Amphotericin B and Filipin Effects on L and HeLa Cells: Dose Response

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Amphotericin B (AmB) and filipin effects on L and HeLa cells were compared by monitoring drug-induced potassium leakage from cells, changes in radioactive uridine incorporation into cellular ribonucleic acid, protein leakage from cells, and cell viability. L cells were much more susceptible to both AmB and filipin than were HeLa cells, but the overall dose response was similar. For AmB, the various effects were easily separable. Potassium leakage occurred at the lowest concentrations of AmB and was reversible. Inhibition of uridine incorporation and loss of viability occurred at intermediate levels, and protein loss occurred at higher levels. In contrast, filipin was much more potent; its effects on potassium leakage were only minimally reversible, and the separation of the permeabilizing effects from complete cell lysis was possible only over a limited concentration range and for a short time.

Amphotericin B (AmB) belongs to the class of polyeone antibiotics, which bind to the sterol component of the membranes of all eucaryotic cells (6, 13). At sufficiently high concentrations, the polyeone antibiotics cause an increase in membrane permeability, with leakage of metabolites and macromolecules from the cell and resultant cell death. Previous studies with fungi and animal cells in culture have shown that at low concentrations AmB can induce nonlethal permeabilizing changes in cell membranes which enhance uptake of certain small molecules (16) and even macromolecules (9). These properties of AmB have been exploited to achieve the penetration into cells of pharmacologically active compounds (9, 12, 17) that do not penetrate the untreated cell membranes.

We have undertaken the present study to compare the nonlethal and lethal effects of AmB on cultured L and HeLa cells, and to determine under which conditions they can be separated and whether the cell damages can be repaired. Similar studies have also been performed with filipin to determine whether permeability changes induced by this polyeone antibiotic are separable from lethality.

MATERIALS AND METHODS

Chemicals. AmB in the form of Fungizone was purchased from E. R. Squibb and Sons, Inc., Princeton, N.J. It was dissolved in sterile water before use. The concentrations of the AmB solutions were calculated on the basis of the amounts of AmB in the Fungizone, as stated by the manufacturer. Filipin, in the form of the unfractonated antibiotic complex, which was 86% pure, was donated by Upjohn Co., Kalamazoo, Mich. The amounts noted refer to the crude product. Filipin was dissolved in methanol before use.

[5-3H]uridine (specific activity, 8 Ci/mmol) was purchased from Schwarz/Mann, Orangeburg, N.Y. L-[methyl-3H]methionine (specific activity, 5.0 mCi/0.071 mg) was purchased from the New England Nuclear Corp., Boston, Mass. Triton X-100 (Triton) was purchased from Packard Instrument Co., Downers Grove, Ill. Lithium dilution was purchased from Instrumental Laboratory, Inc., Lexington, Mass.

Cell culture. HeLa and L 929 cells were maintained and assayed in monolayer in Eagle minimum essential medium supplemented with 0.2 mM of nonessential amino acids, 2 mM glutamine, 10% fetal calf serum, 100 U of penicillin per ml and 100 μg of Kantrex per ml (medium).

Growth of cells. Cells were removed from a monolayer with 0.25% trypsin, seeded in 3 ml of medium at a density of 4.0 × 10⁵ cells per plastic 35-mm-diameter dish, and grown for 17 h. The medium was then removed, and the cells were rinsed once with 2 ml of fresh medium and incubated at 37°C with 1 ml of medium containing the antibiotic to be assayed. The incubation was terminated by aspiration of the medium, and the specific analyses were performed. The results were expressed as percentages of control values obtained for untreated cells.

Because Fungizone contains 41% sodium deoxycholate by weight, the control cells in the AmB experiments were treated with appropriate amounts of sodium deoxycholate. In the filipin experiments, all cells were treated with methanol at a final concentration of 0.2%.
Determinations of intracellular potassium. A standard curve was obtained for 0 to 15 μg of potassium in 4 ml of solution composed of 3.5 ml of lithium diluent, containing 15 meq of Li per liter, and 0.5 ml of 2% Triton. When these potassium concentrations were assayed with a flame photometer (model 143, Instrumentation Laboratory, Inc., Boston, Mass.), linear dependency of reading on potassium concentration was observed. All of our subsequent measurements were done in this concentration range.

