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Antifungal Activity of Amphotericin B Cochleates against *Candida albicans* Infection in a Mouse Model

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Cochleates are lipid-based supramolecular assemblies composed of natural products, negatively charged phospholipid, and a divalent cation. Cochleates can encapsulate amphotericin B (AmB), an important antifungal drug. AmB cochleates (CAMB) have a unique shape and the ability to target AmB to fungi. The minimal inhibitory concentration and the minimum lethal concentration against *Candida albicans* are similar to that for desoxycholate AmB (DAMB; Fungizone). In vitro, CAMB induced no hemolysis of human red blood cells at concentrations of as high as 500 μg of AmB/ml, and DAMB was highly hemolytic at 10 μg of AmB/ml. CAMB protect ICR mice infected with *C. albicans* when the agent is administered intraperitoneally at doses of as low as 0.1 mg/kg/day. In a tissue burden study, CAMB, DAMB, and AmBisome (liposomal AmB; LAMB) were effective in the kidneys, but in the spleen CAMB was more potent than DAMB at 1 mg/kg/day and was equivalent to LAMB at 10 mg/kg/day. In summary, CAMB are highly effective in treating murine candidiasis and compare well with AmBisome and AmB.

Candida albicans, which develops both as a topical and disseminated infection, is the predominant organism that causes fungal disease, although other fungal pathogens, such as *Aspergillus fumigatus*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, and other *Candida* species, are also important and, under some circumstances, may predominate. These organisms have become major nosocomial causes of morbidity and mortality in immunocompromised individuals [11] and thus opportunistic fungal infections are a major cause of nosocomial disease (1, 16, 20), Candidemia is a more serious condition affecting the immunocompromised patient, although not as frequently with human immunodeficiency virus-infected patients. Disseminated candidiasis affects in excess of 120,000 patients annually and has a high overall mortality rate (5, 20).

The treatment options for disseminated fungal infections are extremely limited. The most rapidly acting and potent agent for treating disseminated disease remains amphotericin B (AmB) (5), an amphiphatic molecule possessing a hydrophilic polyhydroxyl chain along one axis and a lipophilic polyene hydrocarbon chain on the other. AmB has an exceptional low solubility in water and in many organic solvents, but it associates well with lipid membranes. Because AmB formulations such as Fungizone (desoxycholate AmB; DAMB) have multiple severe toxicities, including renal insufficiency (4), lipid-based formulations have been developed. These particulate suspensions reduce AmB toxicity but must be used at higher doses than Fungizone.

Cochleate delivery vehicles are a novel lipid-based system that has potential for the delivery of AmB. Cochleates are stable phospholipid-cation precipitates composed of simple, naturally occurring materials (for example, phosphatidylserine and calcium). They have a unique multilayered structure consisting of a large, continuous, solid, lipid bilayer sheet rolled up in a spiral, with no internal aqueous space. Cochleates were first reported by D. Papahadjopoulos as an intermediate in the preparation of large unilamellar vesicles (13). The unique structure of cochleates provides protection from degradation for associated "encochleated" molecules. Since the entire cochleate structure is a series of solid layers, components within the interior of the cochleate structure remain intact, even though the outer layers of the cochleate may be exposed to harsh environmental conditions or enzymes. Cochleates can be stored in cation-containing buffer or lyophilized to a powder, stored at room temperature, and reconstituted with liquid prior to administration. Lyophilization has no adverse effects on cochleate morphology or biological functions. The lipid-based cochleate delivery system was shown to achieve protein and peptide antigen delivery for vaccine applications (10).

In this study, the feasibility and efficacy of cochleates as a delivery vehicle for AmB was assessed by examining the in vivo activity of AmB cochleates (CAMB) against *C. albicans* in a systemic mouse infection model. The activity of the new CAMB system is compared to two commercially available AmB preparations, Fungizone and AmBisome (liposomal AmB; LAMB).

(These results were presented in part at the 39th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, Calif., 1999.)

MATERIALS AND METHODS

Drug, lipids, cells, and animals. AmB was purchased from USP (Rockville, Md.). LAMB and DAMB were purchased from a hospital pharmacy. Dioleoylphosphatidyl serine (DOPS) was obtained from Avanti Polar lipid (Birmingham, Ala.). All other chemical reagents were of analytical grade and were obtained from Sigma Chemical Co. (St. Louis, Mo.). Agar was purchased from Gibco. ICR mice weighing 25 to 30 g were obtained from the University of Texas. *Candida* sp. isolate NIH-A (93-343) was obtained from the American Type Culture Collection.

Cochleate preparation. Cochleate controls were prepared by dispersing DOPS powder with TES [*N*-tris (hydroxymethyl)methyl-2-aminoethanesulfonic acid] buffer (NaCl, 100 mM; histidine, 2 mM; TES, 2 mM) in a ratio of 10 mg of lipid/ml. The cochleates were formed by the slow addition (10 μl) of calcium chloride (0.1 M) to the suspension of liposomes at a molar ratio of lipid to

