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Interactions of Liposome-Incorporated Amphotericin B with Kidney Epithelial Cell Cultures

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SUMMARY

The polyene antibiotic amphotericin B (AmB) is profoundly cytotoxic to both fungal cells and mammalian cells. We have previously shown that the incorporation of AmB into phospholipid vesicles can markedly reduce the toxicity of the drug for mammalian cells (erythrocytes) without changing its antifungal potency [*Mol. Pharmacol.* **31**:1–11 (1987)]. Because the primary site of *in vivo* toxicity of AmB is the kidney, here we investigate the effects of free AmB and liposomal AmB (L-AmB) on LLCPK1 cells, a porcine kidney cell line with many characteristics typical of proximal tubule cells. Acute exposure (2 hr) to free AmB inhibits protein synthesis and causes cell detachment and protein loss in LLCPK1 cells, with an IC_{50} of about 30 μg/ml. By contrast, certain formulations of L-AmB have little effect on protein synthesis/protein loss at concentrations of up to 2 mg/ml. The action of liposomes in protecting against acute AmB toxicity extends to effects on sugar transport and on cellular morphology in differentiated cultured kidney cells. Thus, the IC_{50} for inhibition of sodium-stimulated glucose transport by free AmB is 1.5 μg/ml whereas concentrations of L-AmB up to 1 mg/ml do not inhibit this process. However, chronic exposure of cells to L-AmB results in profound toxic effects as manifested by changes in cellular transport functions and cell morphology. Our results suggest that extended periods of proximity between cells and liposomes permit the transfer to toxic amounts of AmB. This may be of importance to the therapeutic use of AmB, for which protracted courses of drug administration are common.

The mechanism of action of the antifungal polyene antibiotic AmB is thought to involve its ability to complex with sterols in cell membranes, thus inducing the formation of hydrophilic pores (1–3). This leads to the leakage of ions and, at higher drug concentrations, of larger molecules resulting ultimately in cell death (4–6). The selectivity of AmB towards fungal cells is believed to be due to the fact that AmB has a somewhat higher affinity binding to ergosterol, the predominant sterol in fungal cell membranes, than to cholesterol, the major sterol of mammalian cells (1). Recently, it has been shown that the toxicity of AmB towards mammalian cells can be greatly diminished by incorporation of the drug into phospholipid vesicles (7, 8). This also allows the use of higher drug doses *in vivo*, leading to improved antifungal therapy (9–12). The mechanism of the reduced toxicity of L-AmB has been shown to involve the phospholipid composition of the liposome (7, 8, 13). Thus, we have shown that L-AmB made from saturated lipids does not induce cation efflux from preloaded red blood cells but does induce ion fluxes and cytotoxicity in fungal cells (8). By contrast, liposomes prepared from unsaturated lipids proved to be as toxic as free AmB to erythrocytes (8). Because red cells are highly specialized nondividing cells, we wished to compare the effects of AmB or L-AmB in a more representative cellular situation that might offer additional insights into the pharmacological and toxicological behavior of these drug formulations *in vivo*.

One limiting factor in the treatment of systemic fungal infections in patients is the severe kidney toxicity of AmB, which is mainly due to the impairment of proximal tubular cell functions (1). Therefore, we have chosen a pig kidney epithelial cell line (LLCPK1) as an *in vitro* model because this cell line is thought to originate from the proximal tubule of the kidney as judged by the expression of marker enzymes and of specific transport capabilities (14). This cell line is especially interesting as a model for studying AmB-related effects because, upon reaching confluency, the cells develop a sodium-dependent glucose transport system (15–18). This transport system is dependent on the Na^+/K^+ gradient across the cell membrane (18) so that substances that interfere with this ion gradient should also influence the transport of glucose. The glucose transport process can be easily measured using a glucose analog (α-AMG) that is taken up by cells in the same way as glucose

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ABBREVIATIONS: AmB, amphotericin B; L-AmB, liposomal amphotericin B; α-AMG, α-methylglucopyranoside; DOPG, dioleoylphosphatidylglycerol sodium salt; DOPC, dioleoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DOPE, dioleoyl-phosphatidylethanolamine; DMPG, dimyristoyl-phosphatidylglycerol; EBSS, Earle's balanced salt solution; DMSO, dimethyl sulfoxide.

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but that is nonmetabolizable (19). Another expression of substrate transport in LLCPK1 cells is the formation of domes, fluid-filled blister-like structures that are found in many transporting epithelia (19, 20). Dome formation is dependent on several factors including adhesion to the substratum (18), formation of tight junctions (20), and elevation of intracellular cyclic AMP concentrations (19), but the regulation of "doming" is not fully understood (20). This very complex phenomenon can be used as an indicator for overall cytotoxicity by looking for changes in the time course or extent of dome formation using a quantitative morphological approach.

In addressing questions of toxicity of free or liposomal AmB to kidney cells in culture, we decided to examine a "housekeeping" function such as protein synthesis, which is performed by all cells. We also wished to examine differentiated functions characteristic of kidney epithelial cells; thus, we chose to investigate Na^+-stimulated glucose transport. We also investigated changes in overall cell morphology and in the unique process of dome formation, using both light microscopy and scanning electron microscopy. We anticipated that differentiated functions, especially those involving membrane activities, would be more sensitive indicators of toxicity than housekeeping functions.

Experimental Procedures

Materials

AmB in powdered form was obtained as a gift from Squibb (Princeton, NJ). [^{14}C]α-AMG with a specific activity of 100 mCi/mmol was obtained from Amersham (Arlington Heights, IL). [^{3}H]Leucine (40–60 Ci/mol) was from ICN (Irvine, CA). All lipids used for the preparation of liposomes (DMPC, DMPG, DOPC, DOPG, and DOPE) were obtained from Avanti Polar Lipids (Birmingham AL). Cell culture supplies were bought from GIBCO (Grand Island, NY) and the reagent kit for the protein determinations was from Pierce Chemical Co. (Rockford, IL). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) and were of analytical grade purity.

Preparation of Liposomes

AmB-containing liposomes were essentially prepared as described previously (8). Briefly, the powdered drug was dispersed in methanol by sonication and any undissolved drug was removed by filtration through 0.2-μm Nucleopore filters. To this clear solution, 50 mg of total lipids was added (DMPC/DMPG, 7:3; DOPC/DOPG, 1:1; DOPC/DOPE, 1:1; molar ratios) and the mixture was evaporated to dryness. The film was rehydrated with 0.9% saline and the crude suspension was filtered through 0.6-μm Nucleopore membranes with a Luvet device (21). This aided in the removal of unencapsulated drug and the preparation of a more homogeneous multilamellar liposome population (22). The vesicles were pelleted by centrifugation, washed once, and redispersed in saline. For long term experiments, liposomes were sterilely filtered through a 0.2-μm membrane filter and stored aseptically.

Depending on the lipid composition used, between 20 and 80% of the original amount of AmB could be incorporated into the liposomes. For example, DOPC/DOPE vesicles gave 20% incorporation whereas DMPC/DMPC gave 80%. The total amount of phospholipid was determined as inorganic phosphorous according to the method of Bartlett (23). AmB content was measured spectrophotometrically (405 nm) after dissolving the liposomes in methanol; the blank contained an equivalent amount of phospholipid.

Stability Assay

Sterile liposomes containing about 10 μg/ml of AmB were incubated for up to 5 days in complete culture medium at 37°. At the end of the incubation, samples were diluted 10-fold with saline and centrifuged for 1 hr at 20,000 × g. The supernatant was carefully removed and the pellet was dissolved in methanol. The amount of AmB associated with liposomes was measured at 405 nm and the results were expressed as percentage of control. The control consisted of liposomes treated identically, with the exception that they were centrifuged immediately after addition to the medium (zero incubation time).

Cell Cultures

LLCPK1 cells were kindly provided by Dr. J. Lever, Department of Biochemistry, University of Texas Medical School. The cells were grown in a 1:1 mixture of DMEM and Ham's F-12 medium supplemented with 10% fetal bovine serum, 100 units of penicillin/ml, and 100 μg of streptomycin/ml. Cultures were given fresh medium every 2 days and split in a 1:10 dilution upon reaching confluence. Experimental cultures (non-confluent cultures) were set up in 96-well plates at a cell density of 2×10^4 cells/well and used 2 days after plating. Confluent cultures were obtained by plating cells at the same density (2×10^4/well) and growing them for 5 days. Two days thereafter the cells were used for experiments as confluent monolayers. As described elsewhere (20) confluent cultures of LLCPK1 cells display numerous domes, that is, clusters of cells that protrude away from the culture substratum. This phenomenon is due to transepithelial transport of NaCl followed by passive water movement (19).

