ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 1984, p. 892–897
0066-4804/84/120892-06$02.00/0

Vol. 26, No. 6

Stimulatory, Permeabilizing, and Toxic Effects of Amphotericin B on L Cells

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Received 14 May 1984/Accepted 1 October 1984

High concentrations of amphotericin B (AmB) killed mouse L cells, but low concentrations increased plating efficiency and stimulated the incorporation of labeled precursors into DNA and RNA. Thus, there were two disparate effects of AmB on L cells, stimulatory and toxic, and they occurred in distinct dose-related stages. AmB also affected the permeability of L cells. In dose-response studies, increases in cell membrane permeability, measured as the loss of K^+ ions, occurred along with the stimulation of [^{3}H]uridine incorporation into RNA. In contrast, stimulation of [^{3}H]thymidine incorporation into DNA was only observed in cells recuperating from AmB-induced permeability changes. When the K^+ concentration in the medium was lowered to 0.5 from 4.5 mM, or when 1 mM ouabain was added to the cultures, cell killing was potentiated, but the stimulatory and permeabilizing effects of subtoxic concentrations of AmB were unaffected. Furthermore, etruscomycin, a polyene antibiotic without any permeabilizing effects, nevertheless induced an enhancement of plating efficiency and of incorporation of [^{3}H]uridine into RNA and [^{3}H]thymidine into DNA. Our results suggest that the dose-related stimulatory, permeabilizing, and toxic effects of AmB most probably have distinct mechanisms of action and may be independent of one another.

Amphotericin B (AmB) is a polyene antibiotic which binds to sterols in eucaryotic cell membranes, increasing membrane permeability and eventually leading to cell death (see reference 23 for a review). However, in addition to these effects, AmB has been shown to stimulate several cellular functions. Increases in DNA, RNA, and protein synthesis have been demonstrated in different cell types (7, 9, 16, 17). A link between stimulatory and ion-permeabilizing effects of AmB has been suggested (26), but polyenes which do not induce K^+ leakage from viable cells can potentiate the anticellular action of other agents (1, 2) and also enhance the plating efficiency of *Candida albicans* yeast cells (5).

We undertook the present study to learn more about the relationship between the stimulatory action of AmB and its toxic and permeabilizing effects.

(Part of the results have been presented at the 22nd Interscience Conference on Antimicrobial Agents and Chemotherapy [Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 12th, Miami Beach, Fla., 4–6 October abstr. no. 494, 1982].)

MATERIALS AND METHODS

Materials. AmB, in the form of Fungizone (E. R. Squibb & Sons, Princeton, N.J.), was used in our experiments and was dissolved in water just before use. Sodium deoxycholate alone, in concentrations equal to those in the Fungizone used in our experiments, had no effect on cells or the assays. Etruscomycin (lucensomycin) was donated by P. Barbierie (Farmitalia, Milan, Italy). It was dissolved in dimethyl sulfoxide (Me_2SO) just before use. The concentration of Me_2SO in all of our samples was 0.5% and this level had no effect on the cells or the assays.

Thymidine, ouabain octahydrate, and deoxycytidine were purchased from Sigma Chemical Co. (St. Louis, Mo.). [*methyl*-^{3}H]thymidine (specific activity, 20 Ci/mmol) and [^{3}H]uridine (specific activity 23.4 Ci/mmol), were purchased from New England Nuclear Corp. (Boston, Mass.).

Media. The growth medium was minimal essential medium prepared by the Center for Basic Cancer Research, Washington University School of Medicine. It was supplemented with 10% fetal calf serum, 100 U of penicillin per ml and 100 μg of streptomycin per ml. The concentration of K^+ in growth medium was 4.5 mM. Modified culture media were used in some experiments. In the experiments for determining the effects of low extracellular K^+, minimal essential medium containing 0.5 mM K^+ supplemented with serum and antibiotics was used. NaCl (4.0 mM) was added to this medium to replace the K^+. Growth medium was supplemented with 1 mM ouabain in some experiments.