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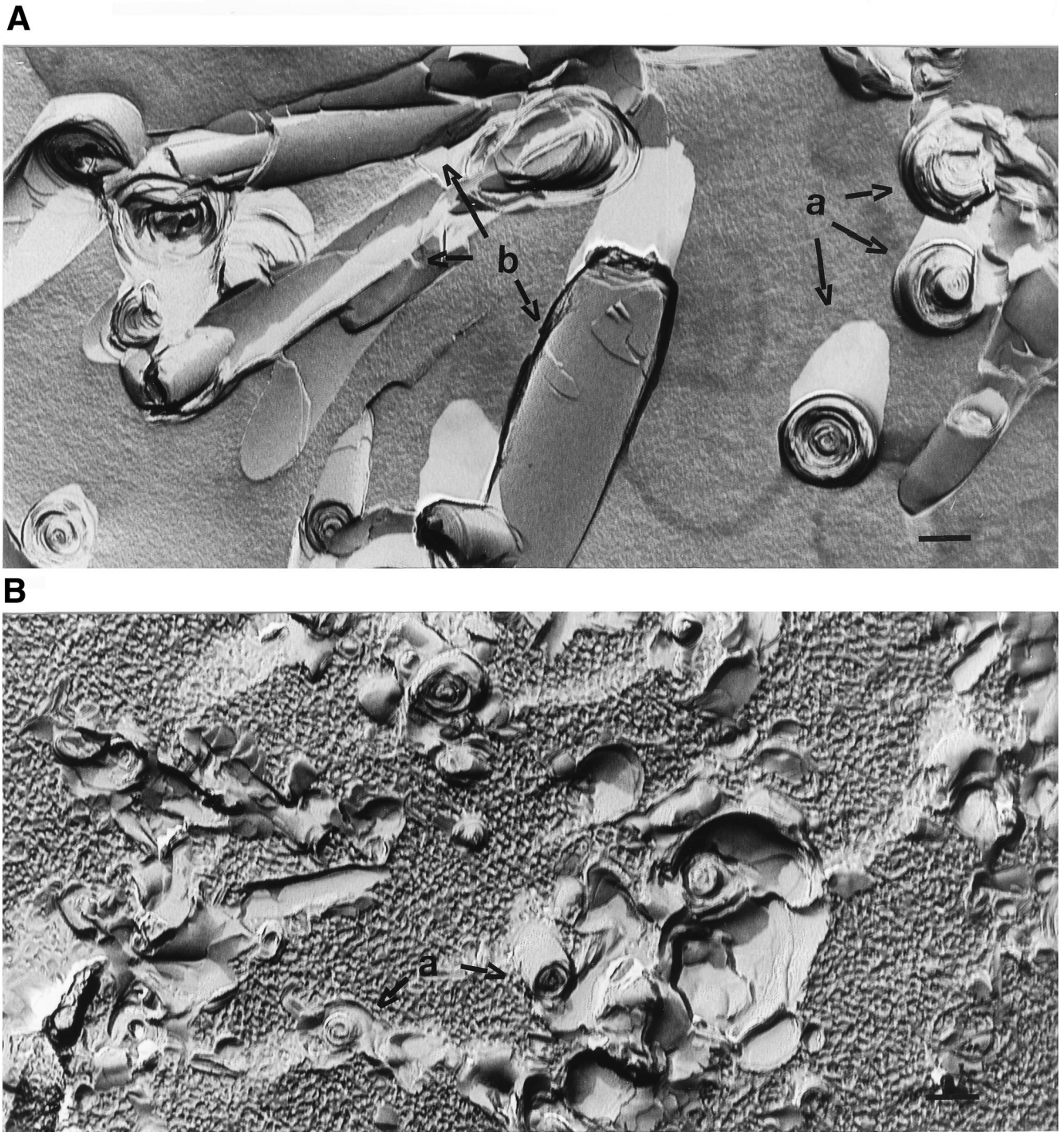

FIG. 1. Freeze-fracture electron micrographs of empty cochleates (A) and CAMB (B) prepared by the trapping film method. Arrows indicate rolled-up cochleate structures. Bar, 275 nm.

calcium of 2:1 with an external excess of 3 mM calcium and then stored at 4°C in the absence of light. The structure of empty cochleates was checked by optical microscopy and freeze-fracture electron microscopy (Fig. 1).

AmB cochleates. DOPS powder was mixed with AmB crystals in a lipid/AmB molar ratio of 10:1 in a sterile polypropylene tube along with TES buffer (pH 7.4) at 10 mg of lipid/ml. After vortexing the mixture three times for 1 min, multilamellar liposomes were formed. The pH of the preparation was increased to pH 11.5 (a pH at which AmB is solubilized) by the addition of 1 N NaOH. Optical microscopy was used to monitor for the absence of AmB crystals and the presence of liposomes (by phase-contrast and polarization techniques). The cochleates were formed by the slow addition (10 μl) of calcium chloride (0.1 M) to the AmB liposome suspension at a molar lipid/calcium ratio of 2:1 with an external excess of 3 mM Ca (Fig. 2). The external pH was adjusted to 7.4 by the addition of 1 N HCl. CAMB were plated onto agar plates to check sterility and then stored at 4°C in the absence of light.

The encochleation efficiency of AmB was assessed as follows. First, 100 μl of the cochleate suspension was centrifuged (6,000 rpm for 20 min at 4°C), and then EDTA (pH 9.5) and 100 μl of Triton X-100 (10%) were added to the cochleate suspension to allow the opening of the cochleates into liposomes and the release of AmB. The pH was adjusted to 11.5 by the addition of 50 μl of 1 N NaOH, and