Protein Loss/Protein Synthesis Measurements

For protein loss measurements, cell protein was uniformly labeled by incubating cells with 1 μCi of [^{3}H]leucine for 48 hr. As shown below, this period is sufficient for steady state labeling of cell proteins. The rates of protein synthesis in LLCPK1 cultures were measured as follows. Wells were preincubated with drug-containing medium for 2 hr. After this time the monolayers were washed with DMEM to remove all AmB and 1 μCi of [^{3}H]leucine/ml in medium was added to all the wells on the plate. After 4 hr at 37° the unincorporated leucine was washed off and the proteins were precipitated with ice-cold 1 M perchloric acid. As shown below, during 4 hr, the incorporation of the label into LLCPK1 cells was linear with time and thus an adequate approximate measure of protein synthetic rate. The precipitate was washed several times with cold 1 M phosphate buffer, pH 7.4, and the proteins were hydrolysed with 1 N NaOH overnight. The solution was transferred into scintillation vials and the activity of the samples was measured by liquid scintillation counting. No correction for quenching was done because the background counts were usually below 70 cpm whereas samples contained several thousand cpm. Cells whose protein synthesis was inhibited by incubation with 50 μM cycloheximide for 24 hr before the experiment served as negative controls. Results on the inhibition of protein synthesis are expressed as the fraction of [^{3}H] leucine incorporation as compared with control cells. Results on recovery of cell protein are expressed as percentage of control.

Sugar Transport

Cells in 24-well plates were plated at a density of 10^5 cells/well and grown for 7 days. After about 2 days confluent monolayers were formed and the appearance of domes could be seen. Because glucose transport ability develops after the cells attain confluency (24), the monolayers were allowed to grow for an additional 5 days. The day before the experiment the cells were replenished with fresh medium.

Verification of the uptake assay. Cultures were washed several times with EBSS without glucose (pH 7.2 at 37°) and incubated with 200 μl of EBSS for 10 min. Then, a 50-μl aliquot of 60 mM α-AMG with 0.14 μCi of [^{14}C]αAMG solution was added to each well, resulting in a 12 mM α-AMG concentration. Uptake was allowed to proceed for different time periods (0.25–6 hr) at 37° and stopped by placing the monolayers on ice and by aspirating the radioactive solution. Blank values were obtained by adding radioactive solution to monolayers placed on ice. All wells were washed three times with ice-cold EBSS (pH 7.2 at 0°). The sodium dependency of the uptake was verified by performing the same assay in the absence of sodium. All Na^+ salts in

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EBSS were isoosmotically replaced by the corresponding potassium salts, except that sodium bicarbonate was replaced by choline chloride. Furthermore, uptake of α-AMG was also monitored in the presence of 0.1 mM phlorizin, a specific inhibitor of Na^+-dependent glucose transport (25). The monolayers were lysed with 0.5 ml of 1 N NaOH overnight and 400 μl of the hydrolyzate was used for liquid scintillation counting without quench correction. Blank values were generally less than 2% of the highest uptake values and were subtracted from all values. Aliquots (50 μl) of the hydrolysate were used for protein determination according to the recommendations of the manufacturer (Pierce). Results are expressed as uptake in terms of cpm of AMG per mg of cell protein versus time.

Dose response curves of free or L-AmB. Free AmB. Drug solutions (range, 0.01–20 μg of AmB/ml) in complete tissue culture medium were prepared by dissolving the appropriate amount of powdered AmB in DMSO and diluting this solution with tissue culture medium. The solutions were freshly prepared on the day of the experiment. The amount of DMSO was kept constant at 1% for all incubations. Control cultures were incubated with 1%, v/v, DMSO in medium. Cells were treated for 2 hr with the drug solutions at 37°. Monolayers were washed with EBSS without glucose and the uptake of α-AMG was followed for 4 hr. After this time, the cultures were processed for liquid scintillation counting and protein determination as described above. Results are expressed as percentage of α-AMG uptake of the control cultures. Experiments were done in triplicate and independently repeated at least twice.

L-AmB. AmB incorporated into liposomes was diluted with tissue culture medium to obtain appropriate drug levels. The solutions were adjusted to the same total amount of lipid and same salt concentration. Therefore, any effects of lipids or saline on α-AMG uptake were taken into account. α-AMG uptake was monitored as described above after a 2-hr exposure of the cells to the drug. For long term experiments, sterile liposomes were added to confluent cultures and the cells incubated for 2 days. Thereafter, their response was measured. For measurement of the ability of LLCPK1 cells to recover from AmB treatment, cells were incubated with 5 μg/ml of free AmB for 2 hr and then allowed to grow for different periods of time in drug-free medium; thereafter α-AMG uptake was measured.

Scanning Electron Microscopy

Cells were plated at 5×10^5 cells/well in six-well plates in which acid-cleaned sterile coverslips had been placed. After reaching confluence (about 1 week) cells were incubated with (a) control medium, (b) 1%, v/v, DMSO-containing medium, (c) 10 μg/ml AmB in medium, (d) empty liposomes (lipid amount as in the drug-containing liposomes), or (e) 1 mg/ml AmB in liposomes. After 2 hr at 37° the cultures were rinsed with warm medium and fixed with 0.5% glutaraldehyde in complete medium at 37°. After 10 min, the medium was replaced by 0.5% glutaraldehyde in EBSS (pH 7.3 at 37°) and incubated for 20 min at 37°. The cells were rinsed with EBSS and treated with 1%, w/v, tannic acid in EBSS at room temperature for 1 hr. The monolayers were carefully washed several times with EBSS to completely remove the tannic acid and then postfixed with 0.5% osmium tetroxide for 30 min at room temperature. After dehydration of the specimens in a graded series of methanol washes at 4°, they were placed in mixtures of amylacetate in methanol. After critical point drying and sputter coating with gold, the specimens were examined in a JEOL 100 CX microscope equipped with a SEM unit.

Light Microscopy

In order to obtain perpendicular sections through monolayers of LLCPK1 cells, we grew the cells on an easily sectionable substratum. Nucleopore filters (2-μm pore size) were boiled for 40 min to remove the wetting agent. The air-dried filters were placed on a coverslip and glued on with melted Parafilm. This rendered the Nucleopore filter impermeable, allowing the formation of domes. On the other hand, the whole assembly could be removed from the coverslip without damaging the monolayer before embedding and sectioning. Because LLCPK1 cells do not attach readily to plain Nucleopore filters,[2] the filters were coated with gelatin that was cross-linked with glutaraldehyde. Sterilization was achieved by placing the covers slips into 70% ethanol for 24 hr. After several rinses with sterile phosphate buffer, the filters were incubated for 1 hr in sterile fetal bovine serum. Due to the gelatin coating, there is probably an enhanced adsorption of attachment-promoting factors, such as fibronectin, allowing a better growth of LLCPK1 cells on these filters. Cells (5×10^5) were plated on top of the coverslips and kept in culture for 1 week. During this time, confluent monolayers developed with abundant domes. These cells were then treated with (a) 10 μg of free AmB/ml, (b) 1% DMSO in medium (untreated control), (c) empty liposomes (total amount of lipid as in condition d), or (d) 0.5 mg/ml AmB in liposomes. After 2 hr at 37° the coverslips were washed with warm medium and the cells were fixed with 0.5%, w/v, glutaraldehyde, pH 7.2, at 37° in medium. In order to preserve lipids, cultures were incubated for 1 hr with 1% tannic acid, w/v, in EBSS with glucose at room temperature. After the complete removal of the tannic acid, the cells were postfixed in 0.5% osmium tetroxide for 1 hr at room temperature. After dehydration in a graded series of methanol solutions, the filters were carefully removed from the coverslip by peeling the Parafilm from the glass. The specimens were embedded in Epon and polymerized and 0.5-μm-thick sections were cut perpendicular to the monolayer. Sections were either stained with 1% toluidine blue in borate buffer or cut thicker (1 μm) and not stained. All sections were examined in a Zeiss Ultrophot II microscope.

Inhibition of Dome Formation

LLCPK1 cells were plated in six-well plates at a density of 5×10^5 cells/well. An acid-cleaned sterile coverslip had been previously placed in each well. After about 2 days, a continuous monolayer was formed on the coverslips with only very few domes. For the free drug, cells were incubated with either 1% DMSO (untreated control) or with three different drug concentrations (0.5, 5, and 10 μg/ml). For L-AmB, five groups were used (untreated cells; cells incubated with empty liposomes; and cells incubated with 0.5, and 10 μg/ml AmB, total lipid concentration being the same in all 4 groups). The cells were cultured for 4 days in the presence of the corresponding drug solutions, the medium being changed after 2 days. Every day, starting from day 0 (base-line level of domes), three coverslips per group were removed and the number of domes in 30 randomly chosen fields of each coverslip was counted using a microscope equipped with Nomarski optics (100× magnification). The data are expressed as number of domes/field versus time for each group.

