ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 1998, p. 767–771 Vol. 42, No. 4
0066-4804/98/$04.00+0
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In Vitro and In Vivo Antifungal Activity of Amphotericin B Lipid Complex: Are Phospholipases Important?

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Received 4 June 1997/Returned for modification 21 July 1997/Accepted 27 January 1998

Amphotericin B lipid complex for injection (ABLC) is a suspension of amphotericin B complexed with the lipids L-α-dimyristoylphosphatidylcholine (DMPC) and L-α-dimyristoylphosphatidylglycerol. ABLC is less toxic than amphotericin B deoxycholate (AmB-d), while it maintains the antifungal activity of AmB-d. Active amphotericin B can be released from ABLC by exogenously added (snake venom, bacteria, or *Candida*-derived) phospholipases or by phospholipases derived from activated mammalian vascular tissue (rat arteries). Such extracellular phospholipases are capable of hydrolyzing the major lipid in ABLC. Mutants of *C. albicans* that were resistant to ABLC but not AmB-d in vitro were deficient in extracellular phospholipase activity, as measured on egg yolk agar or as measured by their ability to hydrolyze DMPC in ABLC. ABLC was nevertheless effective in the treatment of experimental murine infections produced by these mutants. Isolates of *Aspergillus* species, apparently resistant to ABLC in vitro (but susceptible to AmB-d), were also susceptible to ABLC in vivo. We suggest that routine in vitro susceptibility tests with ABLC itself as the test material may not accurately predict the in vivo activity of ABLC and that the enhanced therapeutic index of ABLC relative to that of AmB-d in vivo may be due, in part, to the selective release of active amphotericin B from the complex at sites of fungal infection through the action of fungal or host cell-derived phospholipases.

Amphotericin B has been the agent of choice for the treatment of serious fungal infections for more than 35 years. However, administration of the most common preparation of amphotericin B (a sodium deoxycholate colloidal suspension) is associated with severe, dose-limiting acute and chronic toxicities, particularly nephrotoxicity.

Amphotericin B lipid complex for injection (ABLC) is a suspension of amphotericin B complexed with the lipids L-α-dimyristoylphosphatidylcholine (DMPC) and L-α-dimyristoylphosphatidylglycerol (DMPG) (11). The safety and efficacy of ABLC have been extensively evaluated in laboratory (4–6, 14) and clinical (7, 24, 25) studies. Those studies have shown that ABLC is, in general, markedly less toxic than amphotericin B deoxycholate (AmB-d) and has antifungal activity at least comparable to and sometimes enhanced over that of AmB-d.

Complexation with lipids appears to stabilize amphotericin B in a self-associated state so that it is not available to interact with cellular membranes (the presumed major site of its antifungal activity and its mammalian toxicity) (12, 17). It was previously demonstrated that ABLC is more than 1,000-fold less hemolytic to erythrocytes in vitro than AmB-d and that active (hemolytic) amphotericin B can be released from ABLC by a heat-labile, extracellular fungal product (lipase) (17). It has also been demonstrated that in vitro the MICs of ABLC for certain phospholipase-deficient non-*Candida albicans Candida* species and phospholipase-deficient mutants of *C. albicans* are higher than those of AmB-d (13). In the present study we further evaluated the role of phospholipases in the in vitro and in vivo antifungal activity of ABLC.

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MATERIALS AND METHODS

Organisms. *C. albicans* 2433 was from the American Type Culture Collection (ATCC). The parent strain (strain SC5314) and mutant strains (strains SC15183, SC15184, and SC15185) of *C. albicans* (which were selected for resistance to ABLC after nitrous acid treatment) were a gift from Daniel P. Bonner (Department of Microbiology, Bristol-Myers Squibb Pharmaceutical Research Institute, Wallingford, Conn.) and have been described previously (13) and used in other investigations (8). All isolates were stored frozen at −70°C prior to use. *Aspergillus* isolates were from clinical specimens collected in the United States and sent to the Santa Clara Valley Medical Center, San Jose, Calif., for susceptibility testing. Isolates were identified by conventional macroscopic and microscopic criteria (22).

