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Repair of Membrane Alterations Induced in Baby Hamster Kidney Cells by Polyene Macrolide Antibiotics

BARBARA MALEWICZ,[1] HOWARD M. JENKIN,[1]* AND EDWARD BOROWSKI[2]

The Hormel Institute, University of Minnesota, Austin, Minnesota 55912,[1] and Department of Pharmaceutical Technology and Biochemistry, Technical University, Gdansk, Poland[2]

We studied the correlation between chemical characteristics of 13 polyene macrolide antibiotics and the ability to repair the membrane permeability changes induced by polyenes in BHK-21 cells grown in shaker culture. It had been demonstrated that large-macrolide-ring polyenes with rigid molecules (heptaenes) induced specific membrane permeability pathways which were repaired by the eucaryotic cells under the proper conditions. The influence of environmental conditions on the repair process was examined. Aureofacin trimethylammonium methyl ester derivative was used as a selected representative of polyene macrolides inducing specific pathways. The factors influencing the repair process, monitored by measuring the ability of BHK-21 cells to control K^+ membrane transport, were examined during and after cell contact with the antibiotic. We found that the repair process was dependent upon the temperature, the concentration of the antibiotic, time of its contact with cells, potassium concentration in the medium, and availability of an energy source. The repair process occurred in the presence of cycloheximide, which inhibited protein synthesis in BHK-21 cells. Results showed that the repair process plays an important role in mammalian cell recovery from the toxic effects of polyenes.

The repair process of plasma membrane alterations induced by polyene macrolide antibiotics was discovered by Borowski and Cybulska during their study of the effect of *N*-succinylperimycin on *Saccharomyces cerevisiae* (3). This observation was supported later by other investigators using *Chlorella vulgaris* (2, 4, 17), *Candida albicans* (5), *Histoplasma capsulatum* (1), and animal cells grown in vitro (13, 19). However, the nature of the repair of membrane alterations, as well as essential factors influencing this process, remains unknown.

There is a relationship between the chemical structure of polyenes and the ability of cells to repair polyene-induced membrane changes (4, 17, 18; B. Malewicz and E. Borowski, J. Gen. Microbiol., in press). An appropriate concentration of potassium in the medium is necessary for the repair of polyene-induced membrane alterations (4, 17), although the cells are able by stimulation of K^+ membrane active transport to compensate for K^+ loss through polyene-induced pathways (2, 4, 17). However, the availability of metabolic energy and the high activity of K^+-Na^+ membrane adenosine triphosphatase is required to observe the repair of impaired membranes (2, 4). During the repair process the active transport of metabolites other than K^+ is stopped, but the facilitated diffusion of metabolites into polyene-treated *Chlorella vulgaris* cells occurs at the same rate as into untreated cells (4, 17).

The main pharmacological utility of polyenes is based primarily on the quantitative differences in the affinity of these antibiotics for ergosterol and cholesterol in fungal (pathogen) and mammalian (host) cell membranes, respectively (11); therefore, polyene macrolide treatment affects the pathogen as well as the host. It is important to determine and find differential conditions in which host cells can recover from polyene treatment while pathogenic organisms are inhibited or killed. Fisher and co-workers implicate the repair process as a potential mediator of polyene toxicity (8, 9).

The repair process has not been extensively studied in mammalian cells. Our previous studies (19) showed that baby hamster kidney cells (BHK-21) grown in shaker culture were able to repair membrane alterations induced by amphotericin B and aureofacin and their *N*-glycosyl (NG) and trimethylammonium methyl ester (DMS) derivatives (6, 7). The repair process was observed in a wide range of concentrations of these antibiotics, lower, however, than cytostatic concentrations (19). Kotler-Brajtburg et al. (13) observed the reversibility of K^+ leakage from HeLa cells after amphotericin B treatment in a wide range of polyene concentrations, whereas filipin-treated cells showed reversibility shortly

after treatment and only in very low concentrations of the antibiotic (13).

As a continuation of our previous work (19), this study was undertaken to establish the correlation between the structural characteristics of polyene macrolides and the repair process and to determine factors influencing the repair process, which can lead to an understanding of the nature and the mechanism of this process in mammalian cells. Thirteen antibiotics representing different structural groups of polyene macrolides inducing various types of membrane effects (4) were used. To establish environmental conditions influencing the repair process, DMS-aureofacin was chosen because of its very high selectivity in inducing membrane alterations, enabling full cell recovery, very high biological activity, and very good water solubility (7, 19).

MATERIALS AND METHODS

Cells and media. BHK-21 cells adapted to growth in shaker culture in serum-free medium (10) were used. Cells were incubated at 37°C in a New Brunswick gyratory shaker at 110 rpm and subcultured every 48 to 72 h, using 2.5×10^7 cells per 50-ml medium sample as a starting population. Growth medium consisted of Waymouth 752/1 medium supplemented with 0.2% fatty acid-free bovine serum albumin (Miles Laboratoreis, Inc., Elkhart, Ind.) and buffered with 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (10) at pH 7.4.