Results

Protein Synthesis and Protein Loss

The incorporation of [^{3}H]leucine into acid-insoluble material in LLCPK1 cells is linear for approximately 10 hr and then tends to plateau at about 20–30 hr (data not shown). Thus, initial rates of ^{3}H incorporation during the first few hours should be a measure of protein synthesis rate, whereas incorporation during >30 hr should result in uniform labeling of all cell proteins.

Fig. 1 shows the effect of free AmB on LLCPK1 cells. About 30 μg of AmB/ml inhibits protein synthesis to 50% of that of the control cells if the cells are incubated in serum-containing medium. If AmB is dissolved in serum-free medium, about 10 μg of AmB/ml is sufficient for 50% inhibition of protein synthesis. This 3-fold increase in toxicity is probably due to the fact that AmB becomes bound to the lipoproteins present in serum-containing medium, thus reducing the effective concen-

[2] H. J. Krause and R. L. Juliano, unpublished observations.

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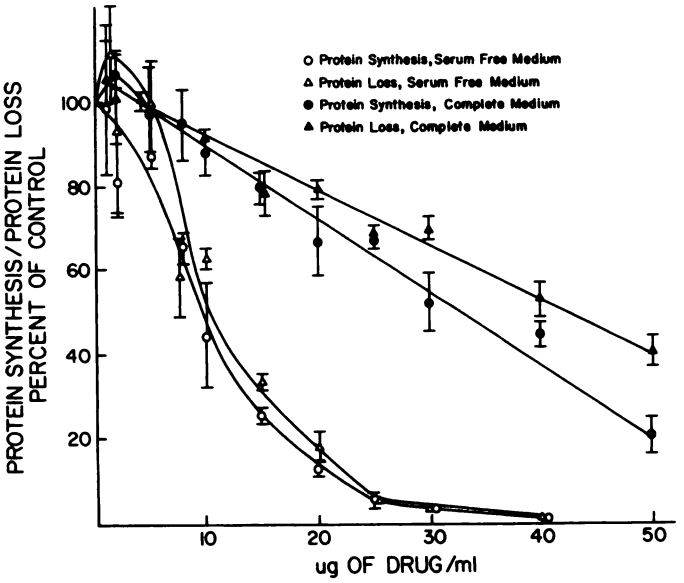

Fig. 1. Effects of AmB on protein synthesis and protein loss. LLCPK1 cells and prelabeled cells were incubated with different drug concentrations for 2 hr in serum-free or serum-containing medium. Thereafter, protein synthesis and protein loss was measured as described in Materials and Methods and expressed as percentage of the control cultures. Results are the mean of six cultures ± standard deviation.

tration of drug. In both cases, the curves of total protein loss are parallel to the curves for inhibition of protein synthesis. Because of this parallelism, the results for the protein synthesis inhibition cannot be corrected for the amount of protein/well, because protein synthesis would then artifactually increase over control levels. By observing the cultures treated with AmB, it could be seen that LLCPK1 cells lose their ability to attach to the substratum. Therefore, the curves denoting protein loss actually reflect the sum of two processes, first, loss of cellular protein due to cell detachment and, second, loss of proteins from porous but still attached cells. Similarly, protein synthesis curves are influenced by the same mechanism. Thus, the values displayed in Figs. 1–3 really reflect the sum of several processes that cannot be readily separated. They are, however, clear manifestations of AmB toxicity.

The time course of AmB toxicity is depicted in Fig. 2. The cells were incubated with 25 μg of AmB/ml and the time course of protein synthesis was followed. As can be seen in Fig. 2, AmB toxicity progresses during the first 2 hr faster than in the interval from 2 to 8 hr. Consequently, a 2-hr incubation period was chosen for all subsequent experiments. Because our pulse label period of 4 hr is rather long compared with the time required to produce drug effects, we wanted to see whether cells, once in contact with AmB for 2 hr, were able to recover. This was not the case, because after exposure to AmB and subsequent washing in drug-free medium, there was a continuous decrease in protein synthesis as well as loss of protein (data not shown). Thus, AmB inhibition of protein synthesis in LLCPK1 cells shows a rapid onset of action and appears essentially irreversible. Exposure of the cells to AmB results in cell detachment, protein loss, and reduction of protein synthetic capability. Removal of excess drug does not reverse these toxic effects; rather, the effects seem to progress. The washing procedure may not remove all of the drug that is bound to cells, but it does remove most of the drug from the cultures.

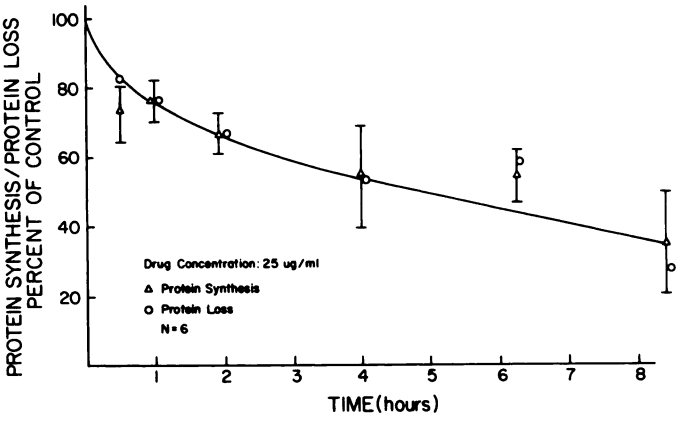

Fig. 2. Time course of AmB effects. Cultures were exposed to 25 μg/ml AmB for different time intervals and protein synthesis/protein loss was determined. Results are the mean of six cultures ± standard deviation.

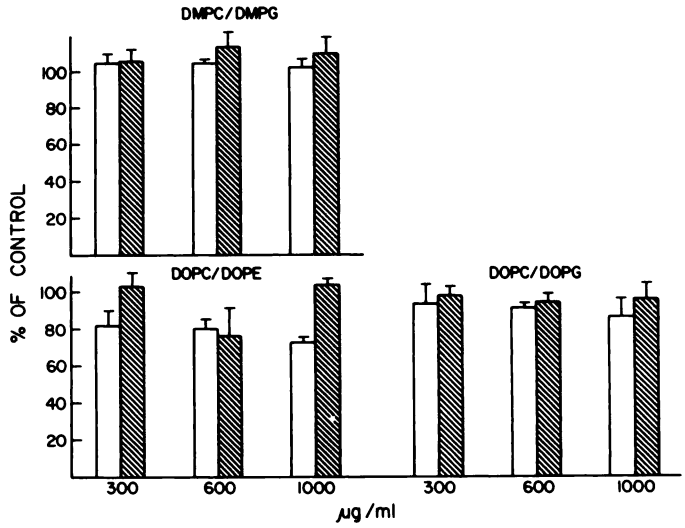

Fig. 3. Effects of liposomal AmB on protein synthesis. Monolayers were incubated with 300, 600, and 1000 μg/ml L-AmB. Control cultures contained the corresponding amount of lipid without added drug. After 2 hr of incubation, protein synthesis/protein loss was determined. Results are the mean of six cultures ± standard deviation. □, Protein synthesis; ▨, protein loss.

Because previous reports have shown that AmB in lipid vesicles is less toxic to red cells than is the free drug (7, 8), we decided to test several forms of liposomal AmB in terms of toxicity to LLCPK1 epithelial cells. Following observations from earlier experiments (8), we selected three different liposomal AmB preparations, which were either (a) nontoxic (DMPC/DMPG 7:3), (b) of intermediate toxicity (DOPC/DOPG 1:1), or (c) quite toxic (DOPC/DOPE 1:1), as measured by effects on Rb^+ efflux from red blood cells.

Because it is known that high concentrations of phospholipids themselves can be cytotoxic in cell culture, we investigated the effects of "empty" liposomes on protein synthesis in LLCPK1 cells. The DMPC/DMPG and DOPC/DOPE liposomes were without effect on [^{3}H]leucine incorporation in LLCPK1 cells at doses of up to 15 mg/ml lipid. Empty DOPC/DOPG liposomes were the only ones that slightly inhibited protein synthesis (85% of control) at a concentration of 15 mg/ml lipid (data not shown). This effect may be due to the removal of cholesterol from the cell membranes by unsaturated lipids

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(26). In order to correct our data for this slight effect, all cells were incubated with the amount of lipid used in the highest liposomal drug concentration (see Materials and Methods).