In vitro susceptibility testing. (i) *Candida*. MICs were determined by a broth microdilution assay modified from the M27P methodology of the National Committee for Clinical Laboratory Standards (15, 20). Briefly, AmB-d (Fungizone; Bristol-Myers Squibb, Princeton, N.J.) was reconstituted according to the package insert and was then further diluted in saline to a concentration of 1,000 μg/ml. ABLC (ABELCET; The Liposome Company, Inc., Princeton, N.J.) was provided as a suspension containing 5.0 mg of amphotericin B per ml in saline and was also diluted in saline to 1,000 μg/ml. The drugs were then serially diluted in RPMI 1640 medium (with L-glutamine and 20 mM HEPES buffer and without sodium bicarbonate) in U-bottom 96-well microtiter plates. The test organism (in RPMI 1640 medium) was added to a final concentration of 0.5×10^3 CFU/ml. The MIC was defined as the lowest concentration that completely inhibited visible growth after 24 h of incubation at 30°C. An additional plate was set up for each organism in medium containing 0.43 U of phospholipase A2, B, or C (Sigma catalogue nos. P3770, P8914, and P7633 respectively; Sigma Chemical Co., St. Louis, Mo.) per ml as well as ABLC or AmB-d. For each organism tested, growth in medium alone and in medium with phospholipase (without ABLC or AmB-d) was confirmed.

(ii) *Aspergillus*. AmB-d (Fungizone) was dissolved in sterile water at a concentration of 1.6 mg/ml in glass vials and was stored in the dark at −20°C. ABLC was diluted with saline to 1.6 mg/ml. Both drugs were then diluted in unbuffered yeast nitrogen base broth containing 0.5% glucose (YNB). Dilutions were twofold from 8 to 0.25 μg/ml in a final volume of 2 ml in 5-ml plastic tubes. Conidia (in YNB) from each test isolate were added carefully with the tip of a pipette to just below the meniscus in each tube to a final concentration of 10^3 cells/ml. Control tubes contained YNB without drug. The inoculum size was confirmed by plating on sheep blood agar plates. The tubes were incubated on a rotary shaker at an angle of approximately 30° from the horizontal with loose caps in ambient air. When growth was evident in the control tubes, the MIC was read (40 to 48 h). The lowest concentration of drug that inhibited visible growth was defined as the MIC.

TABLE 1. Phospholipase-deficient strains of *C. albicans* are resistant to ABLC in vitro

Strain	Phospholipase activity (egg yolk)	% ^{14}C-myristic acid released from ABLC	MIC (μg/ml in RPMI 1640)			
			Without added phospholipase		With added phospholipase	
			AmB-d	ABLC	AmB-d	ABLC
ATCC 24433	+	54.7	0.04	0.04	ND[a]	ND
SC5314	+	53.8	0.16	0.16	0.16	0.16
SC15183	−	28.9	0.32	10.00	0.32	0.32
SC15184	−	14.0	0.16	>10.00	0.16	0.16
SC15185	−	19.7	0.16	2.50	0.16	0.16

[a] ND, not done.

Phospholipase activity. (i) Egg yolk agar. Extracellular phospholipase activity was determined by the method of Price et al. (18) as described previously (13). Briefly, a few colonies from a 1- to 3-day-old agar plate were suspended in distilled water, and their numbers were adjusted to 10^5 cells/ml with a hemocytometer. Ten microliters of the suspension was plated onto Sabouraud dextrose agar containing 1 M NaCl, 0.005 M CaCl, and 8% uncentrifuged Bacto Egg Yolk Enrichment (Fisher Scientific). The plates were incubated at 30°C for 72 h. Isolates that produced extracellular phospholipase showed a distinct, white, opaque zone (precipitate) below and around the colony.