Growth medium supplemented with K^+ to 37 mM was prepared as follows. Waymouth 752/1 medium (K. C. Biologicals, Inc., Lexana, Kan.) purchased as a dry powder without sodium bicarbonate, was dissolved in water, and 10 mM $KHCO_3$ (1 g/liter) was added. The medium was sterilized by filtration through a 0.45-μm Nuclepore membrane. After supplementation of the medium with 0.2% fatty acid-free bovine serum albumin (Miles Laboratories) and 20 mM HEPES buffer, the pH was adjusted to 7.4 with sterile 2 M $KHCO_3$. The final concentration of K^+ in the medium was calculated from the amount of $KHCO_3$ added and total concentration of potassium salts present in the Waymouth medium.

The maintenance medium contained: 5 mM D-glucose, 150 mM NaCl, 0.078 mM $MgSO_4 \cdot 7H_2O$, and 0.5 mM $NaH_2PO_4 \cdot 2H_2O$, pH 7.4. The medium was stored at 4°C.

Intracellular K^+ measurements. Duplicate 2-ml samples of the cell suspension containing from 1×10^6 to 3×10^6 cells were centrifuged 10 min at $250 \times g$ at 25 to 37°C. The supernatant fluid was removed as completely as possible with a Pasteur pipette, and the cells in the pellet were washed generally once with K^+-free maintenance medium. Double washing was required only when the cell suspension contained 37 mM K^+. After the washing and cell sedimentation was completed, the supernatant fluid was removed as above, and the cells were suspended in 4 ml of double-glass-distilled water. Samples were incubated for 1 h at 37°C or kept overnight at room temperature to release all intracellular K^+. After separation of the cell debris by centrifugation for 15 min at $250 \times g$, the K^+ concentration in the supernatant fluid was measured by atomic absorption spectrophotometry (AA 575 series, Varian Techtron Pty Ltd., Springvale, Austria). All conditions in the above procedure were established so that normal BHK-21 cells did not release any K^+ until they were suspended in distilled water. BHK-21 cells grown in shaker culture in serum-free medium are fragile and easily destroyed when not properly handled. The critical conditions for the accuracy of this assay were: pH, ionic strength of the media, speed of cell centrifugation, temperature used during the assay, and temperature of the media used while suspending the cells.

Polyene antibiotics. Aureofacin, candidin, lienomycin, and rimocidin were isolated and purified in the Department of Pharmaceutical Technology and Biochemistry of Technical University (Gdansk, Poland). Amphotericin B and nystatin were obtained from E. R. Squibb & Sons, Inc. (Princeton, N.J.). Filipin was a gift from J. E. Grady, The Upjohn Co. (Kalamazoo, Mich.). Pimaricin was obtained from Mycofarm (Delft, Holland). NG and DMS derivatives of polyene macrolides were prepared according to the procedures of Falkowski et al. (6, 7). The stock solutions of native polyenes were prepared in dimethyl sulfoxide and diluted with growth medium to obtain working solutions of the antibiotics. Final concentration of dimethyl sulfoxide in the cell suspensions did not exceed 0.5%, a concentration which did not influence BHK-21 cell growth nor change permeability of the cell membrane as measured by intracellular K^+. NG and DMS derivatives of polyenes were dissolved in water and further diluted in the medium. All antibiotic solutions were prepared just before use. All concentrations given for DMS-aureofacin were calculated as 100% pure compound. The purity of the DMS-aureofacin (75 to 80%) was determined on the basis of $E^{1\%}_{1\,cm}$ at 379 nm. The remaining 20 to 25% consisted of biologically inactive products from polyene decomposition. DMS-aureofacin stock solution at a concentration of 20 μg/ml was stable for about 1 week when kept at 4°C in darkness (B. Malewicz, H. M. Jenkin, and E. Borowski, unpublished data).

Inactivation of DMS-aureofacin at 37°C. Changes in the biological activity of DMS-aureofacin incubated in growth medium at 37°C were monitored by determining K^+ leakage from the cells. DMS-aureofacin was dissolved at concentrations of 0.1 and 1 μg/ml at 20°C. One set of samples was quickly warmed and stored at 37°C; a second set of samples was quickly chilled and kept at 4°C in the dark. Growth medium without antibiotic was also stored under the same conditions and used as control samples. At different time intervals during incubation, the biological activity of all antibiotic solutions was measured as follows. BHK-21 cells were suspended in the antibiotic solutions (10^6 cells/ml) and incubated at 37°C for 30 min. After incubation, cells were centrifuged (5 min at $500 \times g$) and intracellular K^+ was determined as described above. The amount of K^+ lost by free diffusion from cells incubated in medium with polyene was deter-

mined as described above. The experimental and control results were compared with the amount of K^+ released from cells by a freshly made solution of antibiotic, which expressed the 100% biological activity of polyene.

Measurement of the cell ability to control K^+ membrane transport. BHK-21 cells were suspended at a density of 5×10^5 cells/ml in 50 ml of growth medium; 0.5 ml of properly diluted DMS-aureofacin stock solution was added to obtain the desired amount of antibiotic (calculated as 100% pure compound). Three samples were prepared for each concentration of antibiotic. During cell incubation in the gyratory shaker at 37°C, intracellular K^+ was monitored as described above. The differences in K^+ concentrations obtained for polyene-treated cells and control cells showed the amount of K^+ lost from the cells by free diffusion. The ratio of the K^+ content in treated and control cells expressed the ability of the cells to control K^+ membrane transport and was used to monitor the repair process after polyene treatment (19).