Cell culture. Mouse fibroblast cells, L929, were maintained in monolayer in growth medium; unless otherwise stated, all of the experiments were done in the same medium. Cells were cultured and assayed in a humidified atmosphere of 5% CO_2–95% air at 37°C.

Cell synchronization. All of our experiments were done on synchronized cells. The technique we used involved synchronization at the G_1-S border and was achieved by a double-thymidine block with a scheduling protocol identical to that used by Glassy and Furlong (11). Cells were synchronized in plastic flasks (area, 25 cm^2) in plastic dishes (diameter, 35 mm) with cover slips and in glass scintillation counting vials (purchased from Research Products, Mt. Prospect, Ill.).

The cells were seeded 2 days before beginning the synchronization procedure; the initial inoculum was 1×10^6 cells per flask or 5×10^4 cells per plastic dish or vial. At the start of synchronization, the growth medium was removed, and medium containing 2 mM thymidine was added (5 ml in flasks or 2 ml in plastic dishes or vials). After 16 h of incubation, the thymidine-containing medium was replaced

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by medium containing 10 μM deoxycytidine. After 8 h, fresh medium containing 2 mM thymidine was added for a second 16-h period. At the end of this period, the monolayer was ca. 70% confluent with 95 to 98% cell viability (as determined by trypan blue exclusion). The degree of synchrony was assessed by the pattern of [^{3}H]thymidine incorporation into DNA. Cells were released from the block by replacing the thymidine-containing medium with fresh medium, and they were then used in the experiments.

[^{3}H]thymidine or [^{3}H]uridine incorporation into acid-insoluble pools (DNA or RNA) and [^{3}H]uridine uptake into whole cells. Cells were grown and synchronized in scintillation vials. At the end of synchronization, there were 8.0×10^5 to 1×10^6 cells per vial, and the protein content per vial was 80 to 100 μg. The technique for the growth of cells was that of Fujimoto et al. (10). Measurements of the incorporation of radioactive precursors into acid-insoluble pools by cells were performed as described previously (27). [^{3}H]thymidine or [^{3}H]uridine was added at 0.5 μCi in 1 ml of medium per vial. The uptake of [^{3}H]uridine into the acid-soluble pool was measured in cells rinsed three times with cold 0.1 M $MgCl_2$.

Measurements of plating efficiency. Cells synchronized in plastic flasks were released from thymidine block and removed from the flasks by using a cell scraper and dispersed with a syringe with an 18-gauge needle. Cell dispersions in medium containing 5×10^5 cells per ml were incubated with AmB or etruscomycin for 1 h with occasional mixing. After 1 h, 5-μl portions were removed and diluted with growth medium; 5-ml samples containing 200 cells were then seeded in plastic dishes (diameter, 60 mm). The cells seeded on control plates were untreated or treated with concentrations of deoxycholate equivalent to those introduced with Fungizone or with 5 μl of Me_2SO. In addition, because the highest concentration of antibiotics transferred with the cells from suspensions to culture dishes was 16 ng of AmB per dish or 2 ng of etruscomycin per dish, some control dishes received these concentrations of antibiotics. After 8 days, macroscopic colonies were stained with Giemsa and counted. The plating efficiency was 30 to 35%. The cells in the remaining 995-μl portions were used for K^+ measurements, and there were no differences between untreated cells and any of the controls.

Autoradiography. Synchronized cells grown on cover slips were exposed to either 1.2 or 2.4 μg of AmB in 1 ml of medium for 1 h. The drug was removed, and the cells were rinsed and exposed to 1 ml of medium containing 0.5 μCi of [^{3}H]thymidine. After incubation at 37°C for 2 h in the presence of the labeled precursor, the cells were rinsed several times and fixed in alcohol-acetic acid (3:1) for 15 min. The cover slips were washed, dried, and mounted on slides with Permount. The slides were dipped in Kodak NTB 3 nuclear track emulsion which was diluted 1:1 with warm distilled water. After exposure for various lengths of time (1 to 3 weeks), the slides were developed by standard procedures. Counts of labeled cells were made on ca. 200 to 300 cells.