(ii) Hydrolysis of DMPC in ABLC. ABLC containing ^{14}C-DMPC (Dupont NEN Research Products, Boston, Mass.) was prepared as described previously (16). The final suspension contained 5.3 mM amphotericin B and 6.1 mM phospholipid (~7:3 molar ratio for DMPC and DMPG) with a specific activity of 600 μCi of phospholipid per mmol. For the determination of extracellular phospholipase activity capable of remodeling ABLC, *Candida* organisms were grown at 35°C for 24 h in 50 ml of unbuffered Sabouraud dextrose broth containing 2% dextrose (BBL) in 250-ml flasks with vigorous shaking to ensure adequate aeration. The cells were then removed by low-speed centrifugation followed by filtration through 0.4-μm-pore-size polycarbonate filters. Aliquots of 25 μl of the labeled ABLC were incubated with 3 ml of the cell-free broth for 24 h at 37°C. Following incubation, 7.5 ml of methanol and 3.75 ml of chloroform were added. The resulting monophase formed two phases upon the addition of 3.75 ml each of water and chloroform. As expected, greater than 98% of the total radioactivity was associated with the organic layer. The lower chloroform phase was removed and dried by vacuum rotary evaporation. Approximately 0.2 ml of chloroform was added back, and this volume was then applied to Whatman silica gel 60 thin-layer chromatography plates. Standards of DMPC, myristic acid, and lysophosphatidylcholine (to establish R_f values) were run separately. A chloroform-methanol-water (65:25:4; vol/vol/vol) solvent system was used, and the distribution of radioactivity was assessed by autoradiography with Kodak X-OMAT-AR film. The spots were scraped and assayed for radioactivity by liquid scintillation counting. The remainder of each lane was also scraped to account for any residual radioactivity.

To determine if mammalian tissues produced extracellular enzymes capable of remodeling ABLC, the abdominal aortas of eight anesthetized (sodium pentobarbital) rats were cannulated and the rats were perfused with phosphate-buffered saline. The thoracic aortas were then aseptically removed and placed into Dulbecco's minimal essential medium (DMEM; Gibco, Grand Island, N.Y.) containing 1,000 mg of glucose per liter, 584 mg of L-glutamine per liter, 110 mg of sodium pyruvate per liter, and 10 μg of gentamicin per ml. The arteries were cut into 1-mm sections (a procedure that "activates" the tissue) and placed in the wells of a 24-well tissue culture plate with 1 ml of DMEM per well. The tissues were incubated in air at 37°C for 24 h. Just prior to collecting the supernatants, 2 U of heparin was added to each well (to release membrane-associated phospholipase). The supernatants were pooled and centrifuged at 500 × *g* for 15 min to remove any cellular debris. Medium without any artery sections was incubated, supplemented with heparin, and centrifuged in the same manner. Either calcium (final concentration, 5.6 mM) or EGTA (final concentration, 3.1 mM) was added to aliquots of the supernatant, and then approximately 27 μl of labeled ABLC was added per ml of medium. The labeled ABLC used here was 0.16 mM amphotericin B and 0.19 mM phospholipid (6 mCi of phospholipid per mmol). The ABLC was incubated and the lipids were extracted, separated, and assayed in the same manner as described above for the yeast broths. The artery sections that were used to prepare the supernatants were dried for 3 h at 60°C and weighed. There was approximately 15 mg of dry tissue per ml of medium during the incubation.

