/kg; ▽, ABCD at 6.4 mg/kg; ×, ABCD at 12.8 mg/kg; ○, ABCD at 19.2 mg/kg; ⧖, no treatment.

percent of mice given ABDS at 3.2 mg/kg died as a result of acute toxicity immediately after the first dose.

Figure 2 shows the cumulative survival of mice receiving ABCD compared with survival of untreated controls. Mice in all treatment groups (except ABDS at 3.2 mg/kg, which was acutely lethal) demonstrated ≥50% survival at 49 days. Survival of both ABDS- and ABCD-treated mice was generally dose responsive except for two deaths that occurred in the ABCD group given a dose of 19.2 mg/kg and one death that occurred in the ABCD group given a dose of 12.8 mg/kg on day 6 postinfection (in comparison, the first death in the control groups was on day 11). These deaths, which occurred >0.5 h after dosage administration, eliminated any advantage in the time of survival for mice that received these regimens over that for mice that were in the other treatment groups.

Statistical analysis showed that all treatment groups (except the group that received the acutely toxic ABDS dose) had prolonged survival compared with control groups ($P \leq 0.01$) except the group that received ABCD at 19.2 mg/kg, which was not different. However, the percent surviving in the group that received ABCD at 19.2 mg/kg was significantly ($P < 0.05$) better than that in the controls. All groups had prolonged survival compared with the groups that received the acutely toxic dose of ABDS (3.2 mg/kg). Although survival curves indicated a trend toward better survival with ABCD at 3.2 mg/kg compared with survival after treatment with the highest tolerable dose of ABDS (0.8 mg/kg), the difference was not significant. The survival of mice given ABCD at 3.2 mg/kg was improved over that of mice given ABDS at 0.2 mg/kg, however. Of interest is that the survival of both ABCD- and ABDS-treated mice was similar at equivalent dosages (0.8 mg/kg).

Reduction of residual infection. Table 1 shows the geometric mean $\log_{10}$ of residual organ burdens of *C. neoformans* in surviving animals. All surviving treated mice demonstrated a significant decrease in organ burdens of infection over those in control mice ($P < 0.05$) except for mice that received ABDS at 0.2 mg/kg; survival of mice treated with ABDS at 0.2 mg/kg was not different from that of controls. Consistent with the neurotropism of *C. neoformans*, all surviving animals had considerably higher mean burdens of infection in brains than in the other organs (by approximately 3 or more $\log_{10}$ units in all cases).

Residual infectious burdens were approximately equal among livers, spleens, lungs, and kidneys in ABDS-treated mice. In general, however, ABCD was more effective at reducing organ burdens in livers and spleens than in the other two organs. Total organ burdens of infection after therapy with ABCD at 19.2 mg/kg were reduced for livers ($P < 0.05$) and spleens ($P < 0.05$), and total organ burdens of infection after therapy with ABCD at 12.8 mg/kg were reduced for spleens ($P < 0.05$) compared with total burdens in the spleens of mice treated with the highest nontoxic dose of ABDS (0.8 mg/kg). There was no significant difference in organ burdens between treatment with high dosages of ABCD (12.8 or 19.2 mg/kg) and ABDS (0.8 mg/kg) with regard to brains, kidneys, or lungs, although there was a trend toward decreased CFU counts per organ in the brains of mice treated with ABCD at 19.2 mg/kg versus those in the brains of mice treated with ABDS at 0.8 mg/kg.

Table 1 also provides cumulative data on the number of organs sterilized in each group. ABCD at 19.2 mg/kg was the only regimen in the present study that completely cleared both livers and spleens of *C. neoformans* in all surviving

TABLE 1. Recovery of *C. neoformans* from organs of survivors

Treatment (mg/kg)	No. of survivors	Mean $\log_{10}$ CFU (no. of organs sterilized) in:				
		Brain	Liver	Spleen	Lungs	Kidneys
Untreated	1	7.48 (0)	1.51 (0)	0 (1)	2.19 (0)	4.09 (0)
ABDS						
0.8	8	6.11 (0)	2.06 (2)	1.29 (3)	2.18 (1)	2.09 (1)
0.2	5	7.19 (0)	3.19 (0)	3.18 (0)	1.70 (2)	3.16 (0)
ABCD						
19.2	7	5.68 (0)	0 (7)	0 (7)	2.28 (1)	2.09 (4)
12.8	9	6.89 (0)	0.49 (7)	1.18 (5)	2.69 (0)	2.70 (2)
6.4	6	6.75 (0)	1.34 (2)	0.12 (5)	2.75 (0)	1.87 (1)
3.2	10	6.48 (0)	1.86 (2)	1.25 (3)	2.58 (2)	2.06 (0)
0.8	7	6.52 (0)	2.02 (0)	1.35 (2)	2.96 (0)	3.04 (0)

animals. Mice from groups that received high doses of drug cleared the fungus from more organs of infection, however. ABCD at 19.2 or 12.8 mg/kg cleared more livers (seven mice each) than did ABDS at 0.8 mg/kg (two mice) or ABDS 0.2 mg/kg (no mice) ($P < 0.05$ and $P < 0.005$, respectively). ABCD at 19.2 or 12.8 mg/kg also cleared more spleens (seven and five mice, respectively) than did ABDS at 0.2 mg/kg (no mice) ($P < 0.005$ and $P < 0.05$, respectively).