Cell viability. At the end of the incubation period, the medium was removed by suction in experiments with monolayers or by centrifugation for 5 min at $600 \times g$ in experiments done on cell dispersions. Cells were then rinsed three times with 0.1 M $MgCl_2$. The viability of cells was assayed by exclusion of 0.1% trypan blue solution in saline as described earlier (19); however, cells in monolayer were assayed under an inverted microscope, whereas cells in dispersion were counted in a hemacytometer. The cells in monolayer treated with high AmB concentrations had a tendency to round up and to detach from the monolayer. Protein content was therefore monitored by the Folin method (21) to assess the degree of separation of cells from the monolayer. If protein remained constant, then no detachment had occurred. Both the protein measurements and trypan blue exclusion were used to assess viability.

Cell-associated K^+. At the end of the incubation period, the medium was removed, and the cells were rinsed three times with 1-ml portions of 0.1 M $MgCl_2$, and 0.6 ml of 15 mM $LiNO_3$ (lithium internal standard; Fisher Scientific Co., Pittsburgh, Pa.) was added. The samples were boiled for 5 min, followed by cooling to 24°C. The K^+ content in these rinsed cells was measured with a flame photometer (model 30; Corning Glass Works, Corning, N.Y.) as described before (18).

RESULTS

Mouse fibroblasts have been studied because of evidence that AmB can stimulate DNA and RNA synthesis in this cell type (16). The cultures were synchronized because of the recent demonstration that cell sensitivity to AmB was dependent on the stage of cell cycle and was especially pronounced at the beginning of S phase (20).

Effect of AmB on cell viability, cell membrane permeability, and incorporation of [^{3}H]uridine and [^{3}H]thymidine into intracellular acid-insoluble pools. Cells were released from the synchronizing thymidine block and overlaid with fresh medium; after 1 h, increasing doses of AmB were added to replicate cultures for 1 h. Cell viability, cell membrane permeability as measured by K^+ retention after the cells were rinsed, and incorporation of radioactivity were then determined (Fig. 1). Levels of AmB up to 0.6 μg/ml did not significantly affect the retention of K^+ compared with that of untreated cells. The retention of K^+ progressively decreased from 80 to 20% in the control cells as the concentration of AmB was increased to 20.0 from 1.2 μg/ml. It is important to note that we determined K^+ retention in from rinsed cells as a measure of cell membrane permeability. K^+ levels in

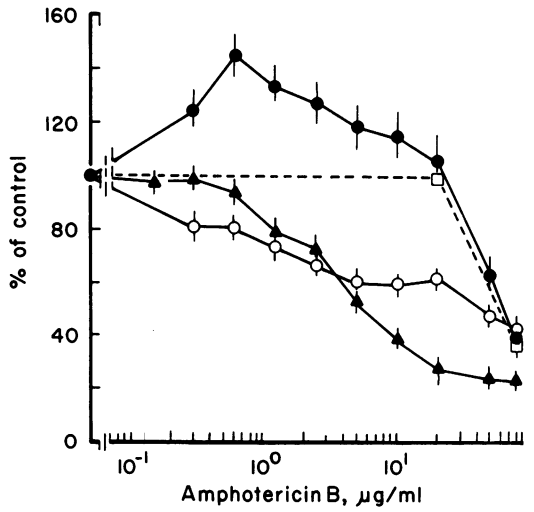

FIG. 1. Effects of incubation of cells with AmB on cell viability (--□--), retention of K^+ after rinsing of cells (▲), and incorporation of [^{3}H]uridine (●) or [^{3}H]thymidine (○) into nucleic acids. Results are expressed as percentage of the value found in untreated cells. All points are means ± standard error of four independent experiments.