Experimental infection models. Fungi were grown on Sabouraud dextrose agar and harvested by washing with sterile saline containing 0.05% Tween 80. The cell suspensions were filtered through sterile gauze to break up the clumps, and the cells were counted with a hemocytometer and diluted to the appropriate concentrations with saline. The inoculum size (sufficient to cause the death of 60% or more of the untreated mice within approximately 4 to 10 days after infection) for each organism was determined in preliminary experiments. For the *Candida* infection model, male BALB/c mice (weight range, 18 to 22 g) were immunosuppressed with 2 mg of cyclophosphamide (Cytoxan; Bristol-Myers Oncology, Princeton, N.J.) administered intraperitoneally on days −4, 0, 4, 8, and 11. For experiments with *Aspergillus*, mice were not immunosuppressed. For both the *Aspergillus* and the *Candida* infection models, infection was by the intravenous route on day 0. Groups of 10 mice were treated intravenously with escalating doses of ABLC or AmB-d (Fungizone) once daily for 4 consecutive days beginning 24 h after infection. A single inoculum preparation was used for each organism, and the groups receiving the two treatments (ABLC or AmB-d) were infected and treated concurrently. Mortality was monitored daily for 28 days.

Statistical analysis. The survival times for mice in the AmB-d group were compared to those for mice in the ABLC group at each dose by the Mann-Whitney U test (26), with significance defined as a P value of <0.05.

RESULTS

In vitro and in vivo susceptibility of phospholipase-positive and phospholipase-deficient *Candida* strains to ABLC and AmB-d. Table 1 presents the phospholipase activities and in vitro susceptibilities of five strains of *C. albicans*. We confirmed that the parent wild-type *C. albicans* strain (strain SC5314) produced extracellular phospholipase detectable on egg yolk agar, whereas three mutants derived from this strain (mutants SC15183, SC15184, and SC15185) did not (8, 13). Strain ATCC 24433 also produced extracellular phospholipase sufficient for detection on egg yolk agar. When ABLC that was made with ^{14}C-DMPC was incubated in cell-free broth in which the five organisms had grown, the highest levels of myristic acid (released from DMPC by phospholipase) were found in the strains with phospholipase activity demonstrable with egg yolk agar. We also confirmed that the ABLC and AmB-d MICs for the strains producing large amounts of phospholipase were identical, whereas the phospholipase-deficient strains appeared to be resistant to ABLC but susceptible to AmB-d. The addition of snake venom phospholipase (phospholipase A2) to the incubation medium restored the activity of ABLC against these mutant strains in vitro. The addition of phospholipase B (from *Vibrio* sp.) or phospholipase C (from *Clostridium perfringens*) also restored the activity of ABLC (data not shown). In these experiments, there did not appear to be a strict concentration-response relationship between DMPC hydrolysis and the MIC, suggesting that the MIC depends on a multiplicity of factors.

Figure 1 presents the results of therapy with ABLC or AmB-d in immunosuppressed mice infected with the parent, wild-type *C. albicans* strain equally susceptible to both drugs in vitro (strain SC5314) or with mutants of this strain that appeared to be resistant to ABLC in vitro (strains SC15183 and SC15184). The maximum tolerated dosage (MTD) of AmB-d in these studies was 0.4 mg/kg of body weight/day, which was slightly less than that found in previous studies of AmB-d in

