Biochimica et Biophysica Acta, 643 (1981) 346—362
Elsevier/North-Holland Biomedical Press

BBA 79227

LIPOSOME-CELL INTERACTIONS

A STUDY OF THE INTERACTIONS OF LIPOSOMES CONTAINING ENTRAPPED ANTI-CANCER DRUGS WITH THE EMT6, S49 AND AE_1 (TRANSPORT-DEFICIENT) CELL LINES

T.M. ALLEN, L. McALLISTER, S. MAUSOLF and E. GYORFFY

Pharmacology Department, University of Alberta, Edmonton, Alberta T6G 2H7 (Canada)

(Received October 30st, (1980)

Key words: Cytotoxicity; Liposome, Anti-cancer drug; Nucleoside transport; Tubercidin

Summary

A study has been made to determine if the cytotoxicity observed when cells in culture were exposed to liposome-entrapped cytotoxic drugs was liposome mediated or resulted from leakage of drug from the liposomes with subsequent uptake of free drug by the cells. In preliminary experiments with the EMT6 cell line in monolayer culture, the cytotoxicity observed when the cells were exposed to a range of concentrations of liposome-entrapped methotrexate, actinomycin D and cytosine arabinoside for a variety of liposome compositions was somewhat less than that observed when the cells were exposed to similar concentrations of free drug. We suspected that the cytotoxicity was mediated via uptake of free drug leaked from liposomes. This was confirmed in experiments involving the EMT6 and S49 cell lines in monolayer or suspension culture, respectively, in the absence and presence of the nucleoside transport inhibitor, 6-((4-nitrobenzyl)thio)-9-β-D-ribofuranosylpurine. Additional experiments were performed on a transport-deficient mutant of the S49 cell line, the AE_1 cell line. No evidence for liposome-mediated cell death could be found in these cell lines when tubercidin 5′-monophosphate was entrapped in either large or small unilamellar liposomes composed of egg phosphatidylcholine/cholesterol (2 : 1), bovine brain phosphatidylserine/egg phosphatidylcholine/cholesterol (8 : 2 : 5) or egg phosphatidylcholine/stearylamine/cholesterol (10 : 1 : 5). Considerable toxicity due to empty liposomes of a variety of compositions was observed in the S49 cell line at high lipid concentrations.

Introduction

In the past several years there have been many reports in the literature on the use of phospholipid vesicles as carriers for introducing biologically active substances into cells in vitro and in vivo. Recent reviews in this area of research include those by Pagano [1] and Poste [2]. The accumulating evidence from the studies of liposome-cell interactions indicates that liposomes are capable of interacting with cells in many different ways, depending on liposomal physical properties and surface charge. These modes of interaction have been described by Poste [2] among others and include: fusion of the liposome with the cell plasma membrane with release of liposome-entrapped contents into the cytosol; endocytosis of liposome and contents with the subsequent possibility of content release from the lysosomes into the cytosol; and absorption of liposomes to the cell surface with either no uptake of liposome contents by the cell, or accompanying alterations in liposome or membrane permeability followed by uptake of released materials by passive diffusion, micropinocytosis or transport processes. A further possibility exists in that liposomes may gradually lose their contents to the medium during the course of the experiment, particularly in the presence of serum components [3—5] and the leaked compounds may be taken up by transport processes. Liposomes are probably interacting with cells via several mechanisms occurring simultaneously. Because of the difficulty in designing experiments in which unambiguous interpretation of the results is possible, it has been difficult to quantitate the contribution of each of the various mechanisms of liposome-cell interactions to the end result.

We have devised an experimental system enabling us to address the following question: Is liposome-mediated uptake of liposome contents into cells (via fusion or endocytosis) occurring to a significant degree, or is cytotoxicity primarily a result of uptake of free materials leaked from liposomes (absorbed or free) into the culture medium?

