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Heat-Induced Superaggregation of Amphotericin B Reduces Its In Vitro Toxicity: a New Way To Improve Its Therapeutic Index

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Superaggregation of amphotericin B (AmB) was previously shown to occur upon heating of solutions at 70°C. In the present study, we demonstrate that heat pretreatment of Fungizone (deoxycholate salt of AmB [AmB-DOC]) solutions induces a drastic decrease in the in vitro toxicity of this antibiotic. Heated AmB-DOC colloidal solutions, which mainly contained superaggregated and monomeric forms of the antibiotic, were strongly less hemolytic than unheated solutions (aggregates and monomers). Thermal pretreatment of AmB-DOC solutions also reduced the toxicity to the cell line HT29, as deduced from two simultaneous cell viability assays (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and lactate dehydrogenase release). These heated colloidal solutions were only slightly less efficient than the unheated ones at inhibiting the growth of *Candida albicans* cells in vitro. Such results suggest that mild heat treatment of AmB-DOC solutions could provide a new and simple solution for improving the therapeutic index of this antifungal agent by reducing its toxicity to mammalian cells.

Amphotericin B (AmB) is a heptaene macrolide antibiotic active against fungi and yeasts. Despite its various acute and chronic toxicities, Fungizone, the deoxycholate salt (AmB-DOC) corresponding to the commercially available product, remains the drug most widely used to treat deep-seated mycotic infections. Due to its structural feature, amphiphilic AmB is poorly soluble in water and leads to self-association in aqueous media (2, 4, 7, 17, 22, 28). The aggregation state of AmB, which was reported to depend on various parameters (16, 19, 26, 27, 30, 32), influences the selectivity of the activity of AmB against ergosterol-containing fungal cells with respect to the toxicity against cholesterol-containing mammalian cells (4, 9, 26, 30). This selectivity can be increased by using liposomal formulations (1, 10, 20, 23, 24, 31, 35). In an aqueous environment, AmB is present as a mixture in equilibrium of monomers and soluble and nonsoluble aggregates. The concentrations of each species depend not only on the total AmB concentration but also on the concentration of the AmB in stock solutions (19, 30). The self-associated soluble form of AmB and the monomers were reported to be more efficient than insoluble aggregates at inducing the permeability of a cholesterol-containing membrane to K^+ (21). Self-associated AmB was shown to increase the permeability of cholesterol-containing egg phosphatidyl choline vesicles to K^+, while both the monomeric and the aggregated forms of AmB modified this parameter in ergosterol-containing liposomes (9). Moreover, the toxic and chemotherapeutic effects in mice were previously demonstrated to be correlated to the aggregation state (3) and to the particle size of intravenous AmB (5). From these results, it appeared that the best strategy for decreasing the cytotoxicity of AmB was to develop new derivatives and/or formulations that would lead to a decrease in the level of aggregation or that would give rise to less toxic aggregates (for reviews, see references 8 and 13). In this field of research, we previously reported that heat treatment of AmB solutions led to an increase in the size of aggregates (19). This heat-induced superaggregation of the antibiotic results from the condensation of monomeric AmB with aggregates. In the present study we compared the in vitro toxicity of AmB-DOC solutions before and after mild heating.

In human erythocytes, induction of permeability to K^+ and hemolysis efficiency were used as biological assays to test the permeabilizing effects and the cytotoxicity, respectively. In the cell line HT29, cell viability was evaluated through the colorimetric assay of mitochondrial oxidative activity with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The toxic effects of the antibiotics at the plasma membrane level were simultaneously evidenced by lactate dehydrogenase (LDH) release. Finally, the efficiencies of these various aggregated forms toward the growth kinetics of fungal cells were also investigated with *Candida albicans* cells. The antifungal effects of short- and long-term exposures to the antibiotic were compared to evaluate the influence of the occurrence of its autoxidation in aqueous medium.

MATERIALS AND METHODS

Chemicals. The salts, solvents, and reagents used in the study were of first-grade purity and were used without further purification. In this study, AmB, used in the form of Fungizone (Squibb, Paris, France), is associated with sodium deoxycholate (AmB-DOC). All biological experiments included a control consisting of DOC at concentrations comparable to that in Fungizone (35% by weight). No difference between the DOC-treated versus untreated cells was noted, and consequently, results were omitted. Millimolar stock solutions of AmB-DOC were prepared in water. Just before use, these AmB-DOC stock solutions was diluted 10-fold with vigorous stirring in pure water (Millipore Milli Q ion exchanger) or in a phosphate-buffered saline (PBS; 155 mM NaCl, 7 mM Na_2HPO_4, 3 mM KH_2PO_4 [pH 7.4]). AmB-DOC solutions were prepared from these latter dilutions that were heated for 20 min at 70°C or unheated. MTT, LDH, NADH, and pyruvic acid were obtained from Sigma Chemical (La Verpillière, France).

AmB calculation and spectroscopic measurements. Absorbance measurements were obtained by using a CARY-1E UV-visible spectrophotometer controlled by a personal computer. The temperature in the quartz cuvette was regulated with a thermostated cuvette holder. The optical path of the quartz cuvettes (0.1, 0.5, 1, and 10 cm) was chosen to obtain absorption values lower than 1.5 unit. Total AmB concentrations were controlled spectrophotometrically at 409 nm after complete monomerization by dilution in methanol. The Rayleigh scattering contribution, which depends on the sizes of the aggregates, was simultaneously monitored in a spectral range at which AmB does not absorb (650 nm).