Toxicity. Besides the acute, lethal toxicity seen with ABDS at 3.2 mg/kg, possible toxicities were also seen with ABCD at dosages that were 16- to 24-fold higher than the maximum tolerated dose of ABDS (0.8 mg/kg). Two deaths occurred in animals that received ABCD at 19.2 mg/kg and one death occurred in a mouse given ABCD at 12.8 mg/kg. Mild renal atrophy was seen in two survivors given ABCD at 3.2 mg/kg; renal atrophy was not seen in survivors from any of the other groups, including mice that received higher dosages of ABCD.

DISCUSSION

Data that demonstrate the efficacy of ABCD or similar cholesterol sulfate carrier systems in systemic fungal infections are accumulating. In a rabbit model of invasive aspergillosis, Patterson et al. (23) investigated the safety and activity of an amphotericin B–cholesterol sulfate complex, which is similar to ABCD, in a model of invasive aspergillosis. In comparison with ABDS, amphotericin B-cholesterol sulfate complex was less effective at equivalent dosages. The therapeutic index of the amphotericin B-cholesterol sulfate complex was sufficiently improved, however, by a fourfold decrease in toxicity, which allowed for treatment with dosages greater than the highest nontoxic dosages of ABDS.

In addition, ABCD has been shown to be efficacious in a murine model of coccidioidomycosis, in which doses of up to 10 mg/kg were given without apparent toxicity (3). Although ABCD was three- to fourfold less effective on a milligram-per-kilogram basis, ABCD was more than five- to eightfold less toxic than ABDS, thereby showing an improved therapeutic index. In a recent study of acute toxicity in mice, the lethal dose of ABCD in uninfected animals was 13- to 19-fold higher than that of ABDS (11).

Although a number of studies have demonstrated the efficacy and safety of lipid-based amphotericin B formulations for the treatment of systemic fungal infections, few have addressed their use against disseminated cryptococcosis. The in vitro activity of amphotericin B intercalated into various lipid carriers against *C. neoformans* (15, 24), although variable, is generally decreased compared with that of ABDS. Hanson and Stevens (13) tested the in vitro activity of ABCD against pathogenic species, including five isolates of *C. neoformans*. Overall, the antifungal activity of ABCD was variable in comparison with that of ABDS. With respect to the *C. neoformans* isolates, however, there was no difference in activity. MICs generally ranged from 0.5 to 1.0 μg/ml for both drugs. That range was confirmed in the present study. In the previous study, another stock of the isolate that we used for challenge, which was tested prior to animal passage, appeared to be more resistant to ABDS. We were unable to confirm such difference with the animal-passaged isolate in the present study.

In a model of murine cryptococcosis which included intraperitoneal, intratracheal, or intracerebral challenges with *C. neoformans*, Graybill et al. (9) compared the efficacy of a liposomal amphotericin B formulation with that of ABDS. At similar doses, the two drugs were found to have equivalent efficacies. Because of decreased toxicity, however, doses of the liposomal amphotericin B formulation that were fivefold higher than the maximum tolerated dose of ABDS were found to be more efficacious regardless of the site of inoculation of the organisms. The activity of a different lipid-complexed form of amphotericin B, designated ABLC (Bristol-Myers Squibb), against cryptococcosis was also demonstrated recently in a murine model (2). In contrast to the study by Graybill et al. (9), in the model of Clark et al. (2), although ABLC was efficacious, ABDS was more effective than ABLC in prolonging survival, even though up to 16-fold higher dosages of ABLC were given.

In our comparative study of activities, ABCD demonstrated efficacy in the treatment of systemic murine cryptococcosis at all dosages studied. In comparison with ABDS, further improvement in the clearance of organisms was seen in mice given ABCD at dosages that were 16- to 24-fold higher than the highest tolerated dose of ABDS. ABCD was generally well tolerated, although some possible toxicities were noted at doses of 12.8 and 19.2 mg/kg, with one and two deaths, respectively. Since the ABCD-treated animals did not die immediately, as did animals treated with ABDS at 3.2 mg/kg, acute cardiorespiratory toxicity seems improbable. Nephrotoxicity also seems unlikely, given the lack of evidence for this in the other animals, most of which received much greater total doses of the drug.

Previous studies comparing ABCD with ABDS for coccidioidomycosis (3) demonstrated that ABCD is three- to fourfold less effective on a milligram-per-kilogram basis. In contrast, in the present study there were no differences in survival or organ burdens of infection when equivalent nontoxic dosage regimens for these amphotericin B preparations (0.8 mg/kg) were used. This suggests that ABCD and ABDS have equal efficacies against disseminated murine cryptococcosis on a milligram-per-kilogram basis. Neither agent, however, was effective in curing animals of brain infection. This underscores the severity of the model, despite a lack of immunosuppression. It is of interest that the organs most effectively cleared of infection by the use of ABCD, i.e., livers and spleens, are those organs where ABCD is known to concentrate in rats (6). This is in contrast to the results of a previous study of ABCD against murine coccidioidomycosis (3). That study noted the rank order of organ clearance of *Coccidioides immitis* to be spleen > lungs > liver.

Recent case reports (1, 4) have documented the successful use of lipid-based amphotericin B formulations in AIDS patients with cryptococcal meningitis. In addition, a preliminary report of a trial of ABLC in the treatment of cryptococcal meningitis indicated a 77% (10 of 13 patients) rate of culture conversion to negative on therapy (10). Our study of ABCD in a murine model of disseminated cryptococcosis demonstrates that ABCD, because of its improved therapeutic index, is a potentially useful agent in the treatment of cryptococcal disease. Clinical trials are needed to establish its role as therapy for cryptococcal disease in humans.

ACKNOWLEDGMENTS

We thank Alon M. Perlman for excellent technical assistance during the course of these studies.

These studies were funded in part by a grant from Liposome Technology, Inc.

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