In addition to naturally occurring nucleosides, many cytotoxic nucleoside analogs enter cells via a nucleoside transport mechanism which has a broad specificity [6—8]. These nucleoside analogs kill cells by interfering with DNA synthesis within the cell. A potent inhibitor of nucleoside transport, 6-((4-nitrobenzyl)thio)-9-β-D-ribofuranosylpurine (NBMPR) has been described which effectively inhibits the transport of nucleosides and cytotoxic nucleoside analogs into cells [6—8]. Cultured cells have been protected against otherwise inhibitory concentrations of antiproliferative nucleosides in the presence of NBMPR [6,8].

In the experiments described below, monolayer cultures of the EMT6 (mouse mammary tumor) cell line or suspension cultures of the S49 (mouse lymphoma) cell line were exposed to either liposome-entrapped or free cytotoxic nucleoside analogs in the absence or presence of NBMPR. In the presence of NBMPR the transport into the cells of cytotoxic nucleoside compounds free in solution can be prevented [6,8], but uptake of cytotoxic materials via liposome-mediated mechanisms such as fusion or endocytosis should not be inhibited. Cell death in these experiments is an indication of uptake of cytotoxic materials by the cells and if uptake of free drug can be prevented in the presence of a nucleoside transport inhibitor, then any cell death observed will

provide a measure of liposome-mediated uptake.

Further experiments were performed using the AE_1 cell line, a mutant of the S49 cell line, which is resistant to uptake to cytotoxic nucleosides because of a deficiency in the transport of purine and pyrimidine nucleosides [9]. The resistance of this cell line might be able to be overcome via liposome-mediated mechanisms of uptake.

Materials and methods

Chemicals

Actinomycin D, cytosine-1-β-D-arabinofuranoside and methotraxate were obtained from Sigma Chemical Co. [3′,5′,9(n)-^{3}H]Methotrexate (25 Ci/mmol) was obtained from Moravek Biochemicals, [5-^{3}H]cytosine-1-β-D-arabinofuranoside (26 Ci/mmol) and [^{3}H]actinomycin D (13.7 Ci/mmol) were obtained from Amersham. Tubercidin, [G-^{3}H]tubercidin (20 Ci/mmol) and NBMPR were a generous gift of Dr. A.R.P. Paterson of the University of Alberta Cancer Research Unit (McEachern Laboratory). Tubercidin 5′-phosphate was obtained from Calbiochem. Cell culture materials and related chemicals were obtained from GIBCO Canada, Burlington, Ontario.

Cell culture

EMT6 cells were derived from a transplantable mouse mammary tumor [10]. The EMT6 tumor was maintained by alternate passage in Balb/c mice and cell culture every 2 weeks as previously described [10]. Monolayer cultures were grown in 250-cm^3 polystyrene flasks in a humidified incubator in a 5% CO_2/air atmosphere at 37°C. Monolayer cultures were grown in Waymouth's medium supplemented with 12.5% fetal bovine serum and antibiotics (100 U penicillin, 100 μg streptomycin and 0.25 μg amphotericin B per ml). Suspension cultures were grown in Eagle's medium with Earle's salts supplemented with 12.5% fetal bovine serum and containing antibiotics, as above.

S49 cells were derived from a mineral oil-induced T-cell lymphoma in a Balb/c mouse [11]. AE_1 cells are a clone derived from mutagenized S49 cells [9]. Both cell types were cultured in suspension in 50-cm^3 screw-cap glass culture bottles at a total volume of 20 ml in a humidified 5% CO_2/air atmosphere at 37°C. Both cell types were grown in Dulbecco's modified Eagle's medium containing 4.5 g/l of D-glucose supplemented with 10% horse serum that had been heat inactivated at 56°C for 30 min. The media also contained 100 U penicillin, 100 μg streptomycin and 0.25 μg amphotericin B per ml.

EMT6 cell experiments

Monolayer cultures. Cells were suspended by a 10 min incubation in 0.05% trypsin in Hank's balanced salt solution containing 0.68 mM EDTA/l at 37°C followed by gentle pipetting. 10^5 cells were pipetted into each 100-mm plastic petri dish and allowed to grow for 24 h. Then 1 ml of Waymouth's medium containing either free drug or drug entrapped within liposomes of various sizes and lipid compositions (see below) was added to each petri dish. Concentrations of drug varied from 10^{-10} to 10^{-5} g/ml. Controls received no drug. Each experiment was performed in triplicate. After 48 h incubation, cells were tryp-

sinized, counted and the percent increase in cell number was calculated using as 100% increase the cell numbers obtained from drug-free controls.