Potassium leakage, determined by the amount of potassium remaining in cells after antibiotic treatment, was measured under two conditions. In the first, we determined the leakage of potassium from cells during the incubation with drug and before rinsing. After cells were incubated for 1 h with AmB or filipin, the medium was removed and the cells were extracted at room temperature with two 400-μl portions of 2% Triton. Of the combined extracts, 0.5 ml was mixed with 3.5 ml of lithium diluent and measured for potassium content. The difference between the potassium content in the extract of untreated, unrinse cells and the extract from untreated cells additionally rinsed three times with 2-ml portions of 0.15 M NaCl (saline) was the amount of potassium originating from the medium, and this value was subtracted from all results.

In the second part of this experiment, we determined the potassium content remaining in rinsed cells. After aspiration of antibiotic-containing medium, cells were rinsed at room temperature three times with 2-ml portions of saline, and then extracted and assayed as before.

Cell viability. To test the viability of cells after exposure to the antibiotics, the medium was removed and the cells were rinsed twice with 2 ml of Hanks solution and incubated for 10 min at 37°C with 0.1% trypsin blue. They were then removed from the monolayer with a rubber policeman and counted in a hemocytometer.

\[ ^{3}H \text{uridine incorporation into acid-insoluble pool (ribonucleic acid (RNA))} \]
To test changes in radioactive uridine incorporation, the cells were exposed to 0.5 μCi of ml \[^{3}H \text{uridine, and the incorporated radioactivity was measured as previously described (J. Kotler-Brajtburg, G. Medoff, G. S. Kobayashi, D. Schlessinger, and A. Atalach, Biochem. Pharmacol., in press).} \]

Methylation of RNA with radioactive methionine. Methylation of RNA with radioactive methionine was carried out according to Zimmerman and Holler (18). Cells were grown for 1 h with 0.5 μg of radioactive methionine per ml. The RNA was then extracted by a standard procedure (15).

Protein measurements. To measure the amount of protein remaining after incubation of the cells with antibiotics, cells were rinsed twice with Hanks solution, and the protein content was determined by a modified Folin method (14), with bovine serum albumin as standard.

RESULTS
Effect of AmB and filipin as a function of dose. Figure 1 shows dose response curves of L and HeLa cells to AmB and filipin as measured by effects on intracellular potassium content, radioactive uridine incorporation, cell viability, and intracellular protein content.

Potassium leakage from L or HeLa cells pretreated with AmB into potassium-free solution occurred at the lowest AmB concentrations employed. When the AmB concentration was increased, the other effects occurred in the same order in L (A) and HeLa (B) cells, but higher concentrations of AmB were required in the latter. The AmB concentrations (micrograms per milliliter) that caused a 50% decrease in each of the following indexes in L cells were: 4 (intracellular potassium content); 36 (radioactive uridine incorporation); 40 (cell viability); and 65 (intracellular protein content).

In HeLa cells, the corresponding values were 20, 56, 115 and 200 μg/ml. A higher concentration of AmB (20 μg/ml) was required to decrease K⁺ content by 50% in unrinse L cells (data not shown) than in rinsed cells.

The response of L (C⁻) and HeLa (D) cells to increasing filipin concentrations showed a different pattern. Below a concentration of 2 μg/ml, no filipin effect was observed. Above this value, potassium content in rinsed and non-rinsed cells (data not shown), protein content, radioactive uridine incorporation, and cell viability all fell at about the same rate. Although the decrease in potassium content in saline-rinsed cells occurred at lower antibiotic concentrations than other effects, the differences were not great and were only apparent in a narrow concentration range. Filipin at 4 μg/ml (for L cells) and 6 μg/ml (for HeLa cells) induced 50% loss of potassium, whereas the other effects were minimal at these concentrations. However, complete loss of potassium occurred at the same concentration as complete cell lysis (20 μg/ml).