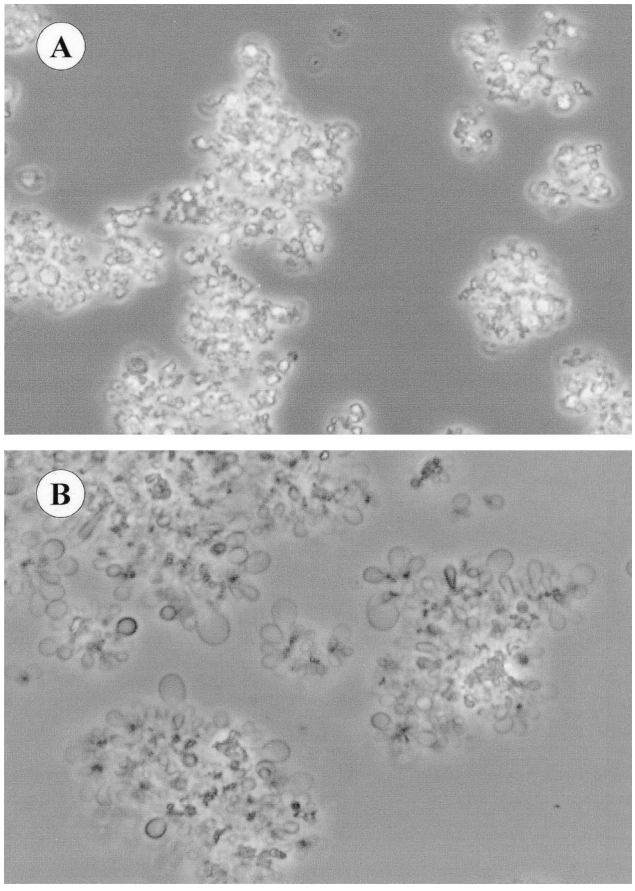

FIG. 2. Phase-contrast optical microscopy of CAMB before the addition of EDTA (A) or after the addition of EDTA (B). Note the formation of liposomes.

the amount of free AmB in the supernatant and pellet was determined by measuring the optical density at 410 nm (OD_{410}). More than 50 separate CAMB formulations were prepared. Spectrophotometer and high-performance liquid chromatography analysis were used to determine the amount of AmB associated within the cochleates. For a given formulation protocol, for example a molar lipid/AmB ratio of 10:1, the amount of the encochleated AmB is highly reproducible (within 3%).

Freeze-fracture electron microscopy. A thin layer of the sample was deposited on a copper holder, quenched in liquid propane, and fractured in vacuo ($<10^{-6}$ T) with a liquid nitrogen-cooled knife in a Balzers 301 freeze-etching unit. The replication was performed with unidirectional Pt-C shadowing; the mean thickness of the metal deposit was ca. 1.5 nm. The replicas were washed with ethanol and water and observed on a Philips EM301 electron microscope.

Stability of AmB cochleates. CAMB were tested for stability for extended time periods at 4°C. Then, 1-ml samples of CAMB were sealed in sterile brown glass vials under nitrogen gas. Samples were stored at 4°C and then analyzed weekly for up to 98 days for the percentage of free AmB as follows. Five samples of 100 μl of CAMB were added to 15-ml polypropylene centrifuge tubes. The tubes were centrifuged at 6,000 rpm for 20 min at 4°C, and the supernatant and pellet were separated. Then, 60 μl of EDTA (pH 9.5) was added to each of the pellets while vortexing them to open up the cochleates (Fig. 2B). Next, 100 μl of 10% Triton X-100 was added, followed by vortexing. The resulting solution was clear yellow. A 50-μl portion of 1 N NaOH was added to bring the pH to 11.5. Then, 10-μl aliquots were taken and diluted in 2.5 ml of methanol and transferred to the spectrophotometer cell. The concentration of free AmB was measured by comparing the absorbance at 410 nm to a standard curve. The amount of free AmB in the supernatant was measured at 410 nm with 2.5 ml of nondiluted supernatant.

Susceptibility testing. *C. albicans* was subcultured onto Sabouraud dextrose agar plates and grown at 35°C for 24 h prior to testing. The broth microdilution method was performed according to the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS) in RPMI 1640 medium (with L-glutamine) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid. Testing was performed in 96-well round-bottom microtitration plates. Cell suspensions were adjusted to give a final inoculum concentration of 0.5×10^3 to 2.5×10^3 cells/ml, which were confirmed by plating them on Sabouraud dextrose plates. The 96-well plates were incubated at 35°C and were read visually after 48 h. The AmB MIC was defined as the lowest concentration of drug that completely inhibited growth.

Hemolytic effect of AmB cochleates on human blood cells. Human blood was drawn by venous puncture into heparinized tubes. Plasma and buffy coat were removed by centrifugation at 3,000 rpm for 10 min at 10°C, and the erythrocytes were washed three times with phosphate-buffered saline (pH 7.4). The red blood cells were then suspended in the same buffer to give a suspension of 1% hematocrit ($N = 8 \times 10^7$ to 9×10^7 cells/ml). The hemolytic activity was determined by adding 2 ml of the suspension to the same volume of solution or by dispersion of the cochleates in TES buffer. After 1 h of incubation in a shaking water bath at 37°C, the tubes were centrifuged to remove the unhemolyzed cells. The degree of hemolysis was determined by comparing the amount of hemoglobin released in the supernatant (OD_{540}) with 0% hemolysis in saline and 100% hemolysis in water. The percentage of hemolysis was calculated as follows: hemolysis (%) = $100\ (OD_{test} - OD_{saline})/(OD_{water} - OD_{saline})$.