Effect of temperature on the repair process. Cells were treated with 0.1 μg of DMS-aureofacin per ml for 30 min at 37°C and transferred into polyene-free growth medium previously held at 4, 10, 27, and 37°C. Cells were incubated at these temperatures, and duplicate samples were taken from each culture for intracellular K^+ assay. The K^+ content in polyene-treated cells was compared with that in untreated cells incubated at the same temperature.

Effect of extracellular source of energy on the repair process. Cells were incubated in the growth medium (containing 1 mg of glucose per ml) with 0.1 μg of DMS-aureofacin per ml for 30 min and transferred into polyene-free growth medium or into growth medium without glucose. Other characteristics of the media were identical. Cells were incubated as described above. Intracellular K^+ content was determined and compared with the K^+ content in untreated cells incubated under the same conditions.

Effect of cycloheximide on the repair process. Cells were preincubated with 2 μg of cycloheximide per ml for 90 min and treated with DMS-aureofacin (0.05 and 0.1 μg/ml). Under these conditions, protein synthesis was inhibited as measured by the lack of the incorporation of ^{14}C-labeled amino acid mixture. Intracellular K^+ was measured during cell treatment and compared with the K^+ content in untreated cells preincubated with cycloheximide. The same procedure was used for polyene-treated and untreated cells which were not pretreated with cycloheximide.

Effect of K^+ on the repair process. Cells were treated with different concentrations of DMS-aureofacin for 30 min and transferred into antibiotic-free growth medium containing standard (2.5 mM) or high (37 mM) K^+ concentrations. Intracellular K^+ was measured and compared with intracellular K^+ content of untreated cells incubated under the same conditions.

Cell viability. Cell viability was determined by cell cloning and by the trypan blue dye exclusion test. For the latter test, 0.5 ml of the cell suspension was mixed with 4 ml of phosphate-buffered saline at pH 7.4, and 0.5 ml of 0.5% aqueous solution of trypan blue was added. Samples were kept for 5 min at room temperature before cells were counted with a model 6300 A Cytograph (Bio/Physics Systems, Inc., Mahopac, N.Y.). All BHK-21 cell suspensions used for the experiments were 99% viable. Cell cloning was performed as follows. A sample of cell suspension was diluted with the appropriate medium to obtain 50 and 100 cells/ml. Two milliliters of the cell suspensions was placed into each of six wells (35 mm in diameter). Plates were incubated at 37°C in a humidified incubator with 5% CO_2. On the 3rd day of incubation, 1 ml of fresh medium was added and cells were incubated for 5 more days. Afterwards, cells were stained with crystal violet and cell colonies were counted under low-power magnification (×100).

Protein. Cell protein was determined by the method of Lowry et al. (16). Fatty acid-free bovine serum albumin was used as standard protein.

RESULTS

Effect of temperature on the repair process. The purpose of this experiment was to determine whether the repair process of polyene-induced membrane alterations is an enzymatically controlled reaction or is based only on physicochemical rearrangement of lipid components in the plasma membrane. The experimental conditions allowed for the highest degree of membrane alterations that were completely reversible without apparent secondary changes observed in the cells. Cells were treated with DMS-aureofacin for 30 min at 37°C, required for membrane-polyene interaction (18). To terminate further changes in the membranes, cells were transferred into polyene-free medium and the repair process was observed at various temperatures by monitoring intracellular K^+ (19). Results showed that the repair process was dependent on temperature (Fig. 1). When the temperature was decreased from 37 to 27°C, the time required for complete cell recovery was increased. Membrane repair did not occur at 4°C, and only partial cell recovery was observed at 10°C. These results suggested that the repair of polyene-induced membrane alterations was an enzymatically controlled reaction. However, large differences between the repair at 10 and 27°C could also suggest that some physicochemical properties of membrane are essential for this process.

Effect of an external source of energy on the repair process. To observe the effect of energy deficiency on the repair process, cells were taken from the logarithmic phase of growth, when external glucose is the main source of metabolic energy. Cells were treated with polyene at normal growth conditions for 30 min, and the repair process was monitored after cells were transferred into antibiotic-free growth medium or into the same medium with glucose omitted. All other factors influencing cell metabolism were identical in the two media. The repair

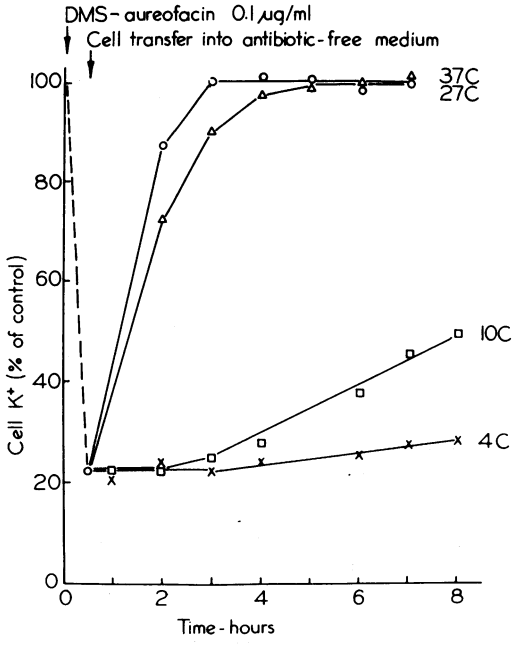

FIG. 1. *Effect of temperature on the repair process. BHK-21 cells (10^6 cells per ml) were treated with DMS-aureofacin (0.1 μg/ml) for 30 min at 37°C. Then the cells were transferred into an antibiotic-free medium and incubated at various temperatures. During the incubation time 2-ml samples of cell suspension were taken, and intracellular K^+ was measured after cell washing with K^+-free medium. Intracellular K^+ was calculated as percentage of K^+ in control samples which were incubated under the same conditions but were not treated with antibiotic. Each point represents the mean obtained from five independent measurements. The standard deviations of results obtained did not exceed ±3.2%.*