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The spectroscopic properties of the aggregates and the monomeric form were used to calculate the concentration of each species by a previously reported method (19).

In vitro studies: Hemolysis and K^+ leakage measurements with RBCs. Venous blood obtained from healthy volunteers was collected in tubes containing EDTA. The plasma and buffy coat were discarded after centrifugation at 1,500 × *g* for 10 min. Erythrocytes (RBCs) were washed twice with PBS and were then dispersed in PBS at a cell density of 4×10^7 cells per ml. They were used on the same day that they were obtained.

Treatment of RBCs was achieved by adding 0.5 ml of the RBC suspension (4×10^7 cells per ml) to 0.5 ml of AmB-DOC solutions at various concentrations in triplicate. Each sample was incubated at 37°C with stirring. Control AmB-DOC samples (without RBCs) were simultaneously incubated to test the stability of the antibiotic under the same conditions. After a 1-h incubation, samples were centrifuged at 1,500 × *g* for 5 min. The supernatants were collected for analysis of the extent of hemolysis by reading the absorption of the hemoglobin at 575 nm. Results from triplicate experiments were expressed as a percentage of hemolysis with respect to that for the untreated samples. The RBCs were washed twice in PBS before lysis in pure water. The amount of K^+ remaining in the RBCs was determined by flame photometry. The results were presented as the percentage of remaining K^+ compared to that remaining in the untreated control samples. The data dealing with K^+ leakage and hemolysis presented in this report are the means ± standard deviations (SDs) of three measurements. Fits of these data as sigmoid curves were calculated by iterations according to the four-parameter equation:

$$\%K^+ = C_4 + \frac{(C_1 - C_4)}{[1 + ([AmB]/C_2)^{C_3}]}$$

In this expression, C_1 and C_4 correspond to the minimal and maximal values of the sigmoid, respectively; C_2 is the AmB concentration inducing 50% effect, and C_3 is the shape parameter of the curve related to the cooperativity of the phenomenon.

In vitro studies: toxicity against the HT29 cell line. (i) Cell treatment. HT29 cells (HT29 cells are from a human colon cancer cell line) were grown in RPMI 1640 medium (Gibco-BRL, Paisley, Scotland) supplemented with 4 mM L-alanine-L-glutamine (Glutamax), 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 10% fetal calf serum. The fetal calf serum, penicillin, streptomycin, and trypsin were purchased from Gibco-BRL. The cells were transferred twice weekly in fresh complete RPMI 1640 medium and were maintained at 37°C in a humidified 5% CO_2 atmosphere. For the experiments, the cells were seeded in 24-well multiwell dishes in the complete RPMI 1640 medium at a cell density of 3×10^4 cells per well.

Treatment of the cells with heated and unheated AmB-DOC was performed on days 0 and 1 (6 and 30 h after cell seeding, respectively). A stock solution of AmB-DOC (10^{-4} M) was prepared in sterile PBS buffer, and the solution was heated for 20 min at 70°C or unheated. Just before use, these solutions were diluted in RPMI 1640 medium in an AmB-equivalent concentration range of 1×10^{-8} to 2×10^{-5} M. Each concentration was tested in triplicate. The cells were rinsed with RPMI 1640 medium, and 500 μl of these AmB-DOC solutions was deposited in each well. The cells were incubated for 15 h at 37°C in a humidified 5% CO_2 atmosphere. Supernatants were then collected for LDH analysis, and the cells were rinsed twice with 400 μl of RPMI 1640 medium before the MTT viability assay. Since cells treated with DOC alone showed no significant toxicity, results were omitted.

(ii) LDH release. The LDH activity released in the supernatants was determined by measuring the NADH disappearance rate during the LDH-catalyzed conversion of pyruvate to lactate (6). A total of 300 μl of supernatants previously collected from treated cells was added to 2 ml of a 0.5 mM solution of sodium pyruvate in PBS. The enzymatic assay was started by adding 50 μl of NADH (1.5 mM). The disappearance of NADH at 37°C under stirring was determined fluorometrically with a Jobin-Yvon fluorometer (λ excitation = 366 nm; λ emission = 450 nm). The various components contained in the supernatants, which included AmB-DOC, were previously verified have no effect on the fluorescence measurements. Kinetics data were deduced from the linear part of the kinetics for times under 6 min. Data, expressed as the nanomoles of NADH consumed per minute, are the means ± SDs of two measurements (two pools of two supernatants for each concentration).

(iii) MTT viability assay. Cell viability was evaluated by the colorimetric assay with MTT by a modified method described earlier (33). After the chemical treatment and washing of the cells, 400 μl of a 0.5-mg/ml MTT solution in RPMI 1640 medium was added to each well. Cells in multiwell dishes were further incubated for 4 h at 37°C in a humidified 5% CO_2 atmosphere. Then, the supernatants were removed and 500 μl of dimethyl sulfoxide was added in order to solubilize the formazan salt. After 30 min at room temperature under mild shaking, the absorbance at 550 nm of each well was measured. Data, expressed as a percentage of the control absorbance (untreated cells), are the mean ± SDs of four independent measurements.