Suspension culture. Cells from trypsinized monolayer cultures of EMT6 cells were suspended in Eagle's medium with Earle's salts at a concentration of 10^5 cells per ml and agitated at 37°C. Free cytosine-1-β-D-arabinofuranoside or liposome-entrapped cytosine-1-β-D-arabinofuranoside at a total concentration of $2 \cdot 10^{-5}$ g/ml of medium (44 μM cytosine-1-β-D-arabinofuranoside and approx. 1 μmol phospholipid/10^5 cells) was added to the suspension culture at zero time. Cell survival was estimated by taking 1 ml samples at various time intervals after addition of drug. Appropriate dilutions were made and cells were re-plated for cloning in plastic petri dishes. Cells were incubated in a humidified 5% CO_2/air atmosphere at 37°C for 8—10 days. The resulting colonies were fixed, stained and counted. Controls contained no drug.

S49 and AE_1 cell experiments

S49 and AE_1 cells were suspended in Dulbecco's modified Eagle's medium containing 10% heat-inactivated horse serum at a concentration of $0.5—1 \cdot 10^5$ cells/ml. Each incubation mixture was done in triplicate. S49 and AE_1 cells were incubated in the presence of free or liposome-entrapped tubercidin or tubercidin 5′-phosphate. Duplicate cultures of cells incubated with free or liposome-encapsulated drug were incubated in the presence of 10 μM NBMPR. Controls were incubated in the presence of either phosphate-buffered saline or liposomes of the same size and composition as those used in the experiment, but containing no drug (empty liposomes) or in the presence of 10 μM NBMPR alone. Cell numbers were estimated by counting duplicate samples from each culture in a Coulter counter.

Lipids and liposome preparation

Egg phosphatidylcholine was purchased from Makor Chemicals Ltd. (Jerusalem). Phosphatidylserine was purchased from Avanti Biochemicals, Inc. (Birmingham, AL). Cholesterol, distearoyl phosphatidylcholine and stearylamine were purchased from Sigma Chemical Co. All lipids were found to be chromatographically pure by thin-layer chromatography. Small unilamellar vesicles were prepared by the following technique: 20 mg of phospholipid were taken to dryness under vacuum in a rotary evaporator. Remaining traces of organic solvent were removed by placing the lipid on a high vacuum pump for 1—2 h. The dried lipid was suspended in either 1 ml of sterile phosphate-buffered saline, pH 7.4, for 'empty liposome' controls, or 1 ml of phosphate-buffered saline containing the appropriate dissolved drug in the following concentrations: methotrexate, 89 mM; actinomycin D, 1.6 mM; cytosine-1-β-D-arabinofuranoside, 220 mM; tubercidin, 10 mM, tubercidin 5′-phosphate, 30 mM. Approx. 10^6 cpm of ^{3}H-labelled drug were added to each sample, with the exception of tubercidin 5′-phosphate where no ^{3}H-labelled drug was available. The lipid/drug mixture was vortex mixed and sonicated to clarity in a bath-type sonicator (30—180 min) for small unilamellar vesicles. Sepharose CL-4B chromatography of these liposome preparations followed by phosphate analysis has indicated that less than 10% of the total lipid in these preparations is present as multilamellar liposomes. Non-entrapped drug was separated from

liposome-entrapped drug on a 1 × 40 cm Sephadex G-50 column equilibrated in sterile phosphate-buffered saline, pH 7.4. Reverse-phase evaporation liposomes were made according to the technique of Szoka and Papahadjopoulos [13]. Large unilamellar vesicles were made by the following technique: 1 ml of drug solution was applied to the top of a 2.5 × 15 cm Sephadex G-50 column followed immediately with 1 ml of a solution of drug with 16 mg phospholipid in sodium deoxycholate at a phospholipid : deoxycholate molar ratio of 1.0. The column was then eluted in phosphate-buffered saline, pH 7.4, at a flow rate of approx. 10—12 ml per h. Large unilamellar vesicles containing entrapped drug eluted in the void volume. Free drug remaining associated with the liposomes was removed by a second chromatographic procedure over a 1 × 40 cm Sephadex G-50 column. Captured volume by this method was 4—6 μl/μmol phospholipid.