process was much slower and reduced by 40% in the glucose-free medium (Fig. 2). Lack of external glucose during the experiment had no effect on membrane permeability of untreated cells as measured by changes in intracellular K^+. All samples contained at least 98% viable cells at the end of experiment, i.e., 8 h posttreatment.

Effect of cycloheximide on the repair process. Cycloheximide was used in this experiment as an effective inhibitor of protein synthesis in BHK-21 cells. Preincubation of BHK-21 cells with 2 μg of cycloheximide per ml for 90 min, which was required for >95% inhibition of protein synthesis, was followed by DMS-aureofacin treatment. Cells pretreated with cycloheximide showed only a short delay for initiation of repair compared with the BHK-21 cells incubated without cycloheximide (Fig. 3). Cells incubated with 0.05 μg of DMS-aureofacin per ml were able to fully recover despite cycloheximide treatment. When 0.1 μg of DMS-aureofacin per ml was used, complete cell recovery could not be observed because cycloheximide treatment (2 μg/ml) induced leakage of intracellular K^+ after 9 h of incubation, thereby making the results difficult to interpret. All observations of the repair process (Fig. 3) were stopped before any permeability changes were observed in cell membranes due to cycloheximide treatment. The results suggested that the repair process does not require de novo protein synthesis.

Effect of K^+ on the repair process and on the toxic effects of polyene. The effect of K^+ on the repair process was observed under various conditions. In the first set of experiments, cells were treated with 0.2 μg of DMS-aureofacin per ml for 30 min and transferred into antibiotic-free medium containing standard (2.5 mM) or elevated (37 mM) K^+. Results showed that cells incubated in the medium with 37 mM K^+ regained the initial amount of K^+ 2 h quicker than

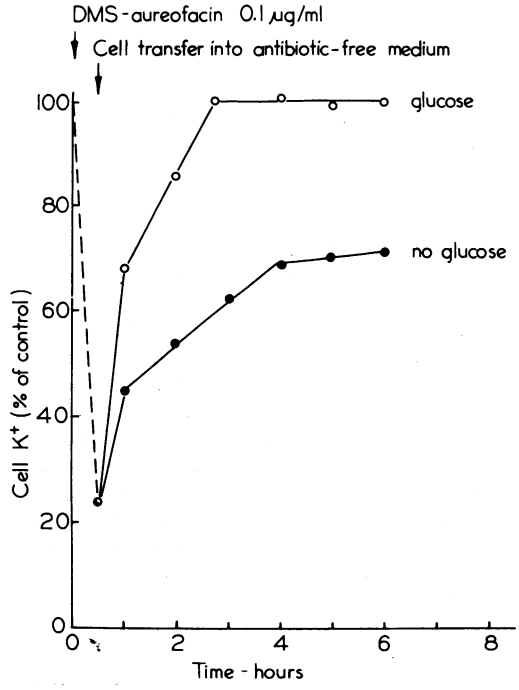

FIG. 2. *Effect of external glucose as a source of energy on the repair process. BHK-21 cells were treated with DMS-aureofacin for 30 min in normal growth medium at 37°C and transferred to an antibiotic-free growth medium or the same growth medium with glucose omitted. Cells were incubated at 37°C in a shaker incubator, and intracellular K^+ was measured. For other experimental conditions, see legend to Fig. 1. Each point on the curves represents the mean obtained from six independent measurements. The standard deviations for all points did not exceed ±3.3%.*

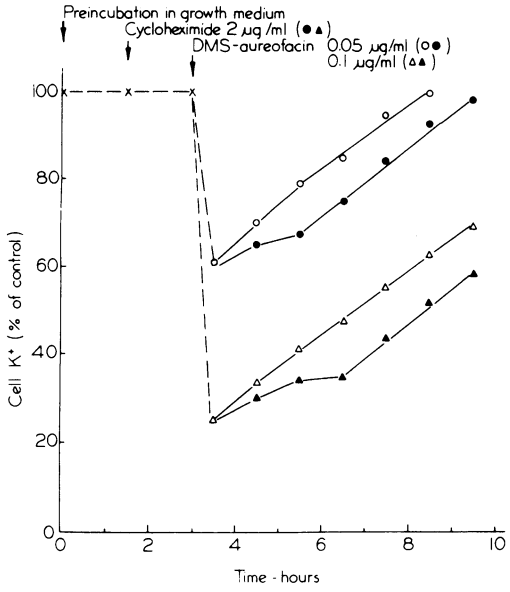

FIG. 3. *Effect of cycloheximide on the repair process. BHK-21 cells were incubated with cycloheximide for 90 min before DMS-aureofacin was added. For other experimental conditions, see legend to Fig. 1. During incubation, intracellular K^+ was determined. Each point represents the mean obtained from four independent measurements. The standard deviations of the results did not exceed ±4.2%.*

the same treated cells incubated with 2.5 mM K^+ (Table 1). Control BHK-21 cells (untreated) maintained intracellular K^+ at the same level, i.e., about 30 μg of K^+ per mg of protein, which is equivalent to 100 mM K^+ (19). Our observations showed that in polyene-treated cells both the intracellular K^+/protein ratio and the percentage of K^+ of the control cells (19) similarly expressed the rate of the repair process (Table 1) and therefore could be used interchangeably. The ratio of cell K^+/protein was a more convenient measure to use in long-term experiments.