If LLCPK1 cells were incubated with AmB liposomes composed of DMPC/DMPG for 2 hr, there was no effect on protein synthesis or overall protein loss, even at extremely high AmB concentrations (40 times the 50% inhibitory concentration of the free drug) (Fig. 3). AmB liposomes composed of DOPC/DOPG or DOPC/DOPE showed small but reproducible decreases in protein synthesis. Thus, as in the case with red cells, encapsulation of AmB in lipid vesicles generally reduces its acute toxicity to epithelial cells, although there are some differences in toxicity with different liposome compositions.

α-AMG Uptake Experiments: Acute and Chronic Effects

If α-AMG uptake is to be used as an indicator of toxic effects on LLCPK1 cells, it is necessary to establish the characteristics of the uptake process under the specific experimental conditions. Fig. 4 shows that α-AMG uptake is approximately linear for up to 1 hr and thereafter levels off. Because we express our results as percentage of maximal uptake capability of control cells per mg of total cell protein, we have chosen a 4-hr incubation period, at which time steady state is achieved by the cells. Furthermore, Fig. 4 documents that the uptake process shows the characteristic behavior of glucose transport by these cells (15–18). The process is Na^+-dependent and can be blocked by phlorizin, a specific inhibitor of Na^+-dependent glucose transport (25).

Acute effects. The influence of free AmB and L-AmB on the α-AMG uptake capability of LLCPK1 cells is depicted in Fig. 5. About 1.5 μg/ml AmB (free drug) causes a 50% reduction of α-AMG uptake after a 2-hr exposure, whereas all L-AmB preparations show markedly lesser effects. Among the liposomal preparations there were differences in acute toxic effects, with DMPC/DMPG liposomes being virtually nontoxic (up to 1 mg/ml AmB), DOPC/DOPG slightly toxic (50% inhibition of α-AMG uptake at about 80 μg/ml (AmB), and DOPC/DOPE being the most toxic (50% inhibition at 20 μg/ml AmB). All liposomal formulations proved to be stable and do not release appreciable amounts of AmB in the incubation medium during 2 hr (Table 1). These observations on α-AMG transport are in agreement with results that were obtained by measuring the Rb^+ efflux from preloaded red blood cells in response to free or

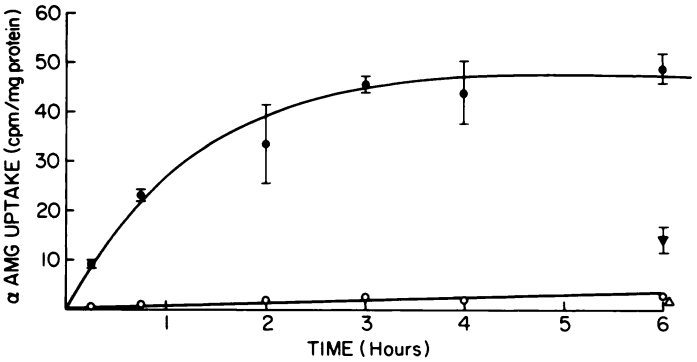

Fig. 4. α-AMG uptake by LLCPK1 cells. Confluent monolayers were incubated with 12 mM α-AMG in the presence or absence of Na^+. Results are mean ± standard deviation of three monolayers. ●, With Na^+; ▼, 6-hr uptake in presence of 0.1 mM phlorizin and Na^+; ○, without Na^+; △, 6-hr uptake in presence of 0.1 mM phlorizin, but without Na^+.

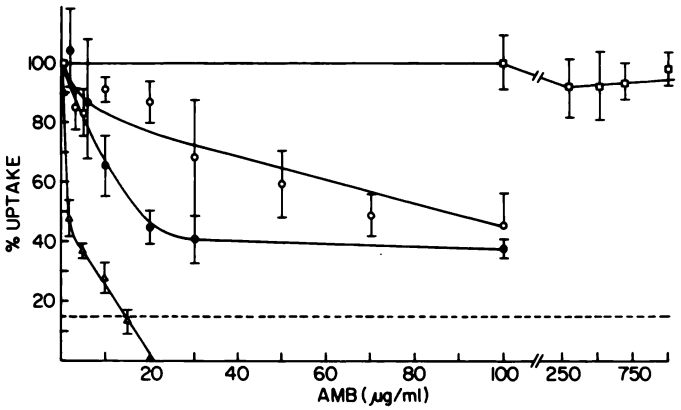

Fig. 5. Acute effects of free or liposomal AmB on α-AMG uptake. Confluent monolayers were incubated with free drug, dissolved in DMSO (1% final concentration), or appropriate amounts of liposomal drug with three different phospholipid compositions. Results are the mean of six monolayers ± standard deviation: controls included 1% DMSO-containing medium or empty liposomes at highest lipid concentration used. Drug exposure, was 2 hr. △, Free AmB; □, DMPC/DMPG, 7:3, liposomes; ○, DOPC/DOPG, 1:1, liposomes; ●, DOPC/DOPE, 1:1, liposomes; – – – 0.1 mM phlorizin.

TABLE 1

Stability of liposomal preparations in tissue culture medium

Sterile liposomes (corresponding to about 10 μg/ml of AmB) were incubated in tissue culture medium for various time intervals at 37°. Incubation was stopped by diluting the medium 10-fold with 0.1 M phosphate buffer, followed by centrifugation for 1 hr at 40,000 rpm. The amount of AmB associated with the pellet was determined spectrophotometrically after dissolving it in 2 ml of methanol. Results are the mean of three samples ± standard deviation and are expressed as percentage of control liposomes, which were diluted and centrifuged directly after the addition of the medium.

t	Amphotericin B retained		
	DMPC/DMPG	DOPC/DOPG	DOPC/DOPE
days		%	
1/12	112.3 ± 2.4	92.1 ± 3.1	93.2 ± 1.6
1/3	110.2 ± 1.5	92.3 ± 2.4	95.0 ± 2.0
1	97.0 ± 14.3	88.9 ± 7.6	81.7 ± 4.6
2	90.5 ± 12.6	85.3 ± 8.2	ND*
3	100.3 ± 4.2	88.1 ± 4.4	ND
4	104.8 ± 14.2	96.3 ± 1.5	ND

* ND, not determined.

L-AmB (8). Thus, the incorporation of AmB into certain liposome formulations produces a marked reduction in acute toxic membrane effects as evaluated by measurement of α-AMG transport.

Chronic effects. If LLCPK1 cells are incubated for 1 or 2 days with free or encapsulated AmB, however, a very different dose-response curve is found. After 1 day, DMPC/DMPG and DOPC/DOPG liposomes begin to influence α-AMG uptake capability at a dose of only 5 μg/ml AmB (Fig. 6A). In addition, the free drug obviously partially loses its activity, inasmuch as 5 μg/ml AmB, which inhibits α-AMG uptake 60% if the cells are incubated for 2 hr, only inhibits 23% after 1 day of incubation. DOPC/DOPE-AmB liposomes are as toxic as the free drug during 1-day incubations whereas the other AmB-containing liposomes are only slightly less toxic (Fig. 6A).

After 2 days of incubation, there are no differences in effects on α-AMG uptake between free and L-AmB (Fig. 6B). Furthermore, the degree of toxicity is reduced in the case of the free drug (10 μg/ml free drug for 2 days produces only a 40% inhibition in α-AMG uptake, whereas incubation with the same concentration of drug for 2 hr reduced α-AMG uptake by 70%).

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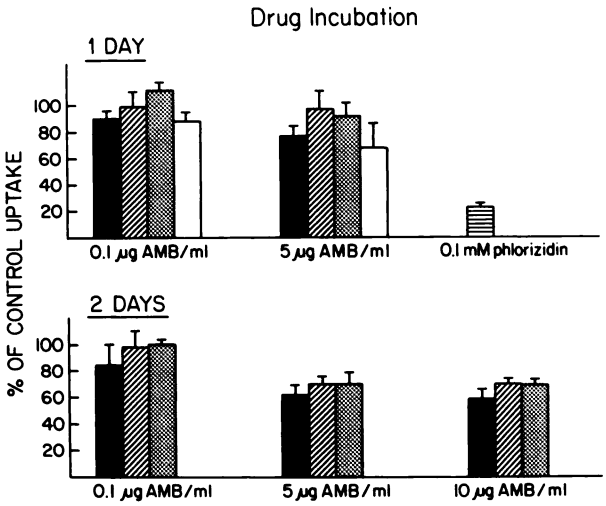

Fig. 6. Long term effects of AmB on α-AMG uptake. Confluent monolayers were incubated with 0.1, 5, and 10 μg/ml AmB, free or encapsulated in sterile liposomes for 1 or 2 days. Thereafter, the α-AMG uptake by the cells was measured as in Fig. 5. Data are expressed as percentage of control (untreated) cultures ± standard deviation, obtained from six monolayers. ■, Free AmB; ▨, DMPC/DMPG, 7:3, liposomes; ⊡, DOPC/DOPG, 1:1, liposomes; □, DOPC/DOPE, 1:1, liposomes; ■, 0.1 mM phlorizin.