In vitro studies: growth kinetics of *C. albicans*. *C. albicans* 444 cells (a kind gift from E. Drouhet, Institut Pasteur, Paris, France) were maintained on Sabouraud dextrose agar and were regularly transferred. Due to the low chemical stability of AmB-DOC in aqueous medium, we analyzed its antifungal efficiency for both short- and long-term exposures.

(i) Effect of short-term exposures to AmB-DOC. A standard inoculum of the culture was transferred to 25 ml of liquid Sabouraud medium. After 16 to 18 h of incubation at 35°C, when the cell population reached confluency, the cell density was adjusted before treatment.

Treatment of *C. albicans* cells was achieved by adding 2 ml of the cell suspension (2×10^6 cells/ml) to 2 ml of AmB-DOC solutions at various concentrations, thus leading to a final inoculum of 1×10^6 organisms/ml. Each measurement was done in triplicate by incubating the samples for 20 min at 37°C with stirring. Control AmB-DOC samples without cells were simultaneously incubated to test the stability of the antibiotic under these conditions. Incubation was stopped by diluting the cell samples in 40 ml of cold PBS. The samples were then centrifuged at 1,500 × *g* for 5 min, and the cells were suspended in 20 ml of liquid Sabouraud medium (at 35°C). The subsequent growth of the cells was then measured as a function of the time of incubation from turbidimetric analysis at 750 nm in the flask culture. Fits of the growth kinetics as sigmoid curves were calculated according to Rodbar's formula. The time required to reach half of the cell density with respect to that at confluency (t_{50}) was obtained from these fits. Data are the means ± SDs of at least three measurements. These values of t_{50} depend on the cell seeding density (CSD), on the fraction of efficient cells able to divide as well as their dividing efficiency. With untreated *C. albicans* cells, a linear relationship was found between 1/CSD and $1/t_{50}$ (data not shown). As a consequence, measurement of t_{50} could provide an estimation of the viable CSD according to the following expression: $1/\text{CSD} = 10^{-6} \times (1.55 - 4.64 \times 1/t_{50})$. Treatment of fungal cells by increasing AmB concentrations decreases the CSD values, as deduced from the increase in the t_{50} values as well as their dividing efficiency (decrease in the slope of the growth curve).

(ii) Effect of long-term exposures to AmB-DOC (IC_{50}s and MFC). The antifungal activity was measured as the concentration causing 50% inhibition of growth (IC_{50}) of *C. albicans*. The minimal fungicidal concentration (MFC) is defined as the concentration that showed more than 90% killing (the two values are expressed as percentages compared to the values for the growth controls). These two concentrations were determined by a standard broth macrodilution method.

C. albicans cells were grown overnight at 35°C in Sabouraud medium, counted, and adjusted to a concentration of 10^6 yeasts/ml before AmB-DOC (Fungizone) exposure. For determination of the inhibitory concentration, the extent of growth was monitored turbidimetrically at 600 nm after 24 h of incubation at 35°C in the presence of the antifungal agent. The IC_{50}s were determined from sigmoid fits of the dose-response curves.

For determination of the fungicidal effect, the MFC was determined by culturing treated samples showing no turbidity in fresh medium after 24 h of incubation at 35°C. The range of AmB concentrations was 0.02 to 5 mM.

RESULTS

Physicochemical properties of the heat-induced superaggregates. As shown in Fig. 1A, the absorption spectrum of AmB-DOC solutions (10^{-5} M) before heating is characteristic of the aggregated species, with a maximum centered at 327 nm and a shoulder centered at about 420 nm. The minor peaks observed at 409, 385, and 363 nm are due to the contribution of the monomeric form (less than 5%). After a 20-min heating and cooling, the sizes of the aggregates increase, as suggested from the increase in the Rayleigh scattered light. The opalescence of this heated solution can be eliminated by centrifugation. Its absorption spectrum shows a new maximum located at 321 nm. A 1-h incubation at 37°C under stirring gives rise to a 5 to 10% decrease of these super aggregated species. Under these incubation conditions, the lowest chemical stability of the other aggregated state leads to a 40 to 50% decrease in the amount of this species. The loss of AmB deduced from these spectral changes can also be followed by using high-performance liquid chromatography analysis (data not shown). This loss is an oxygen-dependent process which does not occur in deaerated AmB-DOC solutions obtained after bubbling nitrogen through the solution. As reported previously (29), it probably corresponds to the autoxidation of the antibiotic.

The aggregated form has previously been shown to be in equilibrium with the monomeric form (9, 19). The spectroscopic properties characteristic of the two aggregation states and of the monomer were used to calculate their relative proportions at various concentrations. As shown in Fig. 1B, the dilution of the unheated AmB-DOC solution from 10^{-4} to