Reversibility of potassium leakage. Potassium content was measured in cells preincubated with antibiotics and then washed free of the drugs and allowed to recover by incubation in antibiotic-free medium for a defined time. The cells were then harvested and rinsed with saline, and the potassium content was measured. Figure 2 shows the decrease in potassium content with different antibiotic concentrations, immediately after exposure and after different recovery times in antibiotic-free medium.

L cells preincubated with AmB (A) at a concentration inducing 90% potassium leakage recovered at 0.5 h and 1 h after exposure in fresh medium. They had lost, respectively, 30 and 10% of their content. HeLa cells incubated with AmB (B) at a concentration inducing 90% po-
tassium loss showed a decrease of only 20% from controls after a 1-h recovery period in fresh medium. L cells incubated with filipin (C) at a concentration inducing 55% potassium leakage still had 70% potassium loss after 1 h of recovery and 20% after 3 h. HeLa cells treated with filipin did not recover after incubation for 1 h in fresh medium (data not shown), and after 3 h there was only slight recovery (D).

Therefore, both L and HeLa cells could recover from the permeabilizing effects of both antibiotics. L cells recovered faster and more completely than HeLa, and the recovery occurred faster and over a wider concentration range when cells were pretreated with AmB than with filipin.

**Effects of time of exposure to the antibiotics.** The effect of the time of exposure of the cells to the antibiotic on changes in permeability to potassium was compared with the effect on incorporation of radioactive uridine (Fig. 3).

Cells preincubated for 8 min with 10 μg of AmB per ml and then rinsed with saline lost 50% of the potassium. After 30 min of incubation, practically all of the potassium had disappeared from the cells. In contrast, the changes in radioactive uridine incorporation induced by the same AmB concentration (10 μg/ml) were more complicated. Initially, an increase in incorporation of radioactivity occurred. An increase of 25% over control values was observed after 20 min, and a maximal increase of 45% was reached after 30 min of incubation with 10 μg of AmB per ml. After this, a gradual decrease in incorporated [3H]uridine followed. When cells were treated with AmB at 20 μg/ml, the increase in [3H]uridine incorporation had a similar pattern, but was present for a shorter time, and the subsequent decrease was more pronounced. The maximal increase in [3H]uridine incorporation observed at 10 and 20 μg of AmB per ml occurred at the same time as when cells were approaching maximal potassium loss. With AmB at 40 μg/ml, no initial increase was observed, and the fall in incorporated radioactivity that occurred later was very marked.

Filipin effects on potassium leakage and [3H]uridine incorporation as a function of incubation time are shown in Fig. 4. L (A) and HeLa (B) cells treated for 15 min with filipin at concentrations of 5 and 8 μg/ml, respectively, released 20% of their potassium. At this time, they incorporated 20 to 30% more radioactive
Fig. 2. Reversibility of the antibiotic-induced permeability to potassium. Cells, L (A and C) or HeLa (B and D), were preincubated for 0.5 h with increasing concentrations of AmB (A and B) or filipin (C and D); they were then rinsed with saline (●) immediately or after additional incubation in antibiotic-free medium for (○) 0.5 h, (△) 1 h, or (□) 3 h. Cells were then extracted with 2% Triton, and the potassium content in the extract was determined.

Fig. 3. Time-dependent AmB-induced decrease in (○) potassium content and in changes of uridine than control cells. As the time of incubation with filipin increased, there was a decrease in [3H]uridine incorporation.