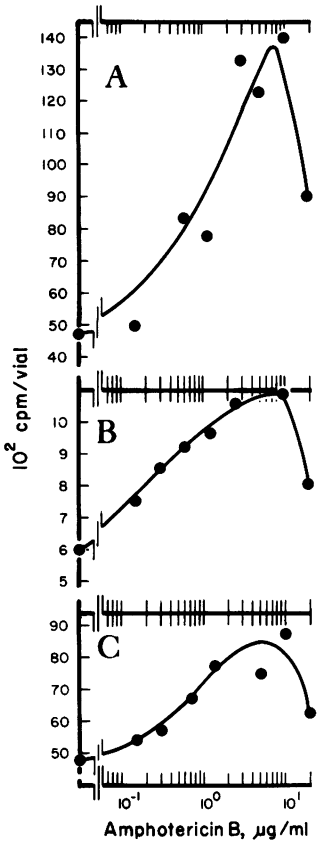

FIG. 2. Effect of incubation of cells with AmB on [^{3}H]uridine uptake into whole cells or incorporation into RNA. Cells in growth medium were incubated for 1 h at 17°C with AmB and [^{3}H]uridine; (A) [^{3}H]uridine uptake into whole cells; (B) [^{3}H]uridine incorporation into RNA. Cells were incubated for 1 h at 17°C with [^{3}H]uridine, rinsed three times with medium, and incubated 1 h at 37°C with AmB; (C) [^{3}H]uridine incorporation into RNA was then measured.

AmB-treated cells before rinsing are substantially higher (17). AmB at concentrations up to 20 μg/ml did not decrease cell viability.

Cells exposed to [^{3}H]uridine during incubations with 0.3 to 10.0 μg of AmB per ml incorporated more radioactivity than did untreated cells. The peak values for incorporation were about 140% of control levels and occurred in cells exposed to 0.6 μg of AmB per ml. The stimulatory effect of AmB on [^{3}H]uridine incorporation under these conditions contrasted with its effect on [^{3}H]thymidine incorporation into DNA, which was inhibited at all concentrations assayed.

Dissociation of [^{3}H]uridine uptake from incorporation into RNA. In an attempt to distinguish AmB-induced RNA synthesis from an increase in the intracellular pools of radioactive uridine, we adapted the approach of Plagemann and Richey (25), who dissociated [^{3}H]uridine uptake at 17°C from subsequent incorporation into RNA at 37°C. One of three typical experiments is depicted in Fig. 2. When cells were exposed to [^{3}H]uridine at 17°C (Fig. 2A), concentrations of AmB from 0.15 to 10.0 μg/ml increased uptake up to 135% of the control values (no AmB). In other experiments the uptake varied from 130 to 170% of the controls. When counts of acid-insoluble material (Fig. 2B) were compared with the counts of acid-soluble material (Fig. 2A), it can be seen that at 17°C, less than 10% of the total radioactivity taken up was in RNA. Thus, more than 90% of the labeled uridine was in the acid-soluble pools.

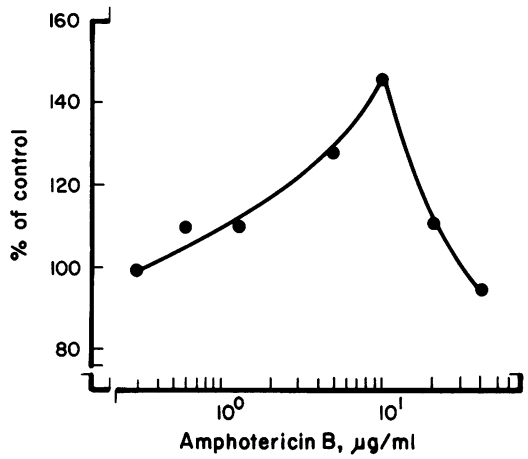

FIG. 3. Effect of preincubation of cells in dispersion with AmB on subsequent formation of colonies in AmB-free medium. The figure presents one of six similar experiments.