However, the toxicity for the liposomal preparations is increased; thus, 10 μg/ml AmB in liposomes (DMPC/DMPG or DOPC/DOPG) for 2 days gives a 30% reduction of α-AMG uptake whereas 2 hr exposure of 1 mg/ml AmB in the same formulations has no effect on α-AMG uptake (Fig. 5).

Thus, upon protracted incubation in culture medium, free AmB loses toxicity, whereas the liposomal preparations of AmB become more toxic. Basically, the two liposomal compositions (DMPC/DMPG, 7:3, and DOPC/DOPG, 1:1) tested were as toxic as the free drug. DOPC/DOPE liposomes could not be tested in the 2-day experiment because these lipids themselves were too toxic to allow an incubation for 2 days (data not shown).

There may be several possible explanations of these results. Because AmB is highly unstable in solution (27), free AmB may become inactivated in the presence of medium, which would account for the loss of activity of free drug after 2 days. In the cases of the liposomal formulations, it is possible that the presence of the cells destabilizes the integrity of the liposomes so that the increase in toxicity is due to release of free drug. Alternatively, the prolonged contact of drug-loaded liposomes with the cell membranes could result in a slow transfer of drug to the cells. In order to explore these questions, we performed the following experiment. Free as well as L-AmB samples at a concentration of 10 μg/ml drug were incubated for 2 days at 37° in spent cell culture medium (which should contain any lipolytic enzymes or other destabilizing factors released by cells); controls included empty liposomes (same amount of total lipid as for drug-containing liposomes). After 2 days, confluent LLCPK1 cells not previously exposed to AmB were incubated for 2 hr with the AmB preexposed to spent medium. The results of this experiment are shown in Table 2. As can be seen, free AmB (10 μg/ml) obviously becomes inactivated in conditioned medium, inasmuch as it produces only a 13% reduction of α-AMG uptake, in contrast to fresh free AmB run as a control, which produces a 56% reduction. Both liposomal preparations

TABLE 2

Response of LLCPK1 cells to free and liposomal drug, previously incubated for 2 days in conditioned medium

Free AmB and L-AmB (all at 10 μg/ml) were incubated in conditioned medium for 2 days at 37°. Confluent cells were treated with these solutions for 2 hr and their α-AMG uptake was measured. Controls included were nontreated cells (1% DMSO or empty liposomes) and cells treated with freshly prepared AmB solution. Results are expressed as percentage of α-AMG uptake of a corresponding control monolayer, mean of six determinations ± standard deviation.

Sample	α-AMG uptake
	% of control cells
Fresh AmB	44.9 ± 3.4
Old AmB[a]	87.7 ± 8.8
DMPC/DMPG with AmB[a]	99.7 ± 8.7
DOPC/DOPG with AmB[a]	95.0 ± 5.4

[a] Incubated with conditioned medium.

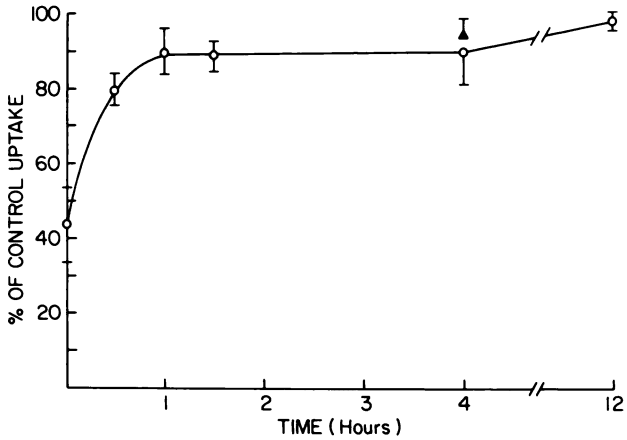

Fig. 7. Recovery of α-AMG uptake capability of LLCPK1. Confluent monolayers were treated with 5 μg/ml AmB. After 2 hr of exposure to the drug, medium was changed, cells were incubated in drug-free medium for different time intervals, and then α-AMG uptake was measured. Results are the mean ± standard deviation of three monolayers.

remained nontoxic, showing no effect on α-AMG uptake. This is consistent with our observations on L-AmB stability in cell-free medium (Table 1), which show virtually no drug release during 48 hr of incubation. Therefore, the AmB does not leak out of liposomes during prolonged incubation under culture conditions but rather prolonged contact with the cell membranes seems to be responsible for the toxicity observed in Fig. 6.

It has been shown, by other investigators (27–30), that AmB-induced toxicity is reversible at low drug concentrations, due to repair mechanisms in the cells. To see whether repair might occur in this cell line as well and might contribute to effects depicted in Figs. 5 and 6, we investigated α-AMG uptake after several hours of recovery, with cells that had been exposed to 5 μg of free AmB/ml for 2 hr. Fig. 7 reveals that, after a recovery period of 1 hr, α-AMG uptake reaches control values. This process is very fast and independent of protein synthesis, because cells with blocked protein synthesis (50 μM cycloheximide) reestablished their α-AMG uptake as quickly as unblocked cells. These observations contrast with the irreversible effects of higher doses of AmB on protein synthesis described previously.

Morphological Data

Scanning electron microscopy. LLCPK1 cells are epithelial cells of polygonal shape with numerous microvilli on the

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apical cell surface (Fig. 8A). Upon reaching confluency, this cell line forms domes, fluid-filled blisters that are characteristic of transporting epithelia (19, 20). A representative monolayer with domes is shown, using scanning electron microscopy, in Fig. 8B. If these cells were treated with 10 μg of free AmB/ml for 2 hr, the cells changed morphology dramatically (Fig. 8C). Cells in the monolayer swell and lose the microvilli on their apical side and the cell membranes are folded. Domes not only collapse but the cells of the domes are highly distorted (Fig. 8D). Fig. 9A shows, by contrast, a monolayer incubated with 1 mg/ml AmB in DMPC/DMPG liposomes. Domes are still intact, although some of the cells have a rounded appearance; in the monolayer some swollen cells that seem detached can be seen (Fig. 9A). No other sign of toxicity could be detected in these monolayers. Control cultures with empty liposomes revealed no morphological difference as compared with cells incubated in medium. This was true for all empty liposomes and for 1% DMSO-containing medium (data not shown). Fig. 9B shows that the morphology of LLCPK1 cells treated with 1 mg/ml AmB in DOPC/DOPG liposomes is more affected than in the case of an equivalent dose of AmB in the DMPC/DMPG liposomes. The collapsed dome, which can be seen in the photomicrograph, consists of rounded cells, the majority of them having microvilli. At higher magnification (Fig. 9C), it becomes clear that cells with bound liposomes show typical signs of AmB-related toxicity such as disappearance of microvilli and invaginated cell membranes; in the background, unaffected cells can be seen. DOPC/DOPE liposomes (1 mg/ml AmB) proved to be the most toxic. The monolayer as well as the domes in the monolayer were heavily affected by the liposomal drug (Fig. 9D). Thus, from the morphological data there are clear differences in toxicity of the various L-AmB formulations, with DMPC/DMPG liposomes being the least and DOPC/DOPE liposomes the most toxic.