Based on the above results, cells were treated with 0.5 μg of DMS-aureofacin per ml, a cytotoxic dose for BHK-21 cells under normal growth conditions (19). Cells were incubated with DMS-aureofacin for 30 min, and further cell membrane alterations induced by polyene were stopped by removing medium containing the antiobiotic and suspending the cells in antiobiotic-free medium with 2.5 or 37 mM K^+. Results showed that the same cell alterations that were toxic at normal growth conditions could be repaired in the medium with the high K^+ concentration (Fig. 4). Polyene-treated cells incubated in the medium containing 2.5 mM K^+ were not able to repair membrane alterations (Fig. 4) and quickly lost their viability (Table 2). The

TABLE 1. *Effect of external K^+ on intracellular K^+ during the repair process of DMS-aureofacin-induced permeability changes in BHK-21 cells*[a]

Time post-treatment (h)	Growth medium with 2.5 mM K^+		Growth medium with 37 mM K^+	
	K^+ as % control[b]	μg of K^+/mg of protein[c]	K^+ as % control	μg of K^+/mg of protein
0	100.0	28.4	100.0	28.7
0.5	16.0	5.6	16.3	5.7
1	39.2	10.2	67.1	19.2
2	58.6	16.2	93.4	26.3
3	76.8	21.3	102.5	29.5
4	90.1	25.2	98.7	28.3
5	101.8	27.9	103.1	28.9
6	99.3	29.1	105.3	28.0

[a] Cells were treated with 0.2 μg of DMS-aureofacin per ml for 30 min at 37°C in normal growth medium and then transferred into antibiotic-free medium containing normal (2.5 mM) or high (37 mM) K^+ concentrations. During the incubation, intracellular K^+ was measured by atomic absorption, and cell protein was determined by the method of Lowry et al. (12).

[b] Mean values obtained from six independent measurements. Standard deviations did not exceed ±3.9%.

[c] Mean values obtained from six independent measurements. Standard deviations did not exceed 1.9 μg of K^+/mg of protein.

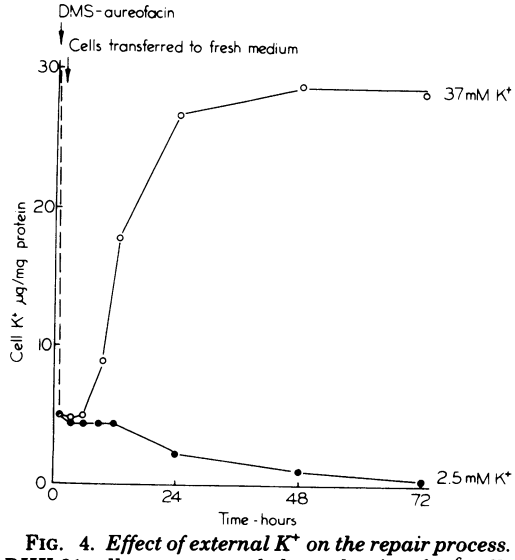

FIG. 4. *Effect of external K^+ on the repair process. BHK-21 cells were suspended to a density of 10^6 cells per ml in growth medium containing 0.5 μg of DMS-aureofacin per ml. After incubation with the antibiotic for 30 min at 37°C in a shaker incubator, cells were centrifuged for 5 min at 250 × g, suspended in an antibiotic-free medium containing physiological (2.5 mM) or high (37 mM) K^+ concentrations, and incubated under normal growth conditions. Intracellular K^+ and cell protein were determined. Each point represents the mean from six independent determinations. The standard deviations did not exceed ±1.6 μg of K^+/mg of protein.*

TABLE 2. *Effect of high K^+ on BHK-21 cell growth and viability changes after DMS-aureofacin treatment*[a]

Time post-treatment (h)	No. of viable cells in culture ($\times 10^5$/ml)[b]			
	2.5 mM K^+ growth medium		37 mM K^+ growth medium	
	Treated	Control	Treated	Control
0	10.1	10.4	10.1[b]	10.4
24	7.5	14.1	10.7	13.6
48	2.1	20.9	17.3	19.7
72	0.8	29.5	23.4	28.2

[a] For cell treatment and all experimental conditions, see the legend to Fig. 4.

[b] Mean values obtained from six independent determinations. Standard deviations did not exceed ±5.4 × 10^4 cells/ml.