The amount of drug trapped in all types of liposome was estimated from the amount of ^{3}H-labelled drug remaining associated with liposomes after Sephadex G-50 chromatography. In the case of tubercidin 5′-phosphate, the concentration of free drug and the amount of drug associated with the liposomes were estimated from the molar extinction coefficient of tubercidin 5′-phosphate (E_{271} = 10 200), following lysis and clarification of the liposome solutions with 0.01% deoxycholate. Phospholipid was measured by using the method of Dittmer and Wells [14].

Results

The results for the effect of liposome-entrapped as against free drug for actinomycin D, cytosine-1-β-D-arabinofuranoside and methotrexate can be seen in Fig. 1A—C. Standard deviation bars have not been included in the figures but in no case was the standard deviation greater than ±10% of the indicated value. The concentration of liposome-entrapped drug in all cases is taken as that concentration which would result if the liposomes were lysed and the drug were free in the medium. As can readily be seen, liposome-entrapped drug was from 20 to 100% as effective in its ability to inhibit cell growth as compared to the same total concentration of free drug. In no case was liposome-entrapped drug more effective than free drug. Cultures receiving empty liposomes had growth characteristics similar to those of controls receiving no drug (results not shown). We suspected that a large proportion of the cell death which we observed might be due to contents leaking out of the liposomes under the influence of fetal bovine serum in the culture medium so, although we had found our liposomes to be relatively non-leaky at 20°C in the absence of serum, we repeated the leakage experiments at 37°C in the presence of culture medium and the results are reported in Table I. As can be seen, liposomes in culture medium are generally more leaky than liposomes in buffer at 37°C. Therefore, over the long incubation time of our experiments, drug was most likely entering the cells as free drug and this could have been masking any liposome-mediated drug uptake which might be occurring.

In order to explore the possibility that liposomes may interact with cells within a few minutes after exposure whereas uptake of leaked drug may only become significant over longer periods of time, it was decided to expose sus-