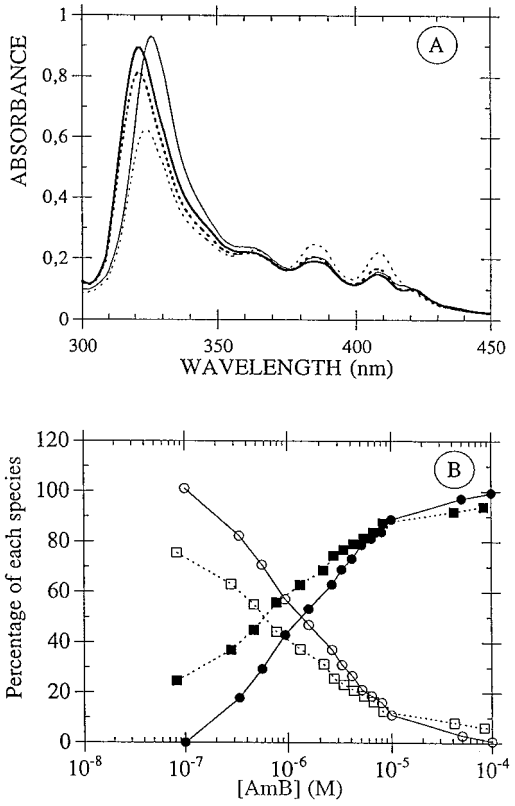

FIG. 1. Physicochemical properties of the aggregates and heat-induced superaggregates in AmB-DOC aqueous solutions. (A) Changes in the absorption properties observed during the heat-induced superaggregation. Absorption spectra of aqueous AmB-DOC solutions (10^{-5} M) obtained from a 10-fold dilution of 10^{-4} M aqueous solutions heated for 20 min at 70°C (thick line) or unheated (fine line). The chemical stability of the two aggregated species after a 2-h incubation at 37°C under stirring were deduced from their absorption spectra (aggregates, fine dotted line; super aggregates, thick dotted line). (B) Thermodynamic stability of the aggregated species upon dilution. Aqueous AmB-DOC solutions (10^{-4} M) heated for 20 min at 70°C or unheated were progressively diluted in water. For each dilution, the concentrations of the monomeric form (λ_{max} = 409 nm), the aggregated form (λ_{max} = 327 nm), and the heat-induced super aggregate (λ_{max} = 321 nm) were calculated from the absorption spectra by a previously described method (19). Equilibrium in unheated AmB-DOC solutions between aggregates (●) and monomers (○) and equilibrium in heated AmB-DOC solutions between super aggregates (■) and monomers (□) are presented.

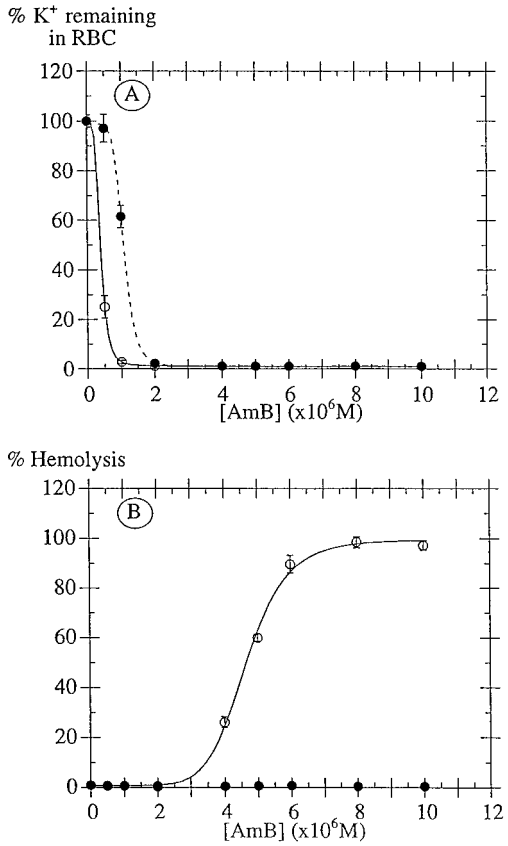

FIG. 2. Dose-response curve of the effect of AmB on the membrane permeability of human RBCs showing the effect of the thermal pretreatment of the aqueous AmB-DOC solution (20 min at 70°C; ●) compared to the effect of no thermal pretreatment (○). Data are the means ± SDs of at least three measurements. (A) Percentage of AmB-induced release of K^+. Data were expressed as percentages with respect to the results for the untreated control cells. (B) Percentage of AmB-induced hemolysis (note that no hemolysis is induced by heated AmB-DOC in this concentration range).

10^{-7} M at room temperature leads to a progressive dissociation of the aggregates into the monomeric form. The half dissociation (50% of aggregates and monomeric form) is observed for a total AmB-DOC concentration of 1 μM. When formed, the superaggregated species is also in equilibrium with the monomer. As shown in Fig. 1A, the dilution of the heated solutions progressively dissociates the superaggregates into monomers. The dissociation of this superaggregated form occurs at a lower concentration than that for the aggregated form. The half dissociation of superaggregates is observed at a total AmB-DOC concentration of 0.5 μM.

In vitro modulation of biological activities by heat treatment of AmB-DOC solutions: thermal modulation of the AmB-induced changes in the permeability of RBC membranes. The leakage of K^+ as well as the hemolysis efficiency that are induced by AmB have been extensively used as indices of polyene toxicity (14, 30). The results of one typical experiment (among three) dealing with the effect of increasing concentrations of AmB on the permeability of the RBC membrane are reported in Fig. 2A and B. After a 1-h incubation at 37°C, micromolar concentrations of AmB-DOC solutions led to a K^+ leakage from the RBCs (Fig. 2A). As estimated from the sigmoidal fit of the experimental data, the AmB-DOC concentration required to trigger 50% leakage is about 0.3 mM. This value is in agreement with that obtained in a previous work (14). Heating of the AmB-DOC solutions for 20 min at 70°C led to a slight decrease in its permeabilizing efficiency (50% leakage was induced with about 1.0 mM AmB).