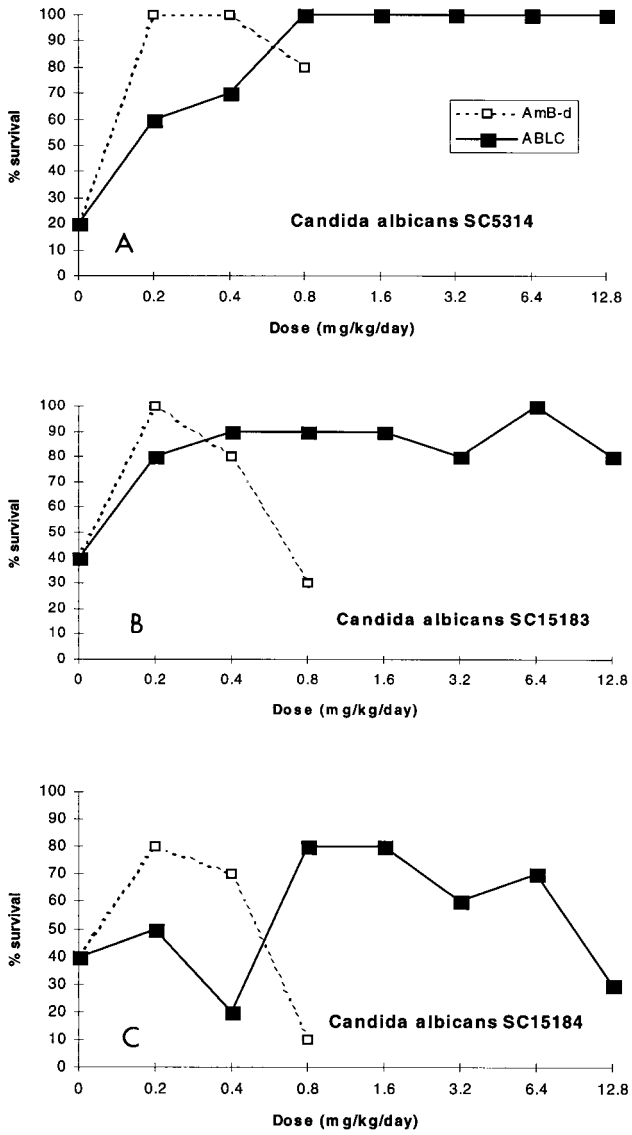

FIG. 1. Comparison of ABLC and AmB-d for the treatment of systemic *C. albicans* infections in immunosuppressed mice. (A) Infection (intravenous) with 2.5×10^4 CFU of a phospholipase-producing strain of *C. albicans* (strain SC5314) per mouse. The median survival time for saline-treated mice was 11.5 days. (B) Infection (intravenous) with 5×10^5 CFU of a phospholipase-deficient mutant of *C. albicans* (mutant SC15183) per mouse. The median survival time for saline-treated mice was 12.5 days. (C) Infection (intravenous) with 10^7 CFU of a phospholipase-deficient mutant of *C. albicans* (mutant SC15184) per mouse. The median survival time for saline-treated mice was 4.5 days.

various murine infection models (0.8 mg/kg/day) (4, 5, 9). Most mice dying of AmB-d toxicity died after receipt of the first or second dose of drug (day 1 or 2), whereas mice dying of fungal infection generally died later (after day 5). The MTD of ABLC was between 6.4 mg/kg/day (mice infected with strain SC15184) and 12.8 mg/kg/day (mice infected with strains SC5314 and SC15183). Thus, the MTD of ABLC was 16- to 32-fold greater than that of AmB-d. Significantly, all three organisms tested were susceptible to ABLC in vivo, regardless of the results of in vitro susceptibility testing. In all cases, it was possible to achieve the same therapeutic effect with ABLC and AmB-d. Although the survival times for mice treated with AmB-d and ABLC at 0.2 mg/kg/day were not significantly different according to the organism with which they were infected, the trends in the overall survival rates suggest that the dosage required for the maximal therapeutic effect was two- to fourfold greater for ABLC than for AmB-d. These larger dosages of ABLC were well tolerated (did not cause early deaths), and this in vivo difference in potency was not related to the in vitro activities of the drugs.

TABLE 2. In vitro susceptibilities of *Aspergillus* isolates to ABLC and AmB-d

Organism and strain	MIC (μg/ml in YNB)	
	AmB-d	ABLC
Aspergillus fumigatus		
DS10AF	1	1
DS93-19	2	1
DS92-270	2	1
DS92-245	2	1
DS92-46	2	>8
Aspergillus flavus		
DS89-37	4	>8
DS89-158	4	>8
DS88-91	4	>8
Aspergillus niger		
DS92-195	1	2
DS93-252	2	2
Aspergillus terreus		
DS92-62	2	>8

