Effect of Polyenic Antibiotics on Ehrlich Ascites and Novikoff Hepatoma Cells

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SUMMARY

The incorporation of thymidine in Novikoff hepatoma cells and Ehrlich ascites cells was severely and irreversibly inhibited by the polyenic antibiotics filipin and lucensomycin, known for their action on phospholipid-cholesterol membranes. No inhibition could be shown on regenerating liver cells or on acellular preparations from tumor cells. Despite the well-known site of action of these antibiotics on cell membranes, which was ascertained by $^{51}$Cr efflux, transcellular migration of glutamate-$^{14}$C through neoplastic cells was not affected. The cells, however, became unable to exclude the acidic dyes used for viability tests. With the use of appropriate concentrations of the polyenes, the uptake of the dye was limited to the nucleus. This behavior was paralleled by a shift in the pH of the transition from orthochromatic to metachromatic staining of the nucleus after methanol fixation.

INTRODUCTION

Previous work from this laboratory (13, 14) has shown that O$_2$ consumption and DNA synthesis by Novikoff hepatoma cells are severely inhibited by filipin, a polyenic antibiotic known for its action on phospholipid-cholesterol membranes (9—11, 15, 18, 24). Regenerating liver cells were less sensitive to this substance. These findings were tentatively correlated to reports in the literature indicating that the composition of membranes from neoplastic cells may differ from that of normal cells (3, 5). In this paper, the action of 2 polyenic antibiotics, filipin and lucensomycin, on thymidine incorporation and membrane permeability of tumor cells was investigated.

MATERIALS AND METHODS

MEM$^1$ (without L-glutamine) was obtained from Grand Island Biological Co., Grand Island, N.Y. Thymidine-6-$^3$H (specific activity, 20,600 mCi/mmmole) and sodium chromate ($^{51}$Cr) in 0.9% NaCl solution (2 mCi/ml, 3.1 μg/ml) were obtained from Radiochemical Centre, Amersham, England; uniformly labeled glutamic acid-$^{14}$C (0.1 mCi/ml, 15 mCi/mg) was obtained from New England Nuclear Corp., Boston, Mass. Lucensomycin (etruscomycin) was kindly supplied by Professor F. Arcamone and Professor M. Ghione of Farmitalia, Milano, Italy; filipin was supplied by Dr. George B. Whitfield, Jr., of The Upjohn Co., Kalamazoo, Mich. The 2 stocks of filipin obtained differed by their content of filipin III, which is the most active isomer (2); Stock A contained 52% filipin III, and Stock B contained 96% (G. B. Whitfield, Jr., personal communication). The samples were stored under N$_2$ at 0° in the solid form. PPO and POPOP were obtained from Nuclear-Chicago Corp., Des Plaines, Ill. Other chemicals were obtained from C. Erba, Milano, Italy, or from E. Merck, Darmstadt, Germany.

Male Sprague-Dawley rats were used as the source of the Novikoff hepatoma, which was studied 6 days after transplantation, and of regenerating liver, which was taken 24 hr after partial hepatectomy (4).

Ehrlich ascites tumor cells were harvested 7 to 8 days after i.p. transplantation in Swiss mice.

Oxygen consumption was measured at 38° in a conventional Warburg apparatus, with 0.015 M glucose and 0.013 M succinate as substrates. An aliquot of the cell suspension was dried at 100° and values were expressed per 10 mg, dry weight (4).

Incorporation studies were carried out as previously described (13) after suitable preincubation in a Krebs-Ringer phosphate solution or in MEM adjusted to pH 7.2 with NaHCO$_3$ by the addition of thymidine-$^3$H, 5 μCi/ml in the case of tumor cells or 20 μCi/ml in the case of the acellular preparation or regenerating liver cells. The pH of the cell suspension was controlled before and after the incubation.

Cell-free preparations for incorporation studies were obtained by homogenizing the 300 X g sedimentsed cells in a Potter-Elvehjem apparatus with an equal volume of distilled water. The homogenate was then centrifuged for 30 min at 40,000 X g, and the supernatant, taken to isosmolarity with concentrated MEM, was used for the experiments. Incubations were performed for 2 hr in the presence of 6 mM ATP; incorporation lasted for 4 hr. Membrane permeability was assayed by measuring the efflux of $^{51}$Cr from cells preloaded by incubation for 30 min at room temperature with 20 μCi/ml of sodium chromate.$^{51}$Cr (25).