Treatment of mice infected with *C. albicans* by AmB cochleates. (i) Survival study. The National Institutes of Health isolate *C. albicans* ATCC 93-343 was used for infection. This isolate was maintained between studies at 4°C on Sabouraud dextrose agar. Before these studies, the isolate was transferred to brain heart infusion broth and grown at 37°C overnight. The inoculum was washed three times in saline, and an aliquot was used for the hemacytometer counting. The inoculum was adjusted to 0.2 ml/mouse, and the count of viable organisms was determined by colony count dilutions. Male ICR mice (30 g, 10 mice/group) were infected through tail vein (intravenously [i.v.]) inoculation of 5×10^6 CFU/mouse (0.2 ml of 2.5×10^7 CFU/ml). At 24 h postinfection, the animals were treated with CAMB at various doses (20, 10, 4, 1, 0.5, and 0.1 mg of AmB/kg) by intraperitoneal (i.p.) injection of 0.2 ml/mouse as a once-daily dose for 10 days. Comparison was achieved with groups of mice receiving DAMB (4, 1, 0.5, and 0.1 mg of AmB/kg) or empty cochleates. The survival of animals was monitored and compared to controls for 30 days postinfection (Fig. 5).

Tissue burden study. For tissue burden studies mice were infected as described above and dosed with CAMB at 10, 1, and 0.1 mg/kg (i.p. route) versus DAMB at 1 and 0.1 mg/kg (i.p. route) and LAMB at 10, 1, and 0.1 mg/kg (i.v. route). Mice were treated daily from days 1 to 7 postchallenge, and the tissue burden was assessed at day 8. The kidneys and spleen were removed and assayed for CFU. Quantitative tissue counts were done by homogenizing the tissue in 2 ml of sterile saline and performing serial 10-fold dilutions onto blood agar plates which were incubated at 35°C for 2 days and then counted.

Statistics. Comparisons were made by the log rank test and Wilcoxon test of life tables. The Sidak's multiple comparison test was used to compare tissue counts. Overall significance was determined at $P < 0.05$ for comparison for two groups, with adjustment of the P values when more than two groups were compared.

In vitro targeting of rhodamine-labeled AmB cochleates to *Candida* cells. Rhodamine-labeled CAMB were prepared as described above for AmB cochleates by using a mixture of phospholipids, DOPS, and rhodamine-linked phosphatidylethanolamine in a molar ratio of 100 to 0.1. In a 12-well microtiter plate, rhodamine-labeled CAMB were added to 0.5 ml of *Candida* suspension at the following final concentrations: 5, 2.5, and 1 μg/ml. Empty cochleates were used as controls, and the plate was incubated at 30°C for 2 h. Analysis was then performed by phase-contrast and fluorescence microscopy.

RESULTS

The addition of calcium ions to sonicated phosphatidylserine AmB vesicles induces fusion of lipid membranes and the formation of planar sheets which eventually coil around an initial point of folding to form cochleate cylinders.

Freeze-fracture electron micrographs (Fig. 1A) show the morphology of standard empty cochleate structures prepared with DOPS. Cross-sections of cochleates show rolled-up bilayers as well as elongated microstructures differing in size and shape. Micrographs of AmB cochleates (Fig. 1B) show rolled-up structures, although the CAMB seemed to be smaller than the empty cochleates.

Optical microscopy was used on a routine basis to assess the CAMB structures. Figure 2 shows typical AmB cochleates prepared by the high-pH method before (Fig. 2A) and after the addition of EDTA (Fig. 2B). By chelating the calcium cations with EDTA, the rolled-up CAMB transform into liposomes (Fig. 2B).

Cochleates based on DOPS associated with more than 98% of added AmB. These formulations were highly stable. There was no apparent release of AmB from the yellow cochleates

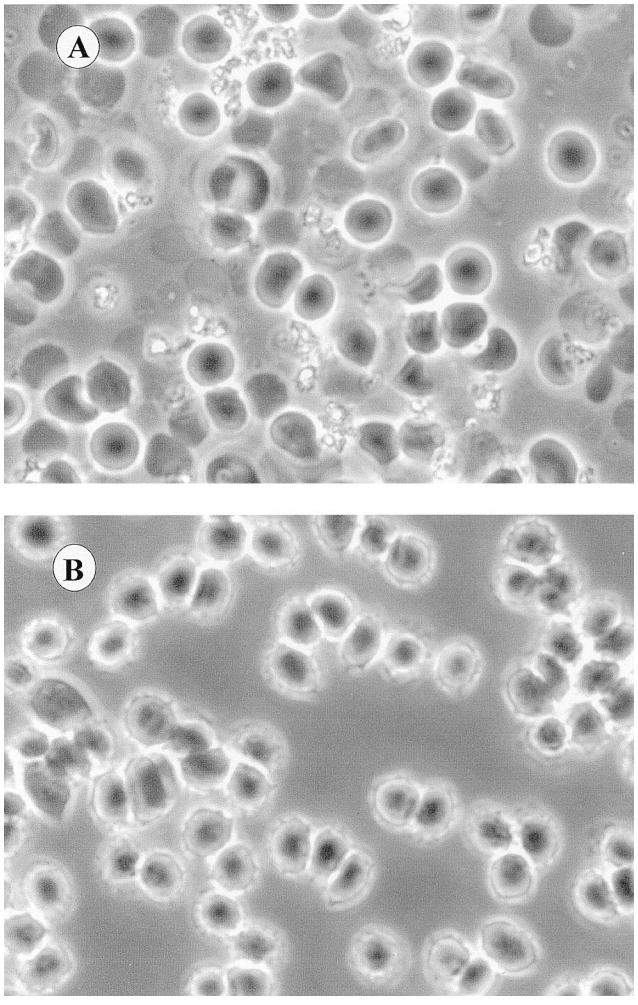

FIG. 3. Phase-contrast micrographs of red blood cells incubated with either 500 μg of CAMB per ml (A) or 1 μg of DAMB (B) per ml.

observed after incubation for at least 3 months at 4°C and for 7 days at 37°C.