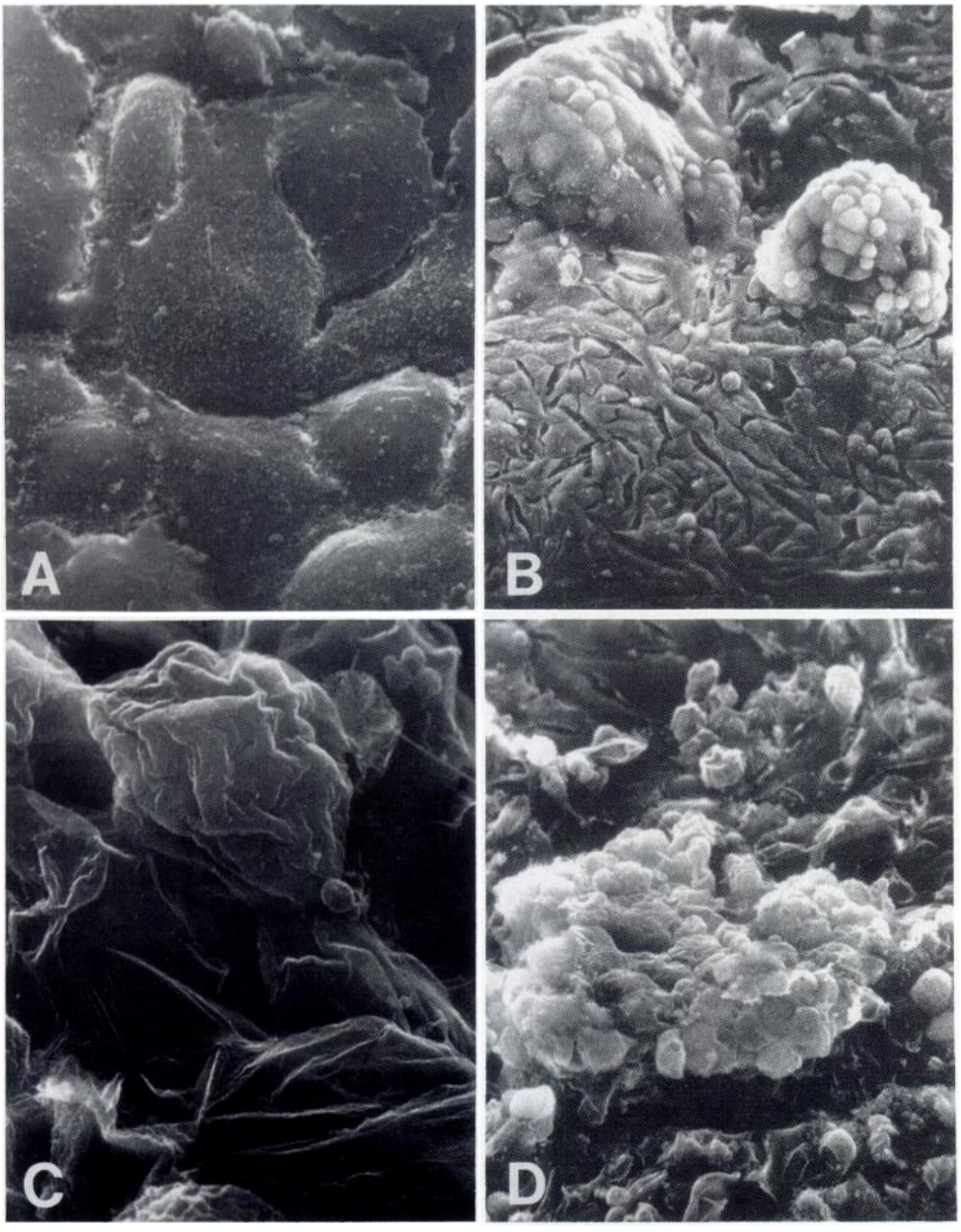

Fig. 8. Acute effects of AmB on cell morphology: scanning electron microscopy. All drug exposures are 2 hr. A, untreated monolayer of LLCPK1 cells. Original magnification 2000×. B, untreated LLCPK1 cells. Note the well preserved domes and monolayer of cells. Original magnification, 660×. C, LLCPK1 cells exposed to 10 μg/ml AmB for 2 hr. Note that cell membranes are folded, cells have polygonal shapes, and microvilli have disappeared. Original magnification, 6600×. D, Collapsed dome after 10 μg/ml AmB. Note that cells are highly distorted. Original magnification, 1100×.

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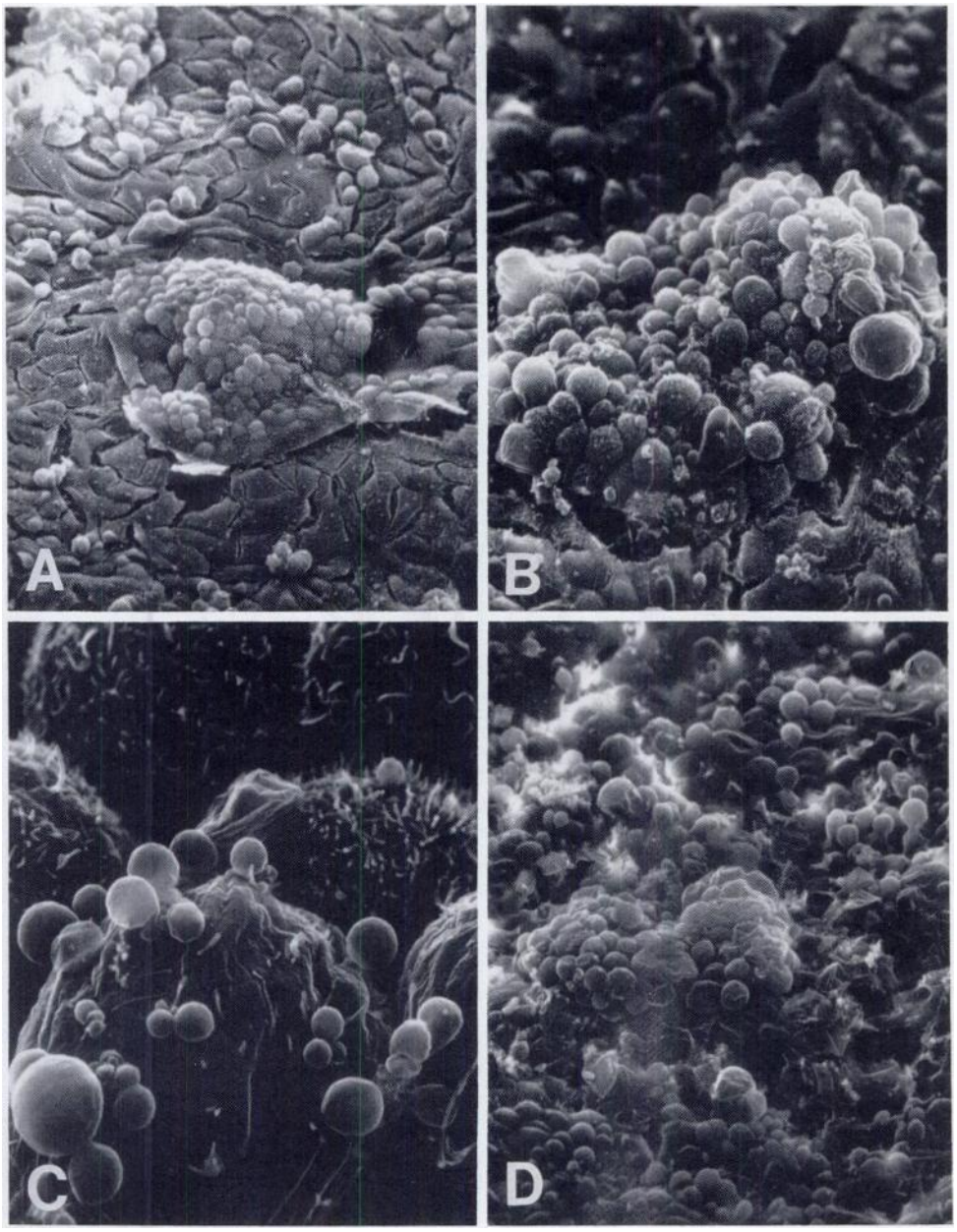

Fig. 9. Acute effects of L-AmB on cell morphology: scanning electron microscopy. All drug exposures are 2 hr. A, LLCPK1 cells incubated with 1 mg/ml AmB in liposomes (DMPC/DMPG). Cells in domes are slightly rounded, with collapsed dome in background. Note some swollen cells in monolayer. Original magnification, 660×. B, Dome in culture treated with 1 mg/ml AmB in liposomes (DOPC/DOPG). Structure is collapsed with some cells lacking microvilli. Original magnification, 1100×. C, Higher magnification of same preparations as B. Liposomes sticking to a damaged cell in dome are visible as spheroids with bright rims; in the background some less affected cells are visible. Original magnification, 11,000×. D, Monolayer incubated with 1 mg/ml AmB in DOPC/DOPE liposomes. All cells are affected by the drug. Original magnification, 660×.