AmB and filipin effects on pulse labeling of RNA. Table 1 presents the incorporation of radioactivity into RNA of L cells pulsed for 20 min with [3H]uridine. Incorporation increased with time of preincubation with AmB up to 2 h and was lower for 20 µg of AmB per ml than 10 µg/ml. The pulse experiments with L cells and filipin showed small increases over control (10%). HeLa cells preincubated 70 min with filipin and then pulsed for 10 min with radioactive uridine incorporated more radioactivity than control cells. The values were in percentages of control: 116% at 5 µg of filipin per ml and 125% at 10 µg of filipin per ml.

To gain a second measure of RNA synthesis independent of pool effects (5), L cells were
grown with radioactive methionine in the medium in the absence or in the presence of 10 μg of AmB per ml. The incubation was stopped after 15, 30, 45, or 60 min. The amount of radioactivity in RNA extracted from control cells, grown without AmB, increased linearly, whereas the radioactivity in RNA extracted from cells grown with AmB was equal to control values up to 30 min of incubation and then fell to 50% of controls.

When L cells preincubated for 1 h with 10 μg of AmB per ml were pulsed with radioactive methionine for 20 min, the radioactivity of extracted RNA was 74% of control values.

**DISCUSSION**

AmB and filipin effects on L and HeLa cells were compared by analyzing potassium leakage, radioactive uridine incorporation, cell viability, and protein leakage. The reversibility of potassium leakage from cells and the responses of potassium leakage and of radioactive uridine incorporation as functions of time were also measured. We found that the different effects of AmB occurred over a wide range of concentrations and that the filipin effects occurred at lower concentrations and over a narrower range.

The effects of AmB and filipin on cell permeability to potassium were reversible, but recovery was faster and more complete from the changes caused by AmB than by filipin. Cells with the highest AmB-induced permeability to potassium recovered completely, whereas filipin-induced permeability of HeLa cells was closely linked to cell destruction. Other investigators with different experimental systems have also observed the two properties of these polynye antibiotics we have noted: the stepwise reversible changes induced by AmB and the all-or-none irreversible effects of filipin (2–4, 7, 11).

A time study of AmB-induced effects on L cells demonstrated that, at low concentrations of the antibiotic, the change in cell permeability to potassium was correlated with an increase in radioactive uridine incorporation. The pulse-label experiments with radioactive uridine and radioactive methionine exclude the possibility that the increase in uridine incorporation resulted from antibiotic stimulation of RNA synthesis. The simplest explanation for our observation is that the greater permeability of cell membrane to uridine results in increased penetration of radioactive uridine into cells or the loss of pool uridine from cells or both. In both these events, the specific activity of the internal [3H]uridine pool would be higher in antibiotic-treated cells, and the radioactive uridine incorporated would be greater than in untreated cells. This increase in radioactivity incorporation was induced by both antibiotics, but the AmB effect was more pronounced and lasted longer.

On the basis of previous work (3, 4), we assume that the various indexes of antibiotic ac-
tion examined by us correspond to different degrees of cell membrane damage. For example, the potassium leakage into potassium-free solution reflects the least perturbing, reversible change in membrane permeability (10). These same changes can also be monitored as increases in radioactive uridine incorporation.

The inhibition of uridine incorporation probably reflects more serious membrane damage. This assumption is in line with work by others (1).

Cell viability was impaired at AmB concentrations equal to or slightly higher than that required to inhibit [3H]uridine incorporation, and finally, protein leakage from cells can be linked to the most severe cell damage equivalent to lysis.

This progressive spectrum of membrane effects was seen with AmB, but not with filipin. With filipin, the separation of the permeabilizing phase from an overall destructive phase was possible only for a short time of incubation and over a narrow range of concentrations.

In regard to clinical applicability of our findings, we conclude that because the toxic and permeabilizing effects of filipin occur over a narrow range, they are not easily separable; and in contrast to AmB, filipin will not be useful to potentiate the action of other pharmacologically active agents. However, it may be that other polyenes maximize the favorable permeabilizing effects without increasing the toxic effects. This would make them even more useful than AmB in potentiating the effects of second agents. It is worthwhile screening other polyene antibiotics for these properties by the methods described in this report.

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