The toxicity of CAMB on human red blood cells was analyzed and compared to that of DAMB. Increasing concentrations of CAMB corresponding to 2.5, 5, 10, 250, and 500 μg of AmB/ml were incubated with a suspension of red blood cells prepared as described in Materials and Methods. Hemoglobin release was quantified by measuring the OD_{540} of the supernatant. NaCl (0.9%) empty cochleates, and empty liposomes were used as negative controls, and distilled water was used as a positive control. CAMB showed no hemoglobin release and, therefore, no hemolysis of red blood cells at all of the tested concentrations, even at the highest concentration of 500 μg/ml. By contrast, DAMB was hemolytic at a concentration of 10 μg/ml, which reflected the presence of the detergent sodium desoxycholate.

When observed by phase-contrast optical microscopy, red blood cell suspensions containing DAMB at 1 μg of AmB/ml reflect the presence of echinocyte shapes (Fig. 3B). This morphological stage is a result of detergent adsorption into the red blood cell membrane and precedes the disruption of the membrane and lysis of the cell (12). Figure 3A is a typical image of red blood cells after incubation with CAMB at 500 μg of AmB/ml. The cells preserved their concave shape, an indication of their healthy state. The micrographs also show the presence of free CAMB unbound to the red blood cells, indicating a lack of interaction between the cochleates and the red blood cell plasma membrane.

TABLE 1. Comparison of MIC and MLC of cochleates for *C. albicans*

Cochleate group	MIC (μg/ml)	MLC (μg/ml)
Empty cochleates	>64	ND[a]
CAMB	0.5	0.5
DAMB	0.5	0.5

[a] ND, not determined.

C. albicans susceptibility to CAMB was evaluated in vitro using NCCLS guidelines as described in Materials and Methods. The MIC and minimum lethal concentration (MLC) of CAMB (0.5 μg/ml) were found to be identical to DAMB (Table 1). Empty cochleates and TES-Ca (6 mM) buffer did not show any activity against *C. albicans*. The targeting of CAMB to *Candida* cells was also shown when rhodamine-labeled CAMB was incubated with *Candida* cells. Figure 4 demonstrates that after a 2-h incubation, the *Candida* cells became

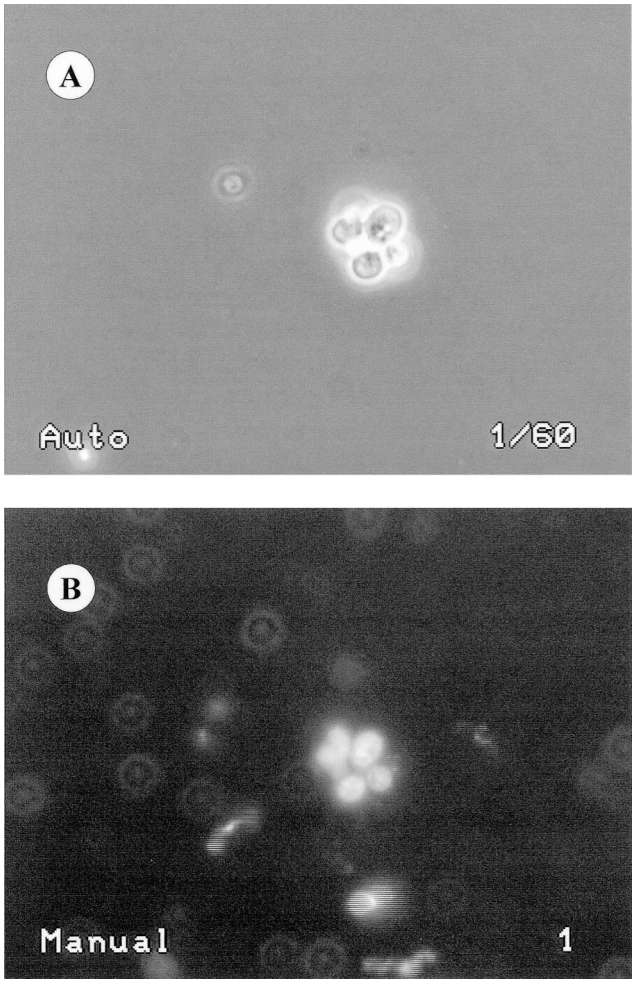

FIG. 4. Uptake of AmB-rhodamine-labeled cochleates by *C. albicans* after incubation for 2 h in Dulbecco modified Eagle medium. Panel A shows the phase-contrast micrographs, and panel B shows the fluorescence micrographs.