Light microscopy. LLCPK1 cells grow as a monolayer of elongated cells, which detach from the substratum forming domes (Fig. 10A). Cells in domes seem not to be different from cells in the monolayer (31); microvilli are confined to the apical surface and numerous junctional complexes can be seen. The existence of tight junctions can be assumed, because otherwise the hydrostatic pressure between substratum and basolateral membrane, which is the cause of domes, could not be maintained. If LLCPK1 cells are treated with 10 μg of free AmB/ml for 2 hr, the morphology changes. The low-power micrograph (Fig. 10B) shows that domes collapse and that the cells are swollen. The volume ratio of the cytoplasm to the nucleus increases for drug-treated cells (note that Fig. 10, A and B have identical magnifications). These effects are likely due to the loss of the semipermeability of the cell membrane, causing water influx due to the higher osmolarity of the cell interior. AmB encapsulated in DMPC/DMPG liposomes at a dose of 0.5 mg/ml did not change the morphology of LLCPK1 cells (Fig. 10C) during short term exposure. Even when viewed at high power, at which liposomes are clearly detected attached to the cell surface, none of the characteristic morphological changes (increase in cell volume, lack of microvilli, collapsed domes) are detected (data not shown). Thus, the liposomal form of AmB prevents the development of domes (see below) but does not cause them to "collapse" during acute exposure. In the case of the more toxic preparation of DOPC/DOPG liposomes (Fig. 10D) domes are collapsed. The cells that have liposomes bound to their surface also change their morphology. Besides affected cells, normal cells without liposomes are visible at higher power (not shown); this suggests that AmB directly transfers to the cells. Control cultures with empty DOPC/DOPG liposomes appeared to be normal (data not shown). Thus, short term exposure to these types of AmB liposomes only marginally affected cell morphology.

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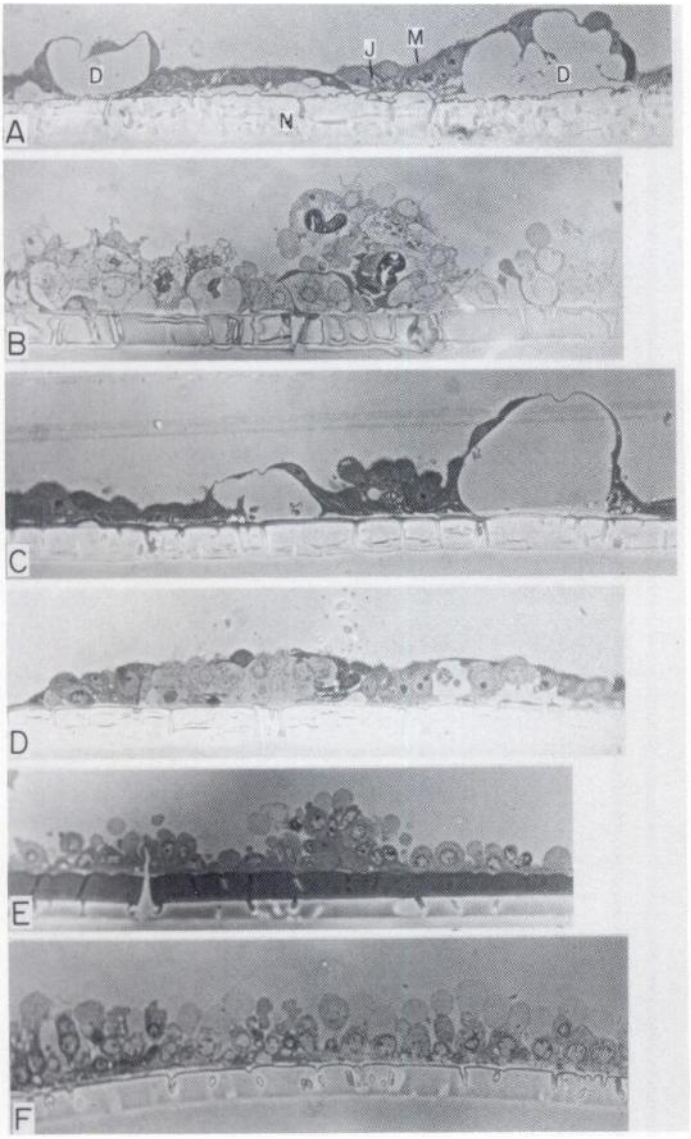

Fig. 10. Acute effects of AmB and L-AmB on cell morphology: light microscopy. Toluidine blue stain; phase contrast; magnification, 250×. All drug exposures are 2 hr. A, Control cells; B, cells treated with 10 μg/ml free AmB for 2 hr; C, monolayer incubated with 0.5 mg/ml AmB in DMPC/DMPG liposomes; D, monolayer exposed to 0.5 mg/ml AmB in DOPC/DOPG liposomes; E, cells treated with empty DOPC/DOPE liposomes; F, cells treated with 0.5 mg/ml AmB in DOPC/DOPE liposomes (lipid concentration as in E). *D*, dome, *J*, junction, *M*, microvilli, and *N*, nucleopore.

In the case of DOPC/DOPE liposomes, empty liposomes alone influence the morphology of the cells (Fig. 10E). Frequently, vacuolized cells can be seen with cell bodies that are elongated. Microvilli are less frequent. Junctional complexes, however, are still visible at higher power (not shown). AmB-containing DOPC/DOPE liposomes change the shape of the cells in a very characteristic way (Fig. 10F). Cells developed a pear-like appearance, with the apical part of the cell generally not as densely stained as the lower part. All cells in the monolayer are affected (Fig. 10F), very similar to the case of cells treated with free AmB (Fig. 10B). Upon closer inspection at high power (not shown), one can see that cells treated with AmB encapsulated in DOPC/DOPE liposomes seem to extrude some parts of their cytoplasm. First, the cells increase their volume with nucleus and organelles confined to the basolateral area; two clearly separated zones are visible in the cells (Fig. 10F). The upper, lighter stained part seals itself with a membrane but is still attached to the residual cell. Finally, an individual vesicle is formed, leaving a residual cell body with nucleus on the filter. This phenomenon could only be seen with the DOPC/DOPE liposomes. Fig. 10F shows that the whole monolayer is affected and that this process occurs only on the apical membrane. In Fig. 10E it can be seen that some of these changes are also induced by empty DOPC/DOPE liposomes, but to a lesser extent. The cause and significance of these interesting effects are not understood at present. Presumably, the membrane-perturbing effects of DOPE, a lipid that can form non-bilayer phases, may contribute to the morphological anomalies seen.

Inhibition of dome formation: a chronic toxic effect. Domes are fluid-filled blisters that are the result of vectorial sodium transport and concomitant water accumulation between substratum and basolateral cell membrane (20). Dome formation is usually seen in differentiated epithelia, but the regulation of this property is not fully understood (19, 20). Fig. 11A shows a monolayer with several domes as viewed with Nomarski optics. Fig. 11, B–D show the effects of different concentrations of L-AmB on dome formation during chronic exposure. In Fig. 11B the cultures were exposed only to DMPC/DMPG liposomes but not AmB; in Fig. 11, C and D, increasing doses of L-AmB were used. Thus, AmB in liposomes seems to impair dome formation in a dose-related fashion. The influence of chronic exposure to free AmB or L-AmB on the induction of dome formation is shown in more quantitative form in Table 3. Nonconfluent monolayers were incubated with different drug concentrations and the cells were grown for 4 days. The number of domes per field was determined. As shown in the table, free AmB slows dome formation in a dose-related manner; 5 μg/ml AmB is sufficient to significantly suppress the number of domes per field. However, L-AmB proved to be as toxic as the free drug in this context, inasmuch as, at 5 μg/ml AmB in DMPC/DMPG liposomes, the number of domes per field diminishes (Fig. 11, Table 3). LLCPK1 cells that have been incubated for 4 days with 10 μg of AmB in DMPC/DMPG liposomes are shown in Fig. 11D. The cells are still confluent and apparently undamaged and the monolayer is not disrupted, but domes are almost completely absent. Therefore, the effects on dome formation closely parallel those on α-AMG uptake during 2 days continuous exposure to the drug (Fig. 6) but are quite different from acute effects on either α-AMG transport or on protein synthesis. The effects of chronic exposure to L-AmB also contrast with our previous morphological observations (Fig. 10) on acute effects of L-AmB, in which the drug had little effect on pre-existing domes.