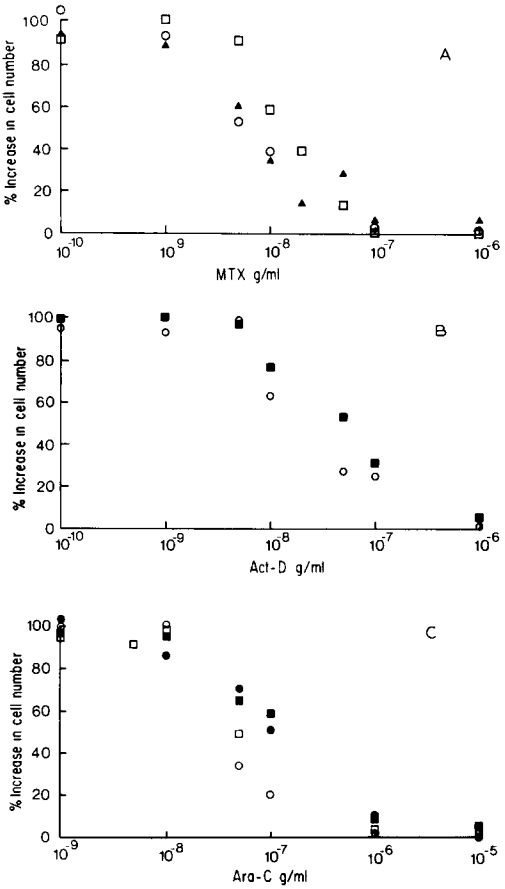

Fig. 1. Percentage increase in cell numbers of EMT6 cell monolayer cultures exposed to free or liposome-entrapped drug. 100% increase was taken as that observed in drug-free controls. Liposome-entrapped drug was present in culture at the same final concentration as free drug for all experiments. A. Cells exposed for 48 h to increasing concentrations of free methotrexate (MTX) (○); methotrexate entrapped in egg phosphatidylcholine/cholesterol (2 : 1) large unilamellar vesicles (▲) or egg phosphatidylcholine/cholesterol (2 : 1) small unilamellar vesicles (□). B. EMT6 cells exposed for 48 h to increasing concentrations of free actinomycin D (○) or actinomycin D entrapped within egg phosphatidylcholine/cholesterol/stearylamine (10 : 5 : 1) small unilamellar vesicles (■). C. EMT6 cells exposed for 48 h to increasing concentrations of free cytosine arabinoside (Ara-C) (○) or to cytosine-1-β-D-arabinofuranoside entrapped within egg phosphatidylcholine/cholesterol (2 : 1) small unilamellar vesicles (□); egg phosphatidylcholine/cholesterol/stearylamine (10 : 5 : 1) small unilamellar vesicles (■) or distearoyl phosphatidylcholine/stearylamine (10 : 1) small unilamellar vesicles (●).

pension cultures of cells to liposome-entrapped or free drug for short periods of time where presumably leakage would not be a large problem. Cells were subsequently washed clear of free drug and of liposomes which had not interacted with, or were not firmly attached to cells, and cell survival was estimated as indicated in Materials and Methods. The results are plotted in Fig. 2. Cytosine-1-β-D-arabinofuranoside entrapped within sonicated liposomes composed of phosphatidylcholine/cholesterol (2 : 1) at a final concentration of 44 μM ($2 \cdot 10^{-5}$ g/ml) was substantially less toxic than free drug at the same concen-

TABLE I

% DRUG LEAKED FROM PHOSPHATIDYLCHOLINE/CHOLESTEROL (2 : 1) SMALL UNILAMELLAR VESICLES AT 37°C IN BUFFER OR CULTURE MEDIUM

Buffer: phosphate-buffered saline, pH 7.4. Waymouth's medium: containing 12.5% fetal bovine serum. Dulbecco's medium: Dulbecco's modified Eagle's medium containing 10% heat-inactivated horse serum. Tubercidin 5′-phosphate: leakage was estimated from extinction coefficient as no radioactive drug was available for this compound.

Entrapped drug	Incubation time (h)	
	2	24
Cytosine arabinoside		
Buffer	6	65
Waymount's medium	14	62
Methotrexate		
Buffer	15	36
Waymouth's medium	29	51
Actinomycin D		
Buffer	35	50
Waymouth's medium	39	60
Tubercidin		
Buffer	83	88
Waymouth's medium	86	90
Dulbecco's medium	85	85
Tubercidin 5′-phosphate		
Buffer	0	0
Dulbecco's medium	0	20

tration, particularly at the longer incubation times. Sonicated liposomes of the same composition, but containing 10 mol% stearylamine were much more toxic than free drug. This could be due to surface absorption of the positively

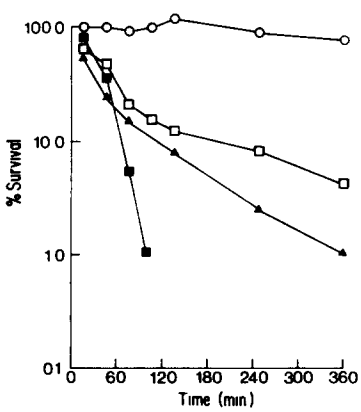

Fig. 2. Percentage survival of EMT6 cells in suspension culture exposed to free or liposome-entrapped cytosine-1-β-D-arabinofuranoside ($2 \cdot 10^{-5}$ g/ml) for 10 min, 30 min, 1 h, 2 h, 4 h or 6 h. % survival was estimated by the ability of the cells to form clones during an 8—10 day incubation. 100% survival was taken as that observed in drug-free controls (○). Free cytosine-1-β-D-arabinofuranoside (▲); cytosine-1-β-D-arabinofuranoside entrapped within egg phosphatidylcholine/cholesterol (2 : 1) small unilamellar vesicles (□); cytosine-1-β-D-arabinofuranoside entrapped within egg phosphatidylcholine/cholesterol/stearylamine (10 : 5 : 1) small unilamellar vesicles (■).

charged liposomes to the cell with subsequent carry-over of the liposomes and their entrapped drug through the washing procedure and into the 8—10 day incubation period. It could also be due to toxicity of the stearylamine itself.