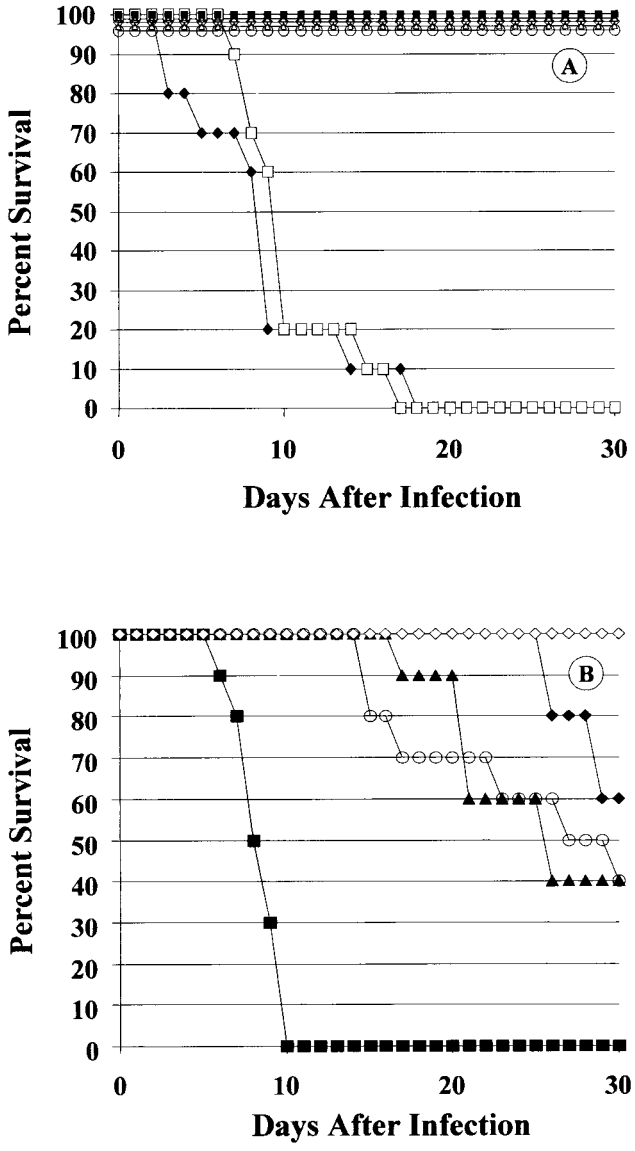

FIG. 5. (A) Survival of mice infected with *C. albicans* and treated either with CAMB at 20 (×), 10 (◇), 4 (●), or 1 (+) mg/kg; with DAMB at 4 mg/kg (▲); or with empty cochleates (□) compared to the untreated control (◆). (B) Survival of mice infected with *C. albicans* and treated either with CAMB at 0.5 (◇) and 0.1 (▲) mg/kg, with DAMB at 0.5 (◆) and 0.1 (○) mg/kg, or with empty cochleates (■).

fluorescent, indicating a transfer of labeled lipid from CAMB to the fungus cells.

CAMB were efficient at inhibiting the growth of *C. albicans* in vivo. The efficacy of CAMB formulations in vivo against *C. albicans* infection was evaluated in infected ICR mice at doses of 20, 10, 4, 1, 0.5, and 0.1 mg of AmB/kg (given i.p.). A comparison was conducted with DAMB doses of 4, 0.5, and 0.1 mg of AmB/kg; empty cochleates; and a negative control. Mice treated with CAMB at 1 to 20 mg/kg showed 100% survival after 30 days (Fig. 5A). In the control group treated with empty cochleates, 80% of the animals died by day 8 of infection, and 100% died by day 16. In a second experiment, lower doses of CAMB (0.5 and 0.1 mg of AmB/kg) were compared with DAMB at the same doses. Empty cochleates displayed almost the same profile as the controls. At a dose of as low as 0.5 mg/kg, 100% survival was observed with CAMB versus 60% with DAMB at the same dose (Fig. 5B). At 0.1 mg of AmB/kg, both cochleates and DAMB showed the same level of survival. For the control group, 100% of the animals died after 10 days. Tissue burden studies were conducted in parallel with the survival studies. Mice were dosed with CAMB at 10, 1, and 0.1 mg/kg (i.p. route) versus DAMB at 1 and 0.1 mg of AmB/kg (i.p. route) and LAMB, a liposome AmB form, at 10, 1, and 0.1 mg/kg (i.v. route). Mice were treated daily from days 1 to 7 postchallenge, and the tissue burden was assessed at day 8. Kidneys and spleens were removed and assayed for the presence of *Candida* cells. In the spleen burden experiments (Fig. 6A), CAMB at a dose of 10 mg of AmB/kg were comparable to LAMB in reducing the fungal colony burden. At 1 mg of AmB/kg CAMB had a better effect on the *Candida* count than LAMB and DAMB, and at 0.1 mg of AmB/kg all three formulations were ineffective. In the kidney (Fig. 6B), all three formulations were comparable at 0.1 and 1 mg/kg in reducing colony counts by 3 orders of magnitude, while at 10 mg/kg CAMB and LAMB had the same profile. For all groups, CFU found in the kidneys and spleens were reduced significantly below the levels found in controls ($P < 0.001$).