In vitro and in vivo susceptibilities of *Aspergillus* isolates to ABLC and AmB-d. Table 2 presents the results of in vitro testing of the susceptibilities of the 11 clinical isolates of *Aspergillus* to ABLC and AmB-d. All isolates were inhibited by 4 μg of AmB-d per ml or less, whereas 5 of the 11 isolates were not inhibited by 8 μg of ABLC per ml. We chose one isolate (*Aspergillus fumigatus* 10-AF) for which the MICs of both AmB-d and ABLC were the same (relatively low) and two isolates (*Aspergillus flavus* 89-158 and *Aspergillus terreus* 92-62) that were not inhibited by ABLC in vitro to evaluate the efficacy of ABLC in vivo. Figure 2 presents the results of those studies. Both AmB-d and ABLC could protect 100% of the mice infected with strain 10-AF. The dosage of AmB-d producing the maximal therapeutic effect was 0.2 mg/kg/day (treatment with AmB-d resulted in a significant increase in survival time compared with that after treatment with ABLC at 0.2 mg/kg/day), while that for ABLC was 0.4 mg/kg/day. The maximum tolerated dose of AmB-d in these mice was 0.8 mg/kg, while that of ABLC was 12.8 mg/kg or greater. For mice infected with the two other *Aspergillus* isolates, it was possible to achieve 70% survival only with the MTD of AmB-d (0.8 mg/kg/day). AmB-d at 0.4 mg/kg/day produced a significant increase in survival time compared with that produced by ABLC at the same dose for mice infected with *A. flavus*, but ABLC was at least as effective, albeit at higher doses, as AmB-d for the treatment of infections produced by both organisms, even though they appeared to be resistant in vitro.

Phospholipase activity of stimulated mammalian vascular tissue. Figure 3 presents the percentage of total radioactivity from ABLC prepared with ^{14}C-DMPC that was recovered as myristic acid or lysophosphatidylcholine after incubation with supernatants from culture medium exposed or not exposed to activated rat vascular tissue. More than 20% of the radioactivity in the organic layer following extraction was recovered as