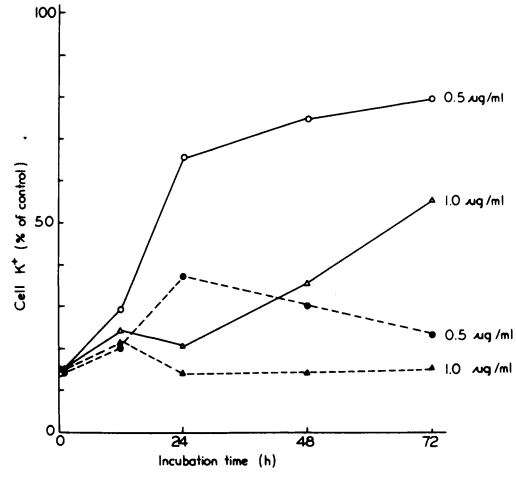

FIG. 5. *Effect of high K^+ concentration on reversal of polyene toxicity in BHK-21 cells. Cells were treated with toxic concentrations of DMS-aureofacin (0.5 and 1 µg/ml) for 30 min under normal growth conditions and transferred into an antibiotic-free medium with 37 mM K^+. During cell incubation at 37°C, the repair process of cells treated with polyene for 30 min was observed (solid line) and compared with the repair process of cells which were continuously incubated with DMS-aureofacin (broken line). For other experimental conditions, see the legend to Fig. 1. Each point represents the mean obtained from six independent measurements. The standard deviations did not exceed ±5.1%.*

same impaired cells incubated in the medium with 37 mM K^+ were able to repair membrane injury (Fig. 4) and afterward were able to grow and multiply as well as the untreated cells (Table 2). Untreated BHK-21 cells grew in 37 mM K^+ medium as well as in 2.5 mM K^+ medium (Table 2). Untreated cells maintained 29 ± 2.1 µg of K^+ per mg of protein during the experiment. The above observations indicate that the toxic effect of DMS-aureofacin could be reversed simply by having a high K^+ concentration in the medium.

Subsequently, we observed the effectiveness of high K^+ on reversing the toxic effect of polyene on BHK-21 cells. The concentration of DMS-aureofacin was increased up to 1 µg/ml, three times greater than the cytostatic concentration (19). Cells were incubated with polyene for 30 min and transferred into antibiotic-free medium with 37 mM K^+, and the repair process was monitored. The repair process was also observed in cells treated with DMS-aureofacin and incubated without a medium change. The results showed that reversibility of the toxic effect of polyene on cells depended upon the time of antibiotic contact with the cell and the polyene concentration (Fig. 5). When cell contact with both doses of polyene was terminated after 30 min by changing the growth medium, cell repair was observed. If the antibiotic was not removed from the medium, little or no repair was observed in cells treated with 0.5 and 1 µg/ml, respectively (Fig. 5). These results suggested that DMS-aureofacin retained its biological activity under these experimental conditions for longer than the 30-min period. Because no information was available on the stability of DMS-aureofacin, we tested the changes in the biological activity of the antibiotic dissolved in growth medium and incubated under the experimental conditions. Results showed that the stability of DMS-aureofacin depended on its concentration in medium and the temperature. Polyene used at 0.1 and 1 µg/ml lost 50% of its ability to release intracellular K^+ within 1.5 and 4 h, respectively, at 37°C, whereas at 4°C in the dark the antibiotic remained stable for at least 24 h (Fig. 6). K^+ concentration in the medium (2.5 or 37 mM) had no effect on antibiotic activity during incubation.

Subsequently we tested 13 polyene macrolides to establish the correlation between their structure and the reversibility of the toxic effect of polyene on cells by extracellular K^+. Representative structural types of polyenes, known to induce in *C. vulgaris* various effects on the repairability of membrane alterations (4, 17), were examined for specificity of action on BHK-21 cells. The selected compounds represented: (i) the "nonspecific immediate" group (4, 17) of neutral and amphoteric polyenes with a small macrolide ring which irreversibly impair the membrane (filipin, rimocidin, and pimaricin); (ii) the "nonspecific progressive" group (4, 17) of polyenes with a large and flexible (due to the low unsaturation in the chromophore) macrolide ring, which induce primarily specific and repair-

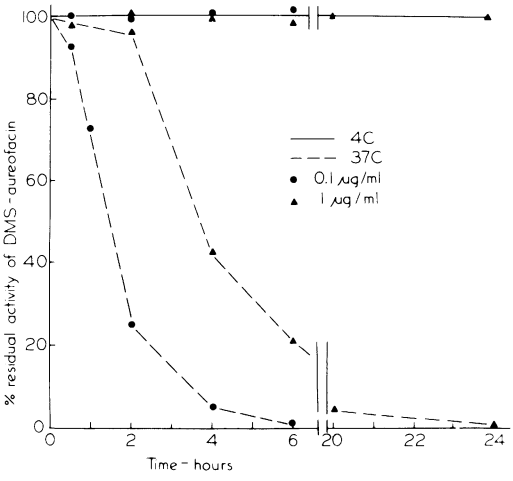

FIG. 6. *Decay of DMS-aureofacin activity. DMS-aureofacin was dissolved in the growth medium at concentrations of 0.1 and 1 μg/ml. One set of samples was incubated at 37°C in a shaker incubator, and the second set was incubated at 4°C in darkness. During the incubation 25-ml portions of antibiotic solutions were removed, and the solution held at 4°C was warmed up quickly to 37°C. BHK-21 cells were suspended in these solutions (10^6 cells/ml) and incubated for 30 min at 37°C in a shaker incubator. Intracellular K^+ was determined after the cells were washed with K^+-free medium. The amount of K^+ released from cells was calculated and compared with the amount of K^+ released from cells by a freshly prepared solution of DMS-aureofacin (0.1 or 1 μg/ml). The latter value was expressed as 100% antibiotic activity. Each point represents the mean from five independent measurements. The standard deviations did not exceed ±4.8%.*