Cells loaded with [^{3}H]uridine and rinsed free of extracellular [^{3}H]uridine with medium maintained the level of their intracellular acid-soluble radioactive pool. When these cells were subsequently incubated at 37°C, they incorporated the radioactive components present in the acid-soluble pool into RNA. Cells loaded with [^{3}H]uridine at 17°C, rinsed, and then incubated for 1 h at 37°C in the presence of AmB formed more radioactive RNA then did cells incubated without AmB (Fig. 2C). Therefore, AmB at concentrations which induce a pronounced increase in membrane permeability, reflected by K^+ depletion in washed cells, caused cells to take up more [^{3}H]uridine (Fig. 2A) and also to make more radioactive RNA (Fig. 2C).

Effect of AmB on plating efficiency. When cells in monolayer were treated with AmB and then freed from the surface with a cell scraper, cell damage occurred. Effects on colony formation were studied with cells in suspension culture to avoid the damage incurred when cells were scraped from the plates. Incubation for 1 h with AmB at concentrations of 0.3 to 1.2 μg/ml did not affect cellular K^+ levels, whereas incubation with 2.5 to 20 μg of AmB per ml resulted in a decrease in K^+ retention (from 70 to 28% of control values; data not shown). Under these conditions, all cells remained viable, and cells preincubated with subtoxic concentrations of AmB subsequently formed more colonies than did untreated cells. One typical experiment in which the maximal increase (45% over control) was seen with cells preincubated with 10.0 μg of AmB per ml is shown in Fig. 3. In other experiments, the maximal increase varied from 20 to 65% over control values in cells preincubated with 0.3 to 5 μg of AmB per ml. Thus, treatment with AmB increased plating efficiency by some unknown mechanism. This result is similar to that seen previously with yeast cells (5).

Effects of preincubation with AmB on cells in growth media with or without added ouabain. Although [^{3}H]thymidine incorporation into acid-insoluble fractions of cells was inhibited in the presence of AmB, thymidine incorporation was increased in cells washed free of AmB and allowed to grow in AmB-free medium (Fig. 4A). However, when cells released from thymidine block were incubated for 1 h with 1.2 or 2.4 μg of AmB per ml or without AmB, rinsed, and then exposed to [^{3}H]thymidine, the percentage of labeled cells as measured by autoradiography was the same (83% of the cells were labeled in both control and AmB-treated cells).

The intracellular level of K^+ has been proposed as an important factor in the response of cells to polyene antibiotics (22). Intracellular levels of K^+ can be lowered by incubating cells in medium deprived of K^+ (24) or by treating the cells with ouabain, an inhibitor of Na^+ K^+ ATPase (8). An added incentive for measuring the effects of ouabain on AmB sensitivity was the proposal that the AmB-induced increase in [^{3}H]thymidine incorporation into a quiescent culture of 3T3 cells might be due to stimulation of Na^+ K^+ ATPase (26).

We compared AmB effects on K^+ levels and incorporation of labeled precursors of RNA and DNA by cells incubated in three different media: growth medium, medium with a lowered K^+ concentration, and medium with added ouabain. At the start of our assays, cells incubated in low K^+ medium contained 85 to 95% of the K^+ found in cells in growth medium and cells in medium with ouabain had 65 to 75% of the level in growth medium. These levels were unchanged in cells not treated with AmB over the course of the assays.

Incorporation of [^{3}H]uridine or [^{3}H]thymidine into acid-insoluble material was similar for untreated cells labeled in growth medium, in medium with low K^+ or medium with ouabain. However, cell sensitivity to the toxic effects of AmB was increased in the experimental media compared with that in growth medium; the threshhold AmB concentrations for cell detachment and loss of viability were 20 μg/ml for cells in growth medium (Fig. 4A) compared with 5.0 μg/ml for cells grown in low-K^+ medium (Fig. 4B), and 1.2 μg/ml in medium containing ouabain (Fig. 4C).