Heat treatment of the AmB-DOC solutions resulted in a much more significant decrease in their hemolysis efficiency (Fig. 2B). Such an effect has been estimated by measuring the amount of hemoglobin leaked into the supernatants of RBCs incubated for 1 h at 37°C with AmB. The fits of these dose-response curves as sigmoids provide the AmB concentrations required to trigger 50% of hemolysis, which are equal in this experiment to 4.7 mM for unheated samples. As shown in Fig. 2B, unheated AmB-DOC solutions are more efficient than heated ones at triggering the hemolysis process in RBCs. While complete hemolysis is observed with 10 μM solutions, heated AmB-DOC remains inefficient at this concentration. RBC lysis occurs at higher concentrations for heated samples (>50 μM) and for longer incubation times (>1 h).

Effect of heat treatment of AmB-DOC solutions on their cytotoxicities for HT29 cells. The MTT assay was particularly

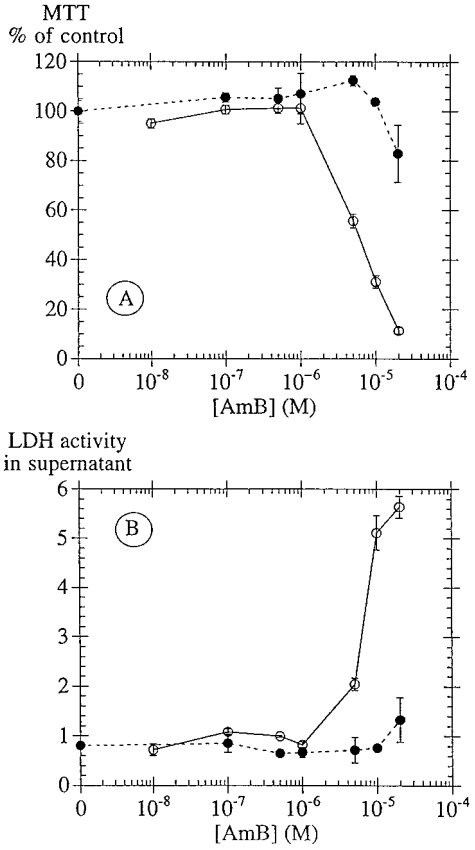

FIG. 3. Effect of thermal pretreatment of AmB-DOC solutions on toxicity to HT29 cells. The cells were seeded at a density of 3×10^4 cells per well in 24-well plastic microplates. After 24 h of growth, they were treated for 16 h at 37°C with unheated (○) or heated (20 min at 70°C) (●) AmB-DOC solutions in RPMI 1640 medium. Each AmB concentration was tested four times. (A) The MTT assay was performed by reading the absorbance at 550 nm for each well. Results were expressed as the percentage of absorbance for the untreated control cells. Data are the means ± SDs of four measurements. (B) LDH activity released in the supernatant is expressed as nanomoles of NADPH consumed per minute. Data are the means ± SDs of two measurements, each corresponding to measurements for two different wells.

relevant at estimating cytotoxic events involving oxidative processes (15), such as in the present case. Treatment of the cells with heated and unheated AmB-DOC was performed immediately or 1 day after the cell seeding, and the MTT assay was executed just after that. The data presented in Fig. 3A were obtained for cells treated with AmB 1 day after seeding. Heated and unheated AmB-DOC solutions were shown to be nontoxic at concentrations lower than 1 μM. Unheated samples were shown to be toxic at higher concentrations (between 1 and 10 μM), while heated samples remained ineffective even at 10 μM. The concentrations required to trigger half of the maximal effect with unheated and heated AmB-DOC solutions were estimated to be 6 and 40 μM, respectively.

As illustrated in Fig. 3B, AmB treatment concomitantly led to a release of LDH in the supernatant, indicating damage at the plasma membrane level. As observed by the MTT test, at 10 μM, heated AmB-DOC does not induce membrane damage, while unheated AmB-DOC at the same concentration strongly alters the membrane, as evidenced by the large increase in LDH release. Microscopic analysis of treated HT29 cells showed that this increase in LDH leakage paralleled marked morphological changes at the plasma membrane level, such as bleb formation. While at 10 μM unheated AmB-DOC strongly induces the blebbing process, after treatment with heated AmB-DOC colloidal solutions at the same concentration, the membranes of HT29 cells remain practically unchanged (data not shown).

The changes in the properties of the cell membrane induced by micromolar concentrations of AmB-DOC solutions are modulated by the cell growth status. Cell toxicity, determined by LDH leakage, bleb formation, and the MTT assay, appeared to be more important when the cells were exposed to the antibiotic just after seeding (data not shown).