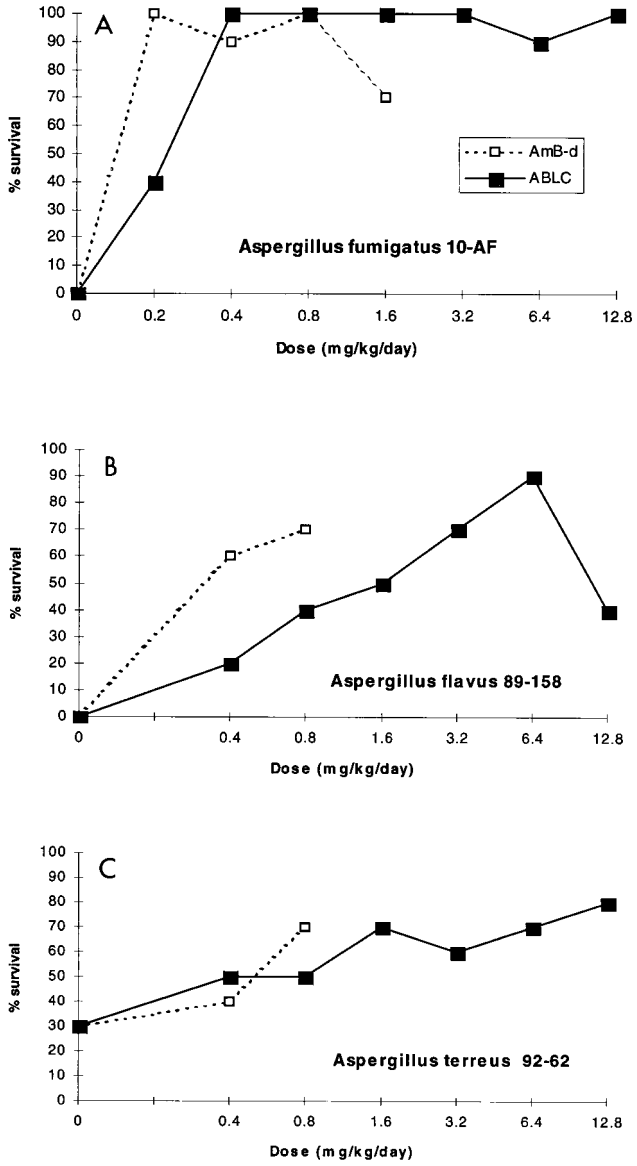

FIG. 2. Comparison of ABLC and AmB-d for the treatment of systemic *Aspergillus* infections in normal mice. (A) Infection (intravenous) with 5×10^6 CFU of *A. fumigatus* 10-AF per mouse. The median survival time for saline-treated mice was 8.5 days. (B) Infection (intravenous) with 2×10^6 CFU of *A. flavus* 89-158 per mouse. The median survival time for mice treated with saline was 6.5 days. (C) Infection (intravenous) with 2×10^7 CFU of *A. terreus* 92-62 per mouse. The median survival time for mice treated with saline was 18 days.

myristic acid when the incubation was performed with the supernatant from culture medium exposed to activated rat artery sections in the presence of calcium. For this sample only, a significant fraction of the radioactivity (~18%) was also in the aqueous layer which corresponds to lysophosphatidylcholine and which is consistent with a nearly quantitative breakdown of DMPC to myristic acid and lysophosphatidylcholine. For samples incubated with medium alone or arteries in the presence of EGTA, greater than 95% of the radioactivity was recovered as intact DMPC (data not shown). Thus, activated rat vascular tissue produces an extracellular, calcium-dependent phospholipase (likely phospholipase A2) capable of remodeling ABLC.

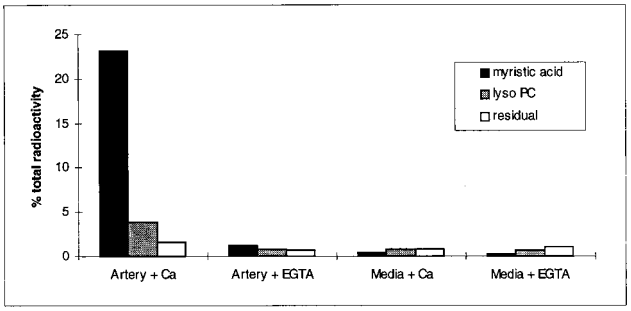

FIG. 3. Percentage of total radioactivity from ^{14}C-DMPC formulated as ABLC that was recovered in the organic layer as myristic acid or lysophosphatidylcholine (lyso PC) following incubation with supernatants from culture media exposed or not exposed to activated rat artery sections.

DISCUSSION

The antifungal activity of ABLC is due to amphotericin B. This antibiotic has been in clinical use for more than 35 years, and the spectrum of its activity, both in vitro and in vivo, has been well characterized. Complexation of amphotericin B with lipids is not believed to change the intrinsic activity of the drug or its mechanism of action. Our data suggest that in in vitro systems, ABLC is not toxic unless the complex is disrupted, allowing the active form of amphotericin B to interact with the target cells. Many yeasts and some molds are known to produce extracellular phospholipases. In some cases (*C. albicans* and *A. fumigatus*) this has been associated with virulence (2, 10). We have shown that the extracellular lipases produced by certain strains of *C. albicans* are able to hydrolyze the major lipid in ABLC, releasing active amphotericin B, and as a consequence, in vitro these strains are just as susceptible to ABLC as they are to AmB-d. We confirmed that mutants of *C. albicans* that were resistant to ABLC in vitro were deficient in extracellular phospholipase production. The addition of exogenous phospholipase to the incubation medium of these strains restored their sensitivity to ABLC. Other factors (e.g., interaction with membrane-bound phospholipases, other components in the medium, or other extracellular products) may also disrupt the amphotericin B-lipid complex sufficiently to allow antifungal activity against some strains. Thus, extracellular phospholipase production appears to be one factor, but may not be the only factor, influencing the in vitro susceptibility of fungal isolates to ABLC.