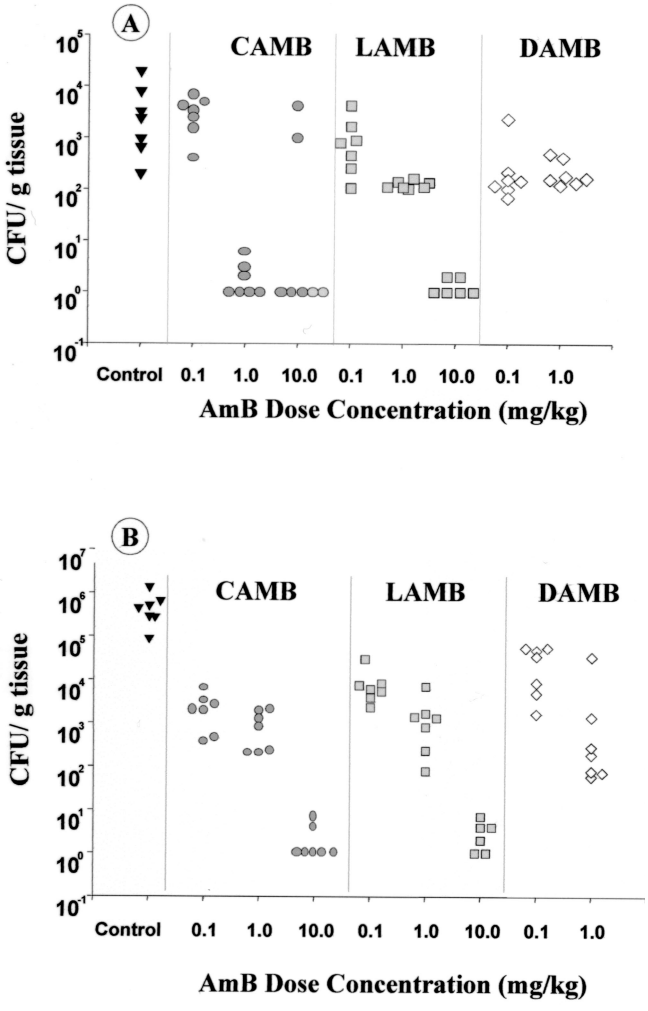

FIG. 6. (A) Spleen tissue burden of mice infected with *C. albicans* and treated with either CAMB (●) at 10, 1, or 0.1 mg/kg; LAMB (■) at 10, 1, or 0.1 mg/kg; or DAMB (◇) at 1 or 0.1 mg/kg compared to controls (▼). (B) Kidney tissue burden for the same groups of mice.

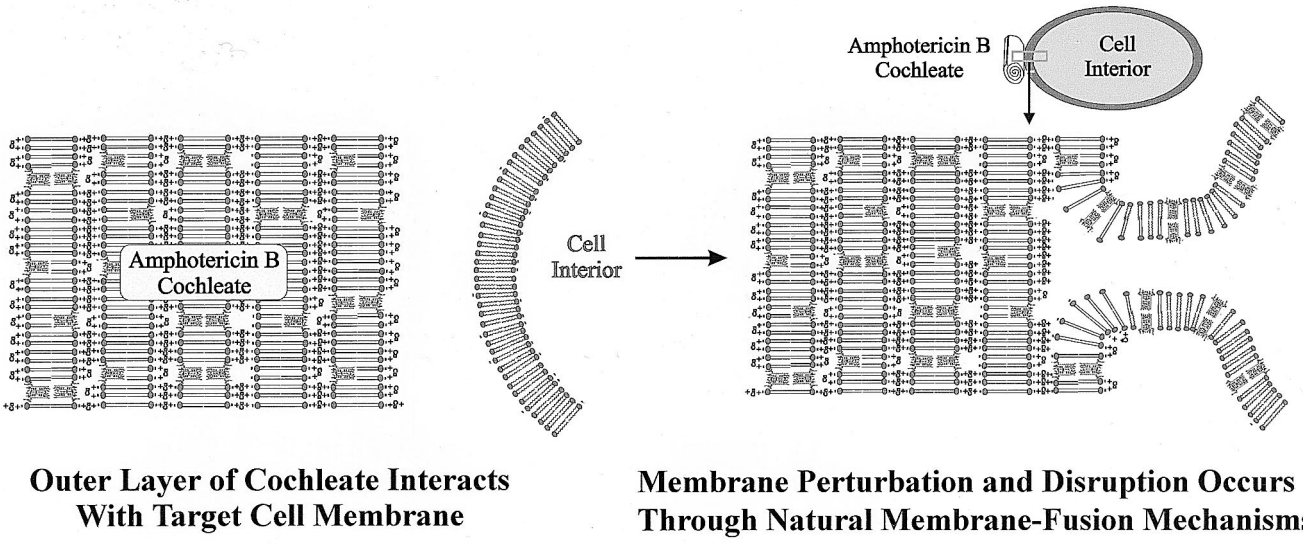

FIG. 7. Schematic of the mechanism of action of CAMB when CAMB bilayers approach a target cell (A) or are fused with a target cell (B).

DISCUSSION

AmB is still the drug of choice for treating the most severe systemic fungal infections (5). It is active against a wide variety of fungal species (7, 9, 19); one hypothesis is that AmB acts by binding to ergosterol present in fungal cells, leading to the formation of transmembrane channels through which the cytoplasmic potassium leaks out, resulting in metabolic disruption and cell death (8). AmB binds with less affinity to cholesterol present in mammalian cells. The toxicity of AmB to critical mammalian organ systems such as the kidney is attributed to the AmB-cholesterol binding.

Several laboratories have formulated AmB into lipid-based delivery systems in order to overcome AmB toxicity problems and to improve the safety of this important drug (3). Although the currently marketed lipid-based AmB systems have improved the safety profile of AmB, these lipid-based systems are less efficacious than DAMB and need to be used at doses substantially higher than Fungizone.

We hypothesized that the encapsulation of AmB into the lipid-based cochleate system would result in stable, nontoxic, highly efficient AmB lipid particles. In earlier investigations on the use of cochleates as a delivery system for DNA, we demonstrated that cochleates can be loaded with DNA, improving the stability and the biological activity of nucleic acids (21).

AmB can be easily embedded into a lipid membrane (3). When producing AmB cochleates, we find that negatively charged DOPS interacts well with the polyenic structure of AmB resulting in mixed bilayers constructed with AmB-DOPS. The addition of calcium induces the fusion of AmB liposomes. Calcium-induced fusion of negatively charged lipid vesicles as the mechanism involved in the formation of cochleates was proposed by D. Papahadjopoulos et al. in 1975. The rearrangement between AmB and phosphatidylserine molecules seems to have no impact on the structure of cochleate (Fig. 1). Sheets of AmB-DOPS appear to roll up into cylinders when a lipid/AmB molar ratio of 10/1 is used. The precise orientation of AmB within the crystalline bilayers of the cochleate is currently under investigation using X-ray analysis.