able membrane alterations with a tendency to extend the membrane changes upon prolongation of cell contact with antibiotics (nystatin A_1 and lienomycin); and (iii) the "specific" group (4, 17) of large-macrolide-ring polyenes with rigid molecules (all heptaenes), which induce repairable membrane alterations. The last group could be divided into two chemical subgroups: one characterized by rigid and stretched molecules (nonaromatic heptaenes, e.g., amphotericin B and candidin), and the second characterized by rigid and bent molecules (aromatic heptaenes, e.g., aureofacin). We also examined NG and DMS derivatives of polyenes which are highly soluble in water and less toxic to mammalian cells than the parent compounds (6, 7, 19). BHK-21 cells were incubated with different concentrations of antibiotics for 30 min at 37°C in normal growth medium (2.5 mM K^+) and then transferred into antibiotic-free growth medium containing 2.5 or 37 mM K^+. Cell survival was measured by a cloning procedure described in Materials and Methods. Each polyene was tested to determine the concentration reducing the number of surviving cells to 50% compared with the control, untreated cells incubated under the same experimental conditions. This concentration was defined as the 50% toxic concentration (TC_{50}). The ratio of TC_{50} values obtained with 37 and 2.5 mM K^+ in the medium characterized the influence of the concentration on the toxic effect of a given polyene (Table 3). The results indicated a lower toxicity of all heptaenes and their NG and DMS derivatives in the presence of the high K^+ concentration, whereas BHK-21 cells treated with nystatin as well as any small-macrolide-ring polyenes showed the same survival at 2.5 and 37 mM K^+.

DISCUSSION

Although the membrane-permeabilizing effect of various structural types of polyene macrolides has been generally recognized (11), the mechanism of killing of eucaryotic cells by polyenes which induce specific channels in the membrane has not been clearly established. The problem is essential because this group of polyene macrolides appears most important for future therapeutic development. The broad range of possibilities for application of these polyenes results from their antifungal, antiprotozoal, antitumor, and some antiviral activities as well as from their other characteristics such as drug potentiation, immunoadjuvanticity, enhancement of the tumoricidal capabilities of macrophages, the stimulation of phagocytosis, and inhibition of prostate gland hyperplasia.

It has been generally accepted that polyenes with a large macrolide ring induce, at least at the beginning of their action, specific membrane pathways which enable potassium efflux from the cells. However, it is not clear whether this is the primary effect leading to secondary lethal metabolic changes after discharge of the potassium gradient or whether other membrane alterations, occurring at high polyene concentration or upon prolonged action, are responsible for the killing effect of these compounds. Kother-Brajtburg et al. (12) and Arnold et al. (1) make a distinction between K^+ efflux-inducing and cell-killing concentrations of polyenes in *S. cerevisiae* and *H. capsulatum*, respectively. Chen et al. (5) and Kotler-Brajtburg et al. (13) favor two different mechanisms for permeabilizing and killing effects of polyene macrolides in *Candida albicans* and mammalian cells, respectively.

The concepts of these two different mechanisms proposed for large-macrolide-ring polyenes result primarily from the observation that

TABLE 3. *Correlation between chemical characteristics of polyene macrolide antibiotics and the influence of K^+ concentration on their toxic effects on BHK-21 cells*

Polyene antibiotic	Chromophore	Type of macrolide ring	Ionic character of polyene	Solubility	TC_{50} (37 mM K^+)/ TC_{50} (2.5 mM K^+)[a]
Amphotericin B	Heptaene	Large, rigid, stretched	Amphoteric	Insoluble	2.5
NG-amphotericin B[b]	Heptaene	Large, rigid, stretched	Slightly acidic	Soluble	9.0
DMS-amphotericin B[c]	Heptaene	Large, rigid, stretched	Basic	Soluble	6.2
Aureofacin	Heptaene	Large, rigid, bent	Amphoteric	Insoluble	3.2
NG-auroefacin[b]	Heptaene	Large, rigid, bent	Slightly acidic	Soluble	4.8
DMS-aureofacin[c]	Heptaene	Large, rigid, bent	Basic	Soluble	9.5
Candidin	Heptaene	Large, rigid, stretched	Amphoteric	Insoluble	2.2
Nystatin A_1	Tetraene	Large, flexible	Amphoteric	Insoluble	1.0
NG-nystatin A_1[b]	Tetraene	Large, flexible	Slightly acidic	Soluble	1.3
Lienomycin	Pentaene	Large, flexible	Basic	Insoluble	1.1
Rimocidin	Tetraene	Small	Amphoteric	Insoluble	1.0
Filipin III	Pentaene	Small	Neutral	Insoluble	1.0
Pirmaricin	Tetraene	Small	Amphoteric	Insoluble	1.0

[a] BHK-21 cells suspended in the growth medium (10^6 cells/ml) were treated with various concentrations of each polyene antibiotic for 30 min at 37°C. Afterwards, cells were centrifuged for 5 min at 500 × *g* and suspended in antibiotic-free growth medium containing 2.5 or 37 mM K^+. Cell repair under both conditions was determined by the cloning procedure described in Materials and Methods. The TC_{50} as compared with that of untreated BHK-21 cells incubated under the same conditions was determined for both K^+ concentrations.