Transcellular migration of glutamate-$^{14}$C through cell-coated Millipore filters was studied in a 2-chamber glass apparatus, as described by Harris and Friedman (7) with the modifications reported by Strom et al. (21), with the use of a K$^+$-free Krebs-Ringer bicarbonate-glutamate solution. After
equilibration at 30°, 14C-labeled glutamate was added at Time 0 to the chamber on side of the Millipore filter cells membrane. Transcellular migration was followed by repetitive sampling of the fluid in the chamber on the other side of the membrane. Paper chromatography in a methanol:acetic acid:water:urea (80:2:17.5:0.5) system (16) of the content of either chamber at the end of the experiments did not show other evident radioactive spots apart from that of glutamate and the amount retained at the origin.

Cell viability was evaluated by the dye exclusion method in a Burker counting chamber with the use of 0.1% trypan blue, 0.025% eosin (23), or 0.015% nigrosine (8) in MEM. For metachromatic staining of the nuclei, the cells, suspended in MEM, were spread on microscope slides, rapidly air dried, fixed for 10 min in methanol, passed through an alcohol series to water, and then stained for 10 to 20 min in 0.02% thionine (C.I. 52000) or 0.02% xylidine Ponceau (C.I. 16255) in 0.1 M glycine buffer at the appropriate pH. They were then washed in water and immediately observed. The pH of the glycine buffer was varied by the addition of appropriate amounts of 0.1 M acetic acid, 0.1 M KH2PO4, 0.1 M KC1 plus 0.1 M HC1, and/or 0.1 M NaOH.

Protein was determined by a biuret procedure (6), with crystalline bovine serum albumin (from Sigma Chemical Co., St. Louis, Mo.) as a standard. DNA and RNA were determined by the diphenylamine and orcinol reactions (17). 14C and 3H crystalline bovine serum albumin (from Sigma Chemical Co., St. Louis, Mo.) as a standard. DNA and RNA were determined by the diphenylamine and orcinol reactions (17). 14C and 3H were estimated as previously described (13, 21) with the use of a Nuclear-Chicago Model 725 liquid scintillation counter; counting efficiency was calculated by a channel ratio method or, when necessary, by internal standardization with 3H-labeled toluene. 51Cr was measured in a Packard Auto-Gamma well scintillation counter. Radioactivity was expressed as dpm. Scanning of paper chromatograms of 14C-labeled material was performed on a Nuclear-Chicago Model 1036 Actigraph II.

Filipin was dissolved in DMSO at a concentration of 11 mg/ml and then diluted with the buffer used in the experiment. Lucensomycin was suspended in the buffer either directly or, when knowledge of precise concentration was required, from a stock solution in DMSO. Whenever DMSO was used as a solvent for the polyenes, control experiments were run with DMSO alone; at the concentrations used (<10 µl/ml) DMSO had, however, generally no significant effect on the various parameters studied.

RESULTS

Effect of Polyenic Antibiotics on Respiration and on DNA Synthesis. As shown in Table 1, the effects of filipin and lucensomycin on O2 uptake by Novikoff hepatoma cells were strikingly different; while filipin caused a severe inhibition of respiration at a concentration of 15 µg/ml, lucensomycin was without effect even at concentrations 60 times higher.

Table 2 shows that lucensomycin caused inhibition of incorporation of thymidine-3H into the DNA of Ehrlich ascites and Novikoff hepatoma cells. The inhibition could not be reversed by thorough washing of the antibiotic-treated cells. DNA synthesis by regenerating liver cells or by acellular preparations from Ehrlich ascites cells was not affected even by very high concentrations of the polyene.

Incorporation of uridine-3H into RNA and of 14C-labeled amino acids into proteins was also inhibited, to a comparable extent, in tumor cells.

Effects on Transport Processes and on Cell Permeability. Neither filipin nor lucensomycin at concentrations up to 300 µg/ml had any effect on transcellular migration of glutamate-14C through Novikoff hepatoma or Ehrlich ascites cells. Also, the susceptibility to K+ ions or to strophanthin remained unchanged (Table 3).

However, the addition of either polyene caused the cells to become permeable to 51Cr (Chart 1) or to dyes such as trypan blue, eosin, or nigrosine.