Other investigators working with different lipid-based preparations of amphotericin B have shown discrepancies between the in vitro activities of these formulations and that of AmB-d (9, 16, 19). The results for the formulations used in the work described here, however, cannot easily be extrapolated to other formulations. Other formulations of AmB-d may contain sterols (which would not be susceptible to phospholipase breakdown) or differ in size, radius of curvature, lipid packing, molar ratio of amphotericin B to lipid, and phospholipid composition, all of which could affect the ability of interfacial catalysts such as phospholipases to interact with the membrane and release active AmB-d.

In this study, when strains that appeared to be resistant to ABLC in vitro (but susceptible to AmB-d) were used to infect mice, ABLC was as effective as AmB-d at prolonging survival. As has been shown previously (4–6), slightly higher doses of ABLC were required to obtain efficacy, but these doses were well tolerated. Furthermore, higher doses of ABLC were required for strains for which the in vitro ABLC MICs were identical to those of AmB-d (*C. albicans* SC5314 and *A. fu-*

migatus 10-AF). In vitro susceptibility studies with ABLC rather than the active ingredient (amphotericin B) alone as the test article did not accurately predict the in vivo activity of ABLC. We suggest that in vitro susceptibility to amphotericin B (dispersed with deoxycholate) may be more useful in predicting the clinical utility of ABLC than in vitro susceptibility testing with ABLC, although confirmation of this will require additional work.

We hypothesize that host tissue-derived phospholipases may be able to hydrolyze the lipid component of ABLC, thus activating ABLC in vivo. Serum-free media in which stimulated rat arteries were incubated were found to contain Ca^{2+}-dependent lipase activity capable of breaking down the DMPC in ABLC to myristic acid and lysophosphatidylcholine. Extracellular phospholipase secretion has been associated with local and systemic inflammation and is an integral part of the host response to infecting microorganisms (23). Since a major source of extracellular phospholipase has been attributed to vascular endothelial and smooth muscle cells (23) and since infection with *Candida* has been shown to stimulate endothelial phospholipase A2 activity in vitro (3), we suggest that endogenous phospholipases may play a role in the activity of ABLC in vivo.

In order for amphotericin B to be selectively released from ABLC at sites of fungal infection by pathogen- or inflammation-induced, host-derived phospholipases, it is necessary for the complex to remain intact until it reaches these sites. Recent work (1) has shown that although ABLC is gradually remodeled in normal rat plasma, most of the complex is removed from the circulation prior to remodeling. It is known that certain fungi are angiotrophic (21) and/or can injure endothelial cells (3). Such injury may disrupt the capillary lining sufficiently to allow ABLC to escape from the bloodstream selectively at sites of fungal infection, where it would then be broken down and would release active amphotericin B.

In conclusion, we have confirmed that certain mutants of *C. albicans* that are resistant to ABLC in vitro but that maintain their susceptibility to AmB-d are deficient in extracellular phospholipase production. The addition of exogenous (snake venom or bacterial) phospholipase to the incubation medium restored the sensitivities of these phospholipase-deficient mutants to ABLC in vitro. In in vivo studies, ABLC was effective for the treatment of experimental infections with these apparently resistant, phospholipase-deficient mutants of *C. albicans*. Certain isolates of *Aspergillus* species apparently resistant to ABLC in vitro (but susceptible to AmB-d) were also susceptible in in vivo models. We were able to show that remodeling of ABLC occurs in vitro in the presence of activated mammalian vascular tissue. Thus, we suggest that (i) standard in vitro susceptibility tests with ABLC itself (rather than the active component, amphotericin B, alone) may not accurately predict the in vivo activity of ABLC and (ii) the enhanced therapeutic index of ABLC relative to that of AmB-d in vivo may be due in part to the selective release of active amphotericin B from the complex at sites of fungal infection or inflammation through the action of phospholipases released by the fungus or activated host cells (phagocytic cells, vascular smooth-muscle cells, or capillary endothelial cells).

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