Discussion

The LLCPK1 cell line provides a useful *in vitro* model for studies of drug effects on kidney cell function, because this cell line can display many morphological and transport characteristics typical of proximal tubule cells (14–20). In this report we have compared effects of AmB, in free form or in liposomal form, both on a basic cellular housekeeping function (protein synthesis) and on differentiated functions (glucose transport and dome formation) in LLCPK1 cells. A central finding is that the incorporation of AmB into liposomes (L-AmB) pro-

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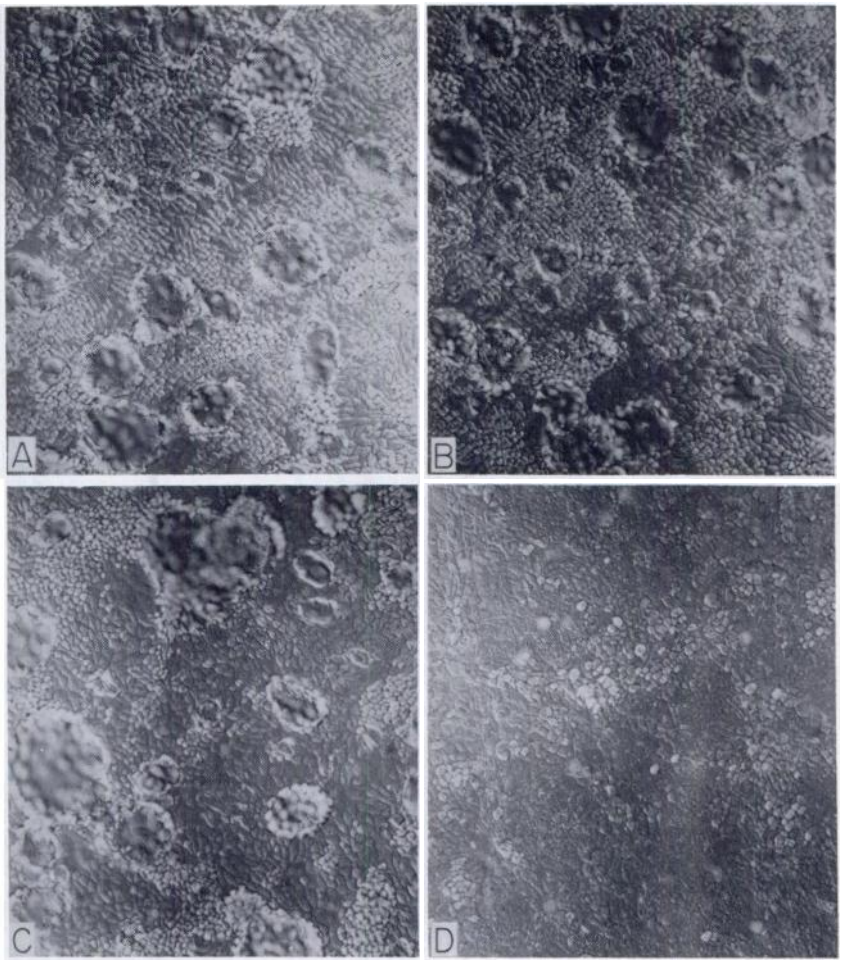

Fig. 11. Inhibition of dome formation. All drug exposures were 4 days. DMPC/DMPG, 7:3, liposomes; Nomarski optics; magnification, 100×. A, Control cells; B, cells incubated with empty liposomes (lipid amount as in liposomes containing 10 μg/ml); C, cells incubated with 0.5 μg/ml liposomal AmB; and D, cells incubated with 10 μg/ml liposomal AmB.

duces a marked reduction in the acute toxicity of the drug as measured by inhibition of protein synthesis or by inhibition of glucose transport or dome formation. In this sense, the effects described here for kidney cells resemble the situation previously described regarding effects of AmB and L-AmB on cation permeability in red cells (7, 8). There are, however, a number of findings presented here that did not emerge during previous investigations.

An important point is that there is a marked difference in the sensitivity of protein synthesis and of glucose transport to inhibition by AmB. Thus the IC_{50} values for inhibition of protein synthesis and of sodium-stimulated glucose transport are 30 and 1.5 μg/ml respectively, under comparable experimental conditions (Figs. 1 and 5). Further, the AmB-induced inhibition of glucose transport was reversible upon removal of the drug (Fig. 7), whereas the inhibition of protein synthesis was essentially irreversible and progressive. AmB concentrations in the low μg/ml range also had marked effects on cell morphology and on the stability of "domes" (Figs. 8 and 10). Thus, AmB at low concentrations causes toxic actions on differentiated functions, perhaps especially those that are membrane related, such as sugar transport and doming; this toxicity seems to be reversible. Other workers have also reported reversible toxic effects of AmB on cell membrane functions (1, 30). At higher AmB concentrations, basic cellular functions such as protein synthesis are inhibited, an irreversible effect that is accompanied by detachment of cells from the substratum and by cell death. Incorporation of AmB in liposomes provides a marked protection (at least 30–60-fold; Figs. 1 and 5) against toxicities to both differentiated functions and housekeeping functions in the acute situation.

A second point is that lipid composition is an important determinant of L-AmB toxicity. Thus, AmB-containing liposomes composed of DMPC/DMPG were less toxic than those composed of DOPC/DOPE or DOPC/DOPG, both in terms of inhibition of α-AMG transport (Fig. 5) and in terms of effects on morphology (Figs. 9 and 10); indeed, DOPC/DOPE vesicles seem to have some interesting toxic effects on cells in the absence of AmB (Fig. 10E). This largely agrees with previous results on induction of cation fluxes in red cells by different L-AmB formulations (8). Interestingly, lipid composition seems to play a lesser role in modulating the protective effects of liposomes at the high concentrations of AmB, which result in inhibition of protein synthesis (Fig. 3). It should be noted that a variety of L-AmB compositions, including the ones used here, retain full potency in terms of cytotoxic effects on fungi; this has been extensively documented by several laboratories (8, 13, 32). Thus, the different lipid compositions explored here have a major effect on the acute toxicity of L-AmB to mammalian cells without affecting antifungal potency in a significant manner.

A third important point is the marked difference in the acute (2 hr) and chronic (1–2 day) toxic effects of L-AmB. Doses of L-AmB that are without effect on α-AMG uptake during 2 hr

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TABLE 3

Effects of AmB or L-AmB on dome formation

Nonconfluent cultures were incubated for 4 days. In the control cultures, dome formation began as the cultures reached confluence. Some of the cultures were exposed to the indicated levels of free AmB or L-AMB during the entire incubation period. Empty liposomes were used as a control for the L-AmB. The results are expressed as mean and standard deviation of the number of domes per field with at least 30 fields evaluated for each point.

[AmB]	Time of culture	Number of domes per field
μg/ml	days	
Free AmB		
0	0	3.0 ± 5
0	1	3.0 ± 5
0.5		1.5 ± 5
5		2.4 ± 5
0		5.6 ± 5
0.5	2	6.0 ± 6
5		1.3 ± 2
0		17.4 ± 16
0.5	3	26.8 ± 14
5		8.9 ± 6
0		51.2 ± 19
0.5	4	58.7 ± 18
5		13.5 ± 7
L-AmB (DMPC/DMPG)		
0	0	0
0		0
0.5	1	0
5		0
0		13.1 ± 8
0.5	2	7.7 ± 7
5		8.8 ± 5
0		38.2 ± 11
0.5	3	34.0 ± 17
5		9.6 ± 6

of drug exposure cause virtually complete inhibition during 48 hr exposure. The liposomal forms of AmB are stable and do not release free drug during 48 hr of incubation under the conditions that prevail in tissue culture; we have documented this both by chemical assay (Table 1) and biological assay (Table 2). This suggests that the chronic toxic effects of L-AmB are caused by a slow transfer of drug from the liposomes to the membranes of the LLCPK1 cells. The mechanism of this transfer process is not fully understood at present; it may or may not require direct contact between cells and liposomes, but it does seem to require that the cells and liposomes be in proximity in the same fluid environment. The biophysical basis for membrane to membrane transfer of polyene antibiotics and the role of membrane lipid composition in this process have been discussed previously (8).

The chronic toxic effects of L-AmB on differentiated functions of kidney epithelial cells may have important implications for the *in vivo* utilization of L-AmB formulations as agents for the therapy of systemic fungal disease. Antifungal therapy frequently requires multiple episodes of intravenous drug administration, often extending over many weeks (1, 12). This would result in prolonged intervals when host tissues would be in contact with L-AmB and thus, in view of our findings, present an opportunity for the expression of chronic toxicity. Offsetting this, the well known distribution characteristics of liposomes administered intravenously, involving accumulation by phagocytic reticuloendothelial cells (macrophages) in liver and spleen with much more modest uptake in the kidney (33), would tend to limit the close proximity between cells and L-AmB that seems to be required for toxicity. Thus, the observed lack of nephrotoxicity of certain formulations of L-AmB *in vivo*, even when given chronically (10–12), may be due to a slow rate of drug transfer between liposomes and cells that precludes acute toxicity during the initial circulation of the injected liposomes, followed by the accretion of the liposomal drug at sites in liver and spleen. There is good evidence (34) that macrophages can take up large amounts of L-AmB without suffering damage; these cells may also be able to inactivate the drug they have accumulated before releasing it. Thus, the enhanced therapeutic index of L-AmB, which is related to its reduced toxicity, may be due to a combination of factors including slow liposome to host cell transfer of drug and the accumulation of liposomes in less critical tissue sites.

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