Cochleates seem to have a nontoxic profile and to improve the safety of toxic drugs. Cochleates are composed of safe products: phosphatidylserine and calcium. Phosphatidylserine is a natural component of all biological membranes and is most concentrated in the brain. Clinical studies to evaluate the potential of phosphatidylserine as a nutrient supplement indicate that it is very safe and may play a role in the support of mental functions in the aging brain (6, 18). Indeed, phosphatidylserine isolated from soy beans is sold in health food stores as a nutritional supplement.

The safety of CAMB in vivo was evaluated in a mouse model after i.p. administration of escalating doses (5, 10, and 25 mg/kg) of CAMB as a daily dose for 10 days. A 100% survival was obtained after 30 days; the animals displayed normal behavior, and their body weight was comparable to that of controls (L. Zarif, I. Segarra, T. Jin, D. Hyra, and R. J. Mannino, Proc. 26th Int. Symp. Controlled Release Bioactive Materials, abstr. P, p. 964–965, 1999).

CAMB were found to be nonhemolytic on red blood cells even at concentrations as high as 500 μg/ml, whereas DAMB was highly hemolytic at 10 μg/ml. One hypothesis would be that cochleates have little or no interaction with red blood cells, in contrast to DAMB, which is AmB formulated with a surfactant, sodium desoxycholate. Although not all surfactants are detergent-like (17) and hemolysis depends on the molecular and ionic structure of a surfactant (12), sodium desoxycholate belongs to the family of anionic surfactant known to be highly hemolytic.

CAMB have been found, in vitro, to have a substantial inhibitory action on the growth of *C. albicans*. The growth of *C. albicans* was inhibited at 0.5 μg/ml by CAMB, as it was by DAMB. The MLC of CAMB was also found to be comparable to that of DAMB (Table 1).

The high efficiency of CAMB in vitro can be explained by the direct interaction and fusion between CAMB and the fungus cell membrane. Cochleates are composed of calcium and negatively charged lipids. The interaction of calcium with negatively charged lipids has been extensively studied. Many naturally occurring membrane fusion events involve the interaction of calcium with negatively charged phospholipids, generally phosphatidylserine and phosphatidylglycerol (14, 15). Calcium-induced perturbations of membranes containing negatively charged lipids and the subsequent membrane fusion events are important mechanisms in many natural membrane fusion pro-

cesses. Hence, cochleates can be envisioned as membrane fusion intermediates.

Our hypothesis on the mechanism by which CAMB interact with the *Candida* cell membrane (apart from the well-known first-pass binding between AmB molecules and ergosterol of the *Candida* cell membrane) is that the calcium-rich, highly ordered membrane of a cochleate first comes into close proximity to the *Candida* membrane, at the junction between the mother cell and the budding daughter cell. A perturbation and reordering of the cell membrane is then induced, resulting in a fusion event between the outer layer of the cochleate and the cell membrane. The precise mechanism by which cochleates fuse with cell membranes is not yet fully understood. Earlier studies by Bangham and Pethica (2) suggest that two membrane surfaces preferentially approach and make contact at regions having a small radius of curvature (<0.1 μm). Other studies have shown that as the radius of curvature of a lipid bilayer becomes smaller the bilayer becomes more thermodynamically unstable. Therefore, the fusion phenomenon between cochleates and cell membranes, promoted by calcium cations, could be occurring at the edge of a cochleate where the radius of curvature is the smallest. This fusion event results in the delivery of a small amount of the encochleated material into the cytoplasm of the target cell. The cochleate could then break free of the cell and be available for another fusion event, either with this or another cell (Fig. 7). Incubation of CAMB prepared with a rhodamine-labeled phospholipid shows the transfer of fluorescence from the cochleates to the *Candida* cells after 2 h (Fig. 4). This suggests that a close interaction has occurred between CAMB and the *Candida* cells. The inhibition of the growth of *Candida* cells by CAMB reported here confirms this hypothesis.

AmB cochleates are highly effective in inhibiting the growth of *C. albicans* in vivo. This is shown by the high percentage of survival and the reduction in colony counts in target organs of *C. albicans*-infected animals following multiple doses of CAMB, administered i.p. Low doses of CAMB (0.5 mg/kg) were sufficient to achieve a complete survival of the mice infected with a large inoculum. In another group of animals, CAMB almost cleared the kidneys when used at 10 mg/kg. CAMB and DAMB at 1 mg/kg have comparable in vivo efficacies, as reflected by the 3-log reduction in colony counts from the kidneys. This is consistent with the results of the in vitro inhibition tests, which showed that at this dose CAMB appear to be comparable to DAMB and LAMB (Fig. 6B). The clearance of *Candida* cells from the spleens of infected animals that received 10 mg of CAMB per kg was identical to that observed in the kidneys (Fig. 6A). At 1 mg/kg, CAMB were able to reduce the colony counts by 3 logs, while DAMB and LAMB showed less than a 2-log reduction. This can be attributed to the possible higher uptake of CAMB by the reticuloendothelial system, resulting in a concentrating of the drug in the liver and spleen.

In summary, CAMB represent a lipid-based vehicle which is well suited to the delivery of AmB to *Candida* cells both in vitro and in vivo. This delivery system warrants further development for possible clinical applications.

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