Thermal modulation of the AmB-induced changes in the growth kinetics of *C. albicans*. (i) Antifungal effect of short-term exposures to AmB. In experiments assessing the antifungal effects of short-term exposures to AmB, the fungal cells were preincubated for 20 min with the AmB-DOC solution. After cell washing, the growth curves were obtained from turbidity measurements by monitoring the absorbance at 750 nm directly in the flask culture (see Materials and Methods). A decrease in the CSD was previously shown to delay the growth of *C. albicans* cells without having an effect on its kinetic rate (data not shown). In contrast, short-term exposures of fungal cells to AmB both delayed and reduced their kinetic rate of growth. The results of one typical experiment (among three), dealing with the effect of an unheated AmB-DOC solution compared to that of a heated AmB-DOC solution on the growth kinetics of *C. albicans*, are presented in Fig. 4.

As shown in Fig. 4A, a 20-min treatment of *C. albicans* cells with increasing AmB concentrations slows their growth kinetics. The slopes of the curves in the exponential part of the growth decrease when the AmB concentration increases. The t_{50} values, which were deduced from the four-parameter fits of growth curves, are reported in Fig. 4C. These t_{50} values increase with the AmB concentration. Treatment of the fungal cells with AmB solutions leads to a decrease in the number of cells able to divide (increase in the t_{50} values) as well as a decrease in their dividing efficiency (decrease in the slope of their kinetics of growth).

As reported in Fig. 4B and C, for concentrations lower than 1 μM, thermal pretreatment of the AmB-DOC solutions does not significantly modify the effect of the antibiotic on the growth kinetics. For higher concentrations (5 μM), the antifungal effect of the antibiotic with short-term exposures is shown to be slightly reduced by the thermal pretreatment, as illustrated by the 20% decrease in the t_{50} values with respect to those for the unheated samples.

Assuming that the increase in t_{50} values was mainly due to the decrease in the number of *C. albicans* cells able to divide after the antibiotic treatment, this surviving fraction was estimated according to the relationship between CSD and values of t_{50} defined in the experimental part of the study. Compared to untreated control cells, the decreases in CSD due to the treatment with 1 and 5 μM unheated AmB-DOC solutions were estimated to be 36 and 47%, respectively. The values of t_{50} are slightly lower for the cells treated with 1 and 5 μM heated AmB-DOC colloidal solutions, corresponding to decreases in CSD of 34 and 45%, respectively.

In the absence of cells, the concentrations of each spectroscopic species from 1 and 5 μM AmB-DOC solutions in water before and after heating at 70°C were independently processed from the absorption data. Assuming the presence of the same percentage of each form in Sabouraud medium with or without cells, comparison of these concentrations and their respective effects on the growth kinetics seems to indicate a slightly higher efficiency of the aggregates on *C. albicans* cells with respect to that of the superaggregated species.

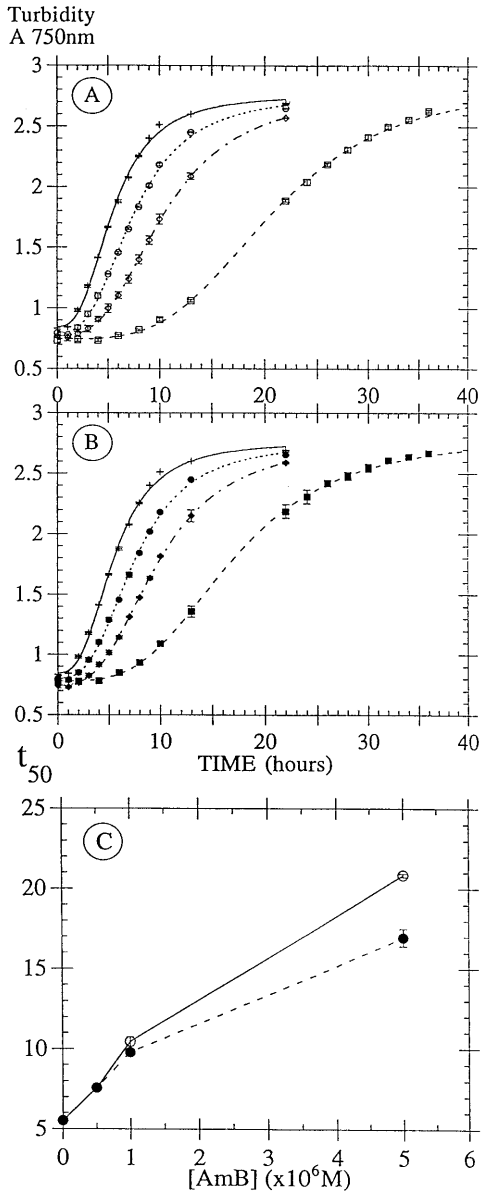

FIG. 4. Effect of thermal pretreatment of AmB-DOC solutions on the growth kinetics of *C. albicans* cells. Fungal cells (10^6 organisms/ml) were incubated for 20 min at 37°C under gentle stirring. After the cells were washed, the kinetics of growth was determined from turbidity measurements by monitoring the absorbance at 750 nm. Fits of these kinetics as sigmoid curve were calculated by the Rodbar formula. The time required to reach half of the cell density with respect to the density at confluency (t_{50}) was obtained from these fits. (A) Growth curves after treatment with unheated AmB-DOC solutions (+, 0 μM; ○, 0.5 μM; ◇, 1 μM; □, 5 μM). Data are means ± SDs of three measurements. (B) Growth curves after treatment with AmB-DOC solutions heated for 20 min at 70°C (+, 0 μM; ●, 0.5 μM; ◆, 1 μM; ■, 5 μM). Data are means ± SDs of three measurements. (C) Comparison of the t_{50} values measured for *C. albicans* cells treated with various concentrations of AmB-DOC solutions unheated (○) and heated for 20 min at 70°C (●). Data are means ± SDs of at least three kinetic measurements.