In low-K^+ (Fig. 4B) and growth medium (Fig. 4A), permeability as measured by the decrease in retention of K^+ in subtoxic AmB concentrations was similar. During a 3-h recovery period, cellular K^+ returned to the values found in untreated cells. As observed above, cells preincubated with subtoxic AmB concentrations also incorporated more [^{3}H]uridine and [^{3}H]thymidine than did untreated cells.

In medium containing ouabain (Fig. 4C), the effect of subtoxic AmB concentrations on K^+ retention was also comparable to the effect in growth medium. Subtoxic concentrations of AmB stimulated incorporation of [^{3}H]uridine and [^{3}H]thymidine to a similar extent as in the other two media.

Thus, cells placed in medium with low K^+ concentrations or in medium with ouabain were more sensitive to AmB toxicity than cells in normal growth medium. In contrast, permeabilizing and stimulatory effects of subtoxic AmB concentrations were comparable to those seen in growth medium.

Comparative effects of etruscomycin. Another way to look for possible linkage between ion flux and stimulation and toxicity was to test etruscomycin, a tetraene which like AmB contains a carboxyl group and a mycosamine function. Unlike AmB, this polyene has no effect on cell membrane permeability apart from cell lysis or killing (18).

Incubation of cell monolayers for 1 h with etruscomycin at concentrations of up to 2.5 μg/ml did not affect the retention of K^+ or cell viability. Concentrations ranging from 5.0 to 20 μg/ml induced an abrupt parallel decrease in the content of cell-associated K^+ (Fig. 5) and in the number of viable cells adherent to the glass (data not shown). Thus, no K^+ leakage, and therefore no increase in cell membrane permeability, was observed in viable cells, and the decrease in K^+ retention by the cells at the end of an incubation with etruscomycin and after 3 h of incubation in etruscomycin-free medium was the same. Preincubation of cells with subtoxic concentrations (0.15 to 2.5 μg/ml) of etruscomycin nevertheless induced increases in [^{3}H]uridine incorporation into RNA and [^{3}H]thymidine incorporation into DNA. The extent of stimulation by etruscomycin was similar to that observed when AmB was used.

For measurements of etruscomycin effects on plating efficiency, cells were transferred from monolayers into suspension. When cells in suspension were treated with etruscomycin, the decrease in cell viability and K^+ retention showed a dose response similar to that in monolayers. Cells incubated 1 h with subtoxic concentrations of etruscomycin (lower than 2.5 μg/ml) and then plated and cultured for 8 days in growth medium formed more colonies than untreated cells. In a representative experiment done in triplicate, cells treated with 0.6 or 1.2 μg of etruscomycin per ml added to 5