After the addition of filipin or lucensomycin, the cells swelled, and the nucleus in particular became more evident (Figs. 1 and 3). A striking phenomenon in the early stages after the addition of the antibiotic was the uptake by the nucleus of acidic dyes such as trypan blue or eosin; when the cells were exposed to various doses of filipin or lucensomycin in the presence of the dye, the nucleus of practically every cell became intensely colored, while the cytoplasm remained clear (Fig. 3). The staining time was, within certain limits, inversely related to the filipin concentration. With filipin III at a concentration of 6 µg/ml, a staining time of 6 min was optimal for differential staining of the nucleus. With filipin III at concentrations above 15 µg/ml, the cells became completely stained at once, without any difference between nucleus and cytoplasm. Lucensomycin had similar, although less sharp, effects.

Metachromatic staining of the cells with thionine in buffered solutions showed a transition from blue to purple around pH 4.2 to 4.3 in the untreated cells; after 5 min of exposure to filipin III, 6 µg/ml, the variation in the staining of the nucleus was shifted 0.7 to 1.0 pH unit toward lower values. Similar results were obtained with xylidine Ponceau.

DISCUSSION

The results reported above and in previous papers (13, 14) show the extreme sensitivity of some tumor cells toward the polyenic antibiotics filipin and lucensomycin.

The former substance was the more active, being able to inhibit both DNA synthesis and cellular respiration, whereas lucensomycin did not affect oxygen uptake. On the other hand, “normal” cells appeared to be completely insensitive to lucensomycin, at least in our experimental conditions, while with filipin it was only a matter of concentrations.

It is generally accepted that polyenic antibiotics increase the permeability of cell membranes, most probably by interaction with cholesterol-phospholipid structures (9, 11, 15, 18, 24). Such a mechanism might account for the effect of these substances on tumor cells. Moreover, disruption of cellular organization abolishes the inhibitory effect of both filipin (13, 14) and lucensomycin (Table 2) on DNA synthesis.

1 Concentration of lucensomycin was checked by determining its absorbance at 305 nm, with ε1%1 cm = 1400 (1).
A rather unexpected result was given by the experiments on the transcellular migration of glutamate-\(^{14}\)C, where no difference between samples with or without the antibiotics was observed. If transcellular migration reflects active and/or facilitated transport, as indicated by the effects of K\(^+\) ions (7, 21) and of strophanthin, the alterations caused by the polyenes would be limited to changes in passive permeability of the plasma membrane.

The nucleus seems to be affected very soon, as is shown by its preferential staining by acidic dyes. This fact clearly shows that some of the amino groups of the histone moiety of the nucleoproteins are available for interaction with the acidic dyes (20). On the other hand, although the experiments in which filipin-treated cells were stained after methanol fixation with thionine or xylidine Ponceau cannot be interpreted as clearly indicating a variation in the pK of nucleoproteins in the nucleus of the living cells (19), they nevertheless show that,
Chart 1. Effect of filipin and lucensomycin on efflux of $^{51}$Cr from Ehrlich ascites cells. Ehrlich ascites cells, suspended in Krebs-Ringer bicarbonate buffer ($3.5 \times 10^6$ cells/ml), were incubated for 30 min at room temperature with $^{51}$Cr-labeled sodium chromate, 20 $\mu$Ci/ml. After being washed, the cells were resuspended in the same volume of buffer and incubated as such (Curve A), with filipin Stock B, 55 $\mu$g/ml (Curve B), or with lucensomycin, 300 $\mu$g/ml (Curve C). Aliquots were withdrawn at 20-min intervals and checked for radioactivity in 1 ml of supernatant at 700 x g.

Fig. 3. Effect of filipin on trypan blue uptake by Ehrlich ascites cells. Filipin (Stock B, 0.6 mg/0.1 ml of DMSO/100 ml of cell suspension in MEM plus 0.1% trypan blue) had been added 6 min previously. x 340.

after exposure of the cells to the polyene, more anionic groups of the nucleic acids in the cell nuclei become available for interaction with the basic dye molecule (22, 26). The effects on the incorporation of thymidine into DNA are already evident very soon after the addition of the polyenes to the cells. This may just be the consequence of an indiscriminated efflux of material from the cytoplasm (10, 12, 18), or it might be due to alteration caused by the polyenes at the level of the nuclear and/or endoplasmic membranes. The different effects of lucensomycin and filipin on respiration may indicate a different mechanism of damage by these polyenes or may be due, more simply, to quantitative differences in solubility or binding.

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