(ii) Long-term antifungal activity. In the experiments assessing the antifungal effects of long-term exposures to AmB, the antibiotic was kept in the incubation medium for 15 h during cell growth. The efficiency of its antifungal effect could be modulated by the progressive loss of AmB resulting from the autoxidation process, which mainly occurred in the unheated solutions. Under these incubation conditions, the results show that there is no difference between unheated and heated AmB-DOC solutions in terms of fungistatic and fungicidal activities (IC_{50} and MFC). In both cases, we found that the AmB IC_{50} was 0.5 mM and that the AmB MFC was 2 mM.

DISCUSSION

Heating (20 min at 70°C) and cooling of the AmB-DOC solution (10^{-4} M) led to significant changes in the aggregation state of the antibiotic. The maximum of the absorption spectrum was shifted from 327 to 321 nm, while superaggregates were formed, as deduced from the strong increase in the scattered light at 650 nm, thus reflecting an increase in the size of aggregates up to flocculation. This superaggregated form which results from condensation of the aggregated form with the monomers was shown to be in equilibrium with the monomeric form independently of the other aggregated form. The dissociation of the superaggregate into the monomeric form occurs at concentrations lower than those for the aggregates. The higher thermodynamic stability of the superaggregated form could influence its association with membranes or lipidic carriers such as lipoproteins.

The heat treatment does not significantly alter the total AmB concentration, and the stability of the heated solutions was shown to be higher than that of the unheated ones. The total concentration of the heated AmB colloidal solutions remains practically unchanged when these solutions were further incubated with stirring for 1 h at 37°C, as in our biological assays. In contrast, autoxidation processes occur from unheated AmB solutions (19, 29), thus leading to a 20% decrease in the total AmB concentration after a 1-h incubation under our experimental conditions. The autoxidation process was previously shown to mainly involve the aggregated form (19).

At low concentrations (<2 μM), AmB induces a reversible permeabilizing effect on RBCs, while at higher concentrations this antibiotic causes RBC lysis. We showed in the present work that the AmB concentration (AmB-DOC) required to trigger 50% K^+ leakage from RBCs is slightly higher when AmB solutions were heated for 20 min at 70°C. At concentrations higher than 1 μM, when the antibiotic was self-associated, AmB was previously demonstrated to form transmembrane channels in cholesterol-containing cell membranes. As shown in the present study, the lower efficiency of the heated AmB-DOC solution at permeabilizing the RBC membrane suggests that the permeabilizing effect also depends on the aggregation state.

Heat treatment strongly decreases the hemolysis efficiency. More than eight times higher concentrations of heated AmB-DOC solutions than unheated AmB-DOC solutions are required to produce 50% hemolysis. Half hemolysis is observed after a 1-h incubation with a 4.7 μM unheated solutions, while heat-treated RBCs remain intact for longer periods of time, even at 50 μM AmB-DOC solutions.

To date, the relationship between these two distinct mechanisms of action of AmB has not generally been admitted or clearly elucidated. However, it is generally assumed that permeabilizing effects are related to the ability of AmB to form transmembrane channels, while the lytic effect could be due to the peroxidative action of AmB at the membrane level (12). This oxidation of unsaturated fatty acids leads to a change in the fragility of the membrane, which becomes more sensitive to the osmotic shock induced by the formation of channels during leakage. Autoxidation of AmB in solution (29) as well as AmB-induced peroxidation of unsaturated fatty acids of the RBC membranes (12) were assumed to be triggered by reactive

oxygen species which could be produced by this antibiotic. The higher stability of the heat-treated samples (mainly as superaggregates) with respect to the unheated ones (mainly aggregates) was reported in a previous study (19) for AmB solutions as well as in the present work for AmB-DOC solutions. These results suggest a lower efficiency of the superaggregated form of AmB compared to that of the aggregated form of AmB at producing reactive oxygen species. In the hypothesis of the peroxidative and lytic actions of AmB, it could also explain the lower lytic efficiency of the heat-treated samples.

The effect of the thermal treatment of AmB in water or AmB-DOC solutions on the growth kinetics of *C. albicans* is much weaker than its effect on RBC membranes. Assuming that the stability of the heat-treated samples (mainly superaggregated forms) reflects their low efficiency at producing reactive oxygen species, this result suggests that the AmB-induced peroxidative process in fungal cells is less relevant than that in RBCs. For long-term exposures, the heat treatment does not change the fungistatic and fungicidal activities of AmB-DOC solutions. For short-term exposures, heat treatment of the AmB-DOC solutions does not change the efficiency of its effect on the growth kinetics of *C. albicans* up to 1 μM AmB, while it induces a 20% decrease in its antifungal activity at 5 μM AmB (with respect to the t_{50} values obtained for the unheated samples).