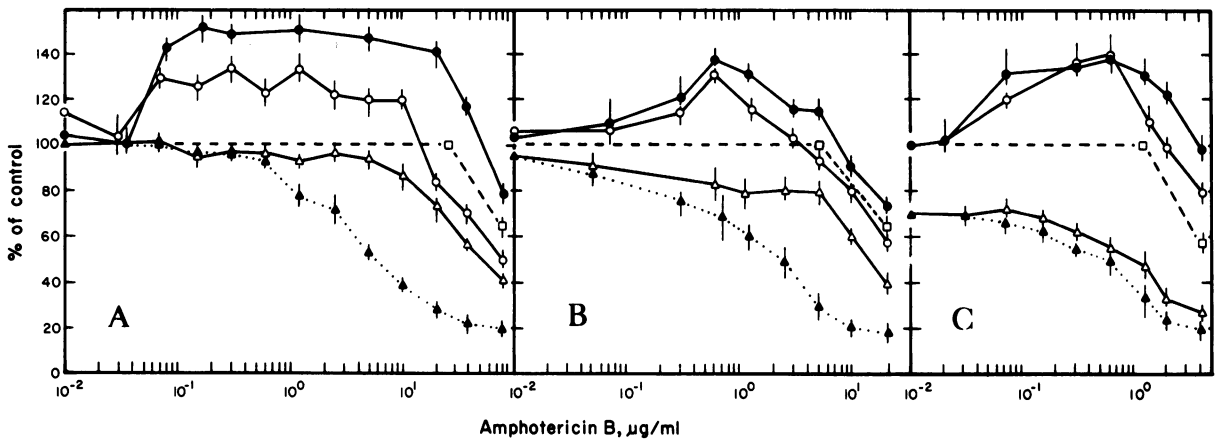

FIG. 4. Effects of preincubation of cells with AmB on subsequent incorporation of radioactive precursors into RNA and DNA and intracellular K^+ content of rinsed cells. Cells in monolayers were released from second thymidine block and overlaid with (A) growth medium, (B) medium with 0.5 mM K^+, or (C) medium with 1 mM ouabain. AmB was added and after 1 h of incubation, cell viability (---□---) and K^+ retention (▲) were measured in some cultures. Other cultures were rinsed three times with the appropriate medium and incubated in the same medium for 3 h. Cells assigned for measurements of radioactivity incorporation into nucleic acids received [^{3}H]thymidine or [^{3}H]uridine. At the end of incubation, retention of K^+ by cells (△), incorporation of [^{3}H]uridine into RNA (●), and incorporation of [^{3}H]thymidine into DNA (○) were measured. Results are expressed as percentage of values found in AmB-untreated cells, incubated in growth medium. All points are means ± the standard error of four independent experiments.

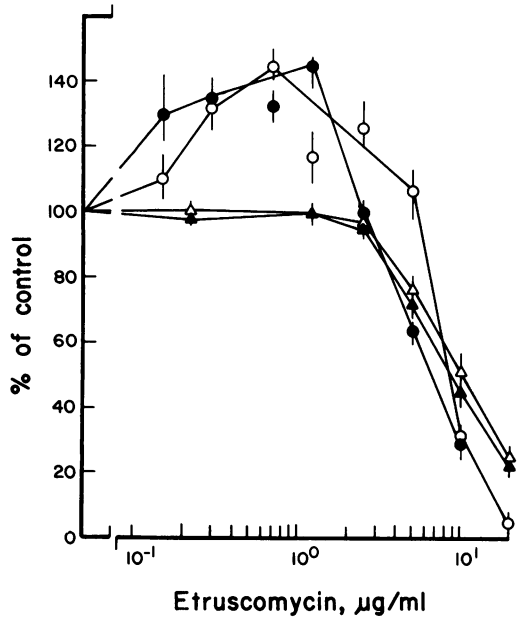

FIG. 5. Effects of preincubation of cells with etruscomycin on subsequent incorporation of [^{3}H]uridine into RNA, [^{3}H]thymidine into DNA, and intracellular K^+ content of rinsed cells. Cells were incubated for 1 h with etruscomycin. One group of samples was then used for measurements of K^+ retention (▲). The remaining cells were rinsed with medium and incubated in fresh growth medium without etruscomycin for 3 h, with [^{3}H]thymidine (○) or with [^{3}H]uridine (●) or without any radioactive precursors. After 3 h, the assays for K^+ (△) retention and incorporation of radioactivity were performed. Results are expressed as percentage of values found in untreated cells. All points are means ± standard error from four independent experiments.

μl of Me_2SO formed, respectively, 39 and 28% more colonies than controls. Thus, the effect of etruscomycin on plating efficiency was similar to that of AmB.

DISCUSSION

Three kinds of effects of AmB on synchronized L cells have been distinguished: (i) stimulatory, measured as an increase in incorporation of [^{3}H]thymidine or [^{3}H]uridine into nucleic acids or as an increase in plating efficiency; (ii) permeabilizing, measured as a decrease in K^+ retention after rinsing viable cells; and (iii) toxic, measured as a decrease in cell viability. In all cases, the stimulatory effects occurred at distinctly lower AmB concentrations than did the toxic effects.