[b] *N*-Glycosyl derivative (*N*-glucamine salt).

[c] Trimethylammonium methyl ester derivative (monomethyl sulfate salt).

there is a dissociation between nontoxic drug concentrations, inducing K^+ efflux, and the cell-killing concentrations (12, 14, 19). We have postulated that this dissociation does not result from two different types of membrane changes but is a consequence of the repair process (19). Also, Fisher et al. (8, 9) suggest that the repair process is potentially an important mediator of the polyene toxic effect on mammalian cells; however, direct evidence has not been reported.

In this paper, we show a direct correlation between repair of antibiotic-induced membrane alterations and reversal of the toxic effects of specific polyenes on mammalian cells. The same membrane alterations induced by DMS-aureofacin were observed as toxic or were fully repaired by the cells under conditions differing only in K^+ concentration (Fig. 4, Table 2). The above results were confirmed by using the different experimental approach for other heptaenes (Table 3). The dependence of the repair process on the external K^+ concentration could be explained as follows. Potassium efflux is the only observed effect of specific polyenes on cells when monitored just after antibiotic treatment (2, 4, 17). Potassium lost by free diffusion is compensated by stimulation of K^+ active transport (2, 4). The degree of compensation depends upon the activity of K^+-Na^+-dependent adenosine triphosphatase as well as on the rate of K^+ leakage (4, 17). Since stimulated K^+ active transport can be limited (4), it may not be sufficient to compensate for K^+ loss through free diffusion. Therefore, the K^+ concentration in the medium is critical for maintaining the physiological level of K^+ in the cells. The important role of the external K^+ concentration in the repair process (Fig. 4, Table 3) could explain the early observation, made by Lampen and Arnow (15), that glycolysis inhibited by nystatin in *S. cerevisiae* can be restored by a high K^+ concentration in the medium. These authors, however, did not report whether any changes in cell viability occurred.

The results presented in this paper clearly showed that the toxic effect of large-macrolide-ring polyenes with rigid molecules (heptaenes) on BHK-21 cells could be reversed by high concentrations of external K^+. These results are in agreement with those previously obtained for *C. vulgaris* and *S. cerevisiae* (3, 4, 17). Small-macrolide-ring polyenes and large-macrolide-

ring polyenes with flexible molecules induced in BHK-21 cells nonspecific membrane alterations; therefore, a high K^+ concentration in the medium had no or negligible effect on cell viability after the treatment. At this stage of our investigations, it is difficult to quantitatively correlate the structural characteristics of various heptaenes with the ability of potassium to decrease drug toxicity and to repair membrane alterations. However, the higher TC_{50} ratio (Table 3) obtained for NG and DMS derivatives, as compared with the parent compounds, suggested that the solubility of polyene in water diminished the toxic effect of these compounds on mammalian cells. Although this suggestion has to be further proven, it is in agreement with the earlier report of Fisher et al. (9).

The presence of metabolic energy was also essential for the repair process. The lack of an easily utilized external source of energy (glucose) as well as conditions inhibiting production of metabolic energy (low temperature) strongly inhibited or even stopped the repair process (Fig. 1 and 2). These results confirmed our previous observations (4, 17) indicating that the toxic effect of polyene is dependent not only on the concentration of antibiotics but also on environmental conditions. The incubation conditions inhibited or even stopped the repair process and, under these circumstances, secondary metabolic and membrane changes followed the primary polyene effect and led to cell death (Fig. 4, Tables 2 and 3). These secondary metabolic changes were responsible for the toxic effect observed after treatment with specific polyenes. Decomposition of the antibiotic terminated its interaction with the membrane (Fig. 6). The repair process started immediately after the antibiotic was removed from the medium (Fig. 4 and 5). This observation and the fact that cycloheximide did not inhibit full cell recovery after polyene treatment (Fig. 3) suggested that no new enzyme protein was required to be synthesized for membrane repair. We did not observe any changes in cell sensitivity to DMS-aureofacin treatment in the presence of cycloheximide (Fig. 3). The temperature dependency of the repair process (Fig. 1) confirmed data obtained under conditions of the poor energy supply (Fig. 2). However, the large discrepancy in the repair rate observed between 10 and 27°C suggests that mobility of the lipid membrane fraction may also be a factor influencing the repair process.

The involvement of metabolic energy in the repair process and the active participation of enzyme systems rather than the physicochemical rearrangement of membrane lipids or drug molecules is supported by other observations. In *C. vulgaris*, the repair of polyene-induced injury occurs only in the presence of oxygen (4, 17). In dengue virus-infected BHK-21 cells, virus multiplication inhibits the repair process of membrane alterations induced by DMS-aureofacin (B. Malewicz, H. M. Jenkin, and E. Borowski, Drugs Exp. Clin. Res., in press).

It has been demonstrated that the formation of specific pathways in the membrane requires metabolic energy (18), most probably to move the antibiotic to the hydrophobic core of the membrane. Therefore, it is reasonable to consider the probability that the repair process is a reverse reaction of antibiotic-membrane interaction. This process could also require metabolic energy to move antibiotic (intact, complexed with membrane constituents, or in metabolized form) in or from the membrane. However, other possible mechanisms of the repair process must also be considered.

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