The aggregation state of AmB is assumed to play a key role in the activity of this antibiotic in various biological systems. Self-associated water-soluble forms of AmB, which are formed at concentrations higher than 10^{-7} M, was previously shown to trigger permeability changes in RBC membranes and to induce cytotoxic events (30). In this work, the higher level of toxicity of the water-soluble aggregated form with respect to that of the non-water-soluble aggregate form was demonstrated in human RBCs after separation of these two forms by centrifugation. The threshold of the concentration beyond which this kind of drug gives rise to the formation of aggregates seems to be the main parameter modulating its biological activity. As a matter of fact, AmB derivatives with an increased concentration of this aggregation threshold have been shown to induce permeability through RBC membranes with a weaker efficiency (data not shown). A recent study, in which molecular design of a homologous series of AmB-oligo(ethylene glycol) conjugates was used, demonstrated the relationship between the aggregation properties, the critical micellar concentration, and the ability to disrupt the integrity of *C. albicans* and RBCs membranes (36). An incremental increase in the size of the oligo(ethylene glycol) moiety was shown to increase both the critical micelle concentration of the macrolide and the concentration required to trigger the hemolysis process. In contrast, the antifungal activity was only slightly influenced by the size of the conjugates. These interesting results point out the important role of the aggregation properties, which are connected to the hydrophobicity of the derivatives, on biological activity.

In the present study, we also compared the in vitro cytotoxicities of unheated and heated AmB-DOC solutions on the HT29 cell line (human colon cancer line). The MTT assay, which involves mitochondrial succinate dehydrogenase activity, was chosen to evaluate cell viability after antibiotic treatment (33). Membrane damage assayed by the leakage of cytosolic LDH was taken as another index of cell viability. LDH release assays as well as assays for bleb formation constitute noninvasive means of documenting membrane damage and cell integrity. It is generally assumed that if the site of cellular damage directly involves the plasma membrane, as in the present case, the results of these two permeability assays are in good agreement with those of the clonogenic assay (15). The cell toxicity measured by the MTT assay, which followed membrane damage evidenced by LDH leakage and bleb formation, was shown to be sevenfold lower with heated (50% lethal dose, 40 μM) than with unheated (50% lethal dose, 6 μM) AmB-DOC solutions. Changes in the membrane properties induced by AmB-DOC affect membrane permeability, as shown by K^+ leakage, and also modify other cellular ion pumps, leading to the inability of the cells to maintain ion homeostasis. In cultured glomerular mesangial cells, AmB was previously demonstrated to induce a concentration-dependent increase in the intracellular calcium concentration (34). Calcium ions, which are involved in the interaction between the basal membrane and the cytoskeleton, greatly influence bleb formation and plasma membrane breaks (25).

Animal cells have different levels of susceptibility to the toxic effects of polyene antibiotics (for a review, see reference 8), depending on the cell origins (animal species and tissues). Moreover, various studies dealing with the toxicities of these antifungal agents on mammalian cells in culture expressed cell viability in the presence of different levels of the agents in the cells. Some of them considered the alterations in membrane integrity such as hemolysis and K^+ leakage, some of them performed the exclusion test, and some of them made ^{51}Cr release measurements, while others analyzed the proliferative ability of the treated cells, including incorporation of [^{3}H]thymidine or [^{3}H]uridine into nucleic acids, protein synthesis assay, clonogenic assay, and plating efficiency assay. The 50% lethal dose for RBCs and HT29 cells treated with unheated AmB-DOC solutions obtained in the present study (4.7 and 6 μM, respectively) are in agreement with data previously reported with various cell lines of fibroblasts (11, 18).

It is now generally assumed that the cytotoxicity of AmB is related to its aggregation, which occurs in aqueous solutions. Consequently, the main strategies for reducing the toxicity of this antifungal treatment deal with the design of new derivatives or formulations of this drug, leading to a decrease in the level of aggregation or giving rise to less toxic aggregates. As part of this latter field of research, we have shown in the present study that the physical change in the aggregation state of AmB, induced by heating at 70°C of AmB-DOC solutions, leads to a decrease in its in vitro toxicity. Rayleigh scattering measurements suggest that superaggregates are larger than the aggregated form. When they are formed, superaggregates were shown to be in equilibrium with AmB monomers. Their dissociation as monomers takes place at AmB concentrations lower than those of the dissociation from the aggregated form.

The superaggregated form was demonstrated to be less efficient than other aggregates at inducing membrane changes in the RBCs, as shown from the hemolysis and K^+ leakage measurements. In the HT29 cell line, the cell toxicity measured by the MTT assay, which paralleled membrane damage evidenced by LDH leakage and bleb formation, was shown to be sevenfold lower with heated than with unheated AmB-DOC solutions. The efficiency of AmB superaggregates compared to that of AmB aggregates at inhibiting the growth kinetics of *C. albicans* cells is only slightly reduced at concentrations higher than 1 μM. In the range of concentrations in plasma (<1 μM), heat treatment does not significantly reduce the antifungal efficiency of AmB. The lower toxicity in mammalian cells could permit larger doses to be given, and consequently, this formulation could be more effective than free AmB against fungal infections. So, our present study suggests that heat treatment of AmB-DOC solutions at 70°C for 20 min could provide a new solution to improving the therapeutic index of this antibiotic by reducing its cytotoxicity to mammalian cells. Further in vivo experiments are being undertaken to determine the effect of

heat treatment on the acute toxicity in mice and the antifungal efficiency of AmB-DOC solutions in experimental murine infections.

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