The increase in [^{3}H]uridine uptake into cells could be dissociated from RNA synthesis by using incubation temperatures of 17 and 37°C (25). We were thus able to document that AmB potentiated both uptake of [^{3}H]uridine into the acid-soluble cellular pool and formation of radioactive RNA. The increase in the production of radioactive RNA probably reflected an increase in RNA synthesis. This explanation is in line with previous experiments and considerations by others (16). However, it is not possible to exclude other ad hoc more complicated interpretations of our data because we have not measured the specific activity of internal pools of [^{3}H]uridine. Since almost all cells in the culture were actively dividing, the increased uptake of [^{3}H]thymidine into DNA after AmB exposure could be due to an accelerated S phase, increased repair synthesis of DNA, or an increased specific activity of the thymidine pool.

Using fibroblast cells starved of serum, Rozengurt and Mendoza (26) tried to relate stimulation of DNA synthesis to the permeabilizing properties of AmB. On the basis of a similarity in dose-response between AmB-induced stimulation of DNA synthesis in density-inhibited fibroblast cells and monovalent cation fluxes, they hypothesized that changes in cellular concentrations of monovalent cations affect DNA synthesis, probably through stimulation of ATPase (26). This concept is in line with the finding that only polyene antibiotics capable of inducing permeability changes are B cell mitogens (12, 13) and are able to stimulate tumoricidal activity of macrophages (6). However, we cannot explain all of our findings on this basis. Increased RNA synthesis occurred in the presence of AmB concentrations which caused a significant increase in cell membrane permeability, but the stimulation of DNA synthesis was found only in almost completely recuperated cells. Furthermore, the effects were clearly separable with etruscomycin; there was no increase in cell membrane permeability and K^+ leakage at levels below those that cause cell death, but subtoxic concentrations of this polyene nevertheless stimulated RNA and DNA synthesis and increased plating efficiency. Therefore, it seems reasonable to assume that the stimulatory action of AmB does not depend on its permeabilizing effect and may have a different mechanism.

The stimulatory and permeabilizing effects were also separable from toxic effects. Low-K^+ concentrations or added ouabain did not affect the stimulation of RNA or DNA synthesis or increase in cell membrane permeability by subtoxic concentrations of AmB, but intensified its toxic action. In growth medium, the cells remained viable and were able to recover from the damage induced by up to 20.0 μg of AmB per ml. In low-K^+ medium, the cells were unable to recover from the damage induced by AmB levels of greater than 5 μg/ml. The ability to recover was impaired even more by the addition of ouabain; in that case, AmB was toxic above 1.2 μg/ml. This analysis of our results is in line with the conclusions of Malewicz et al. (22), who found that the repair of polyene antibiotic-induced damage was dependent upon the concentration of the antibiotic and the K^+ concentration in the medium.

The apparent separation of mechanisms of the permeabilizing and toxic effects of AmB is in agreement with our previous finding (4, 18) and with the work of Hsu-Chen and Feingold (15). These workers derived mutants of *C. albicans* which were resistant to the lytic action of AmB but retained normal levels of susceptibility to the permeabilizing effects of the drug.

The notion of phased biological effects of AmB is consistent with dose-response studies of the physical state of artificial membranes. Analysis of AmB interactions with phospholipid and sterol-phospholipid vesicles by circular dichroism has demonstrated various AmB species or conformers (3, 28). Their appearance can be ascribed to various AmB-membrane interactions starting from adsorption at membrane surfaces (first conformer), through permeabilizing changes (second and third conformers), to membrane disruption.

The stimulatory effects may be especially distinct when they result from interactions of polyenes with nonsterol membrane components. Such an interaction has been postulated in a recent report describing the stimulation of lymphocytes by polyene antibiotics (14). In support of this notion, we have recently found (unpublished data) that AmB treatment of bacteria (which contain no sterols in their cell membranes) leads to no toxic effects even at higher concen-

trations, but again provokes an increase in colony forming ability.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants AI 16228, CA 15665, AI 07015, and AI 07172 from the National Institutes of Health.

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