In order to clarify the mechanism of the EMT6 cell death which we were observing, we attempted to inhibit the cell death due to uptake of free drug which had leaked from liposomes in order to separate this component of cell death from that which was directly liposome mediated. We attempted to protect EMT6 cells against free cytosine-1-β-D-arabinofuranoside by culturing them in the presence of NBMPR, a potent inhibitor of nucleoside transport. In two separate experiments, 5 μM NBMPR was able to increase cell survival in EMT6 monolayer cultures from 3% to an average of 38% in the presence of 0.22 μM cytosine-1-β-D-arabinofuranoside and 10 μM NBMPR increased cell survival to 58%. However, when the concentration of cytosine-1-β-D-arabinofuranoside was raised to 2.2 μM, no protective effect of NBMPR could be seen (Table II). Cell death in the presence of liposomes could be almost eliminated in the presence of 5 μM, or even better, 10 μM NBMPR (Table II) which was highly suggestive that much of the cell death observed in the presence of this type of liposome was due to leakage from the liposomes with subsequent uptake by transport of free drug into the cells.

Uptake of tubercidin into cells can be effectively inhibited [12]. Experiments with the EMT6 cell line in monolayer culture indicated that protection by NBMPR against the cytotoxic effects of tubercidin was more effective than protection against cytosine-1-β-D-arabinofuranoside at an equitoxic dose. Cell death induced by both 0.05 and 0.1 μM tubercidin could be 100% inhibited in the presence of 10 μM NBMPR (results not shown). No significant decrease in cell numbers as compared to control was observed after 72 h in culture when tubercidin was encapsulated in phosphatidylcholine/cholesterol/stearylamine (10 : 5 : 1) sonicated liposomes at a final concentration of 0.1 μM tubercidin

TABLE II

Liposomes were composed of egg phosphatidylcholine (PC)/cholesterol (CHOL)/stearylamine (SA) (10 : 5 : 1) small unilamellar vesicles. Ara C, cytosine-1-β-D-arabinofuranoside.

	EMT6 cell numbers after 24 h in culture (% of control)
NBMPR (5 μM)	99
NBMPR (10 μM)	100
Ara C ($1 \cdot 10^{-7}$ g/ml)	3
Ara C ($1 \cdot 10^{-7}$ g/ml) (5 μM NBMPR)	38
Ara C ($1 \cdot 10^{-7}$ g/ml) (10 μM NBMPR)	58
Ara C ($1 \cdot 10^{-6}$ g/ml)	<1
Ara C ($1 \cdot 10^{-6}$ g/ml) (5 μM NBMPR)	<1
Ara C ($1 \cdot 10^{-6}$ g/ml) (10 μM NBMPR)	<1
PC : CHOL : SA ([Ara C] = $1 \cdot 10^{-7}$ g/ml)	77
PC : CHOL : SA ([Ara C] = $1 \cdot 10^{-7}$ g/ml; 5 μM NBMPR)	92
PC : CHOL : SA ([Ara C] = $1 \cdot 10^{-7}$ g/ml; 10 μM NBMPR)	97
PC : CHOL : SA ([Ara C] = $1 \cdot 10^{-6}$ g/ml)	55
PC : CHOL : SA ([Ara C] = $1 \cdot 10^{-6}$ g/ml; 5 μM NBMPR)	63
PC : CHOL : SA ([Ara C] = $1 \cdot 10^{-6}$ g/ml; 10 μM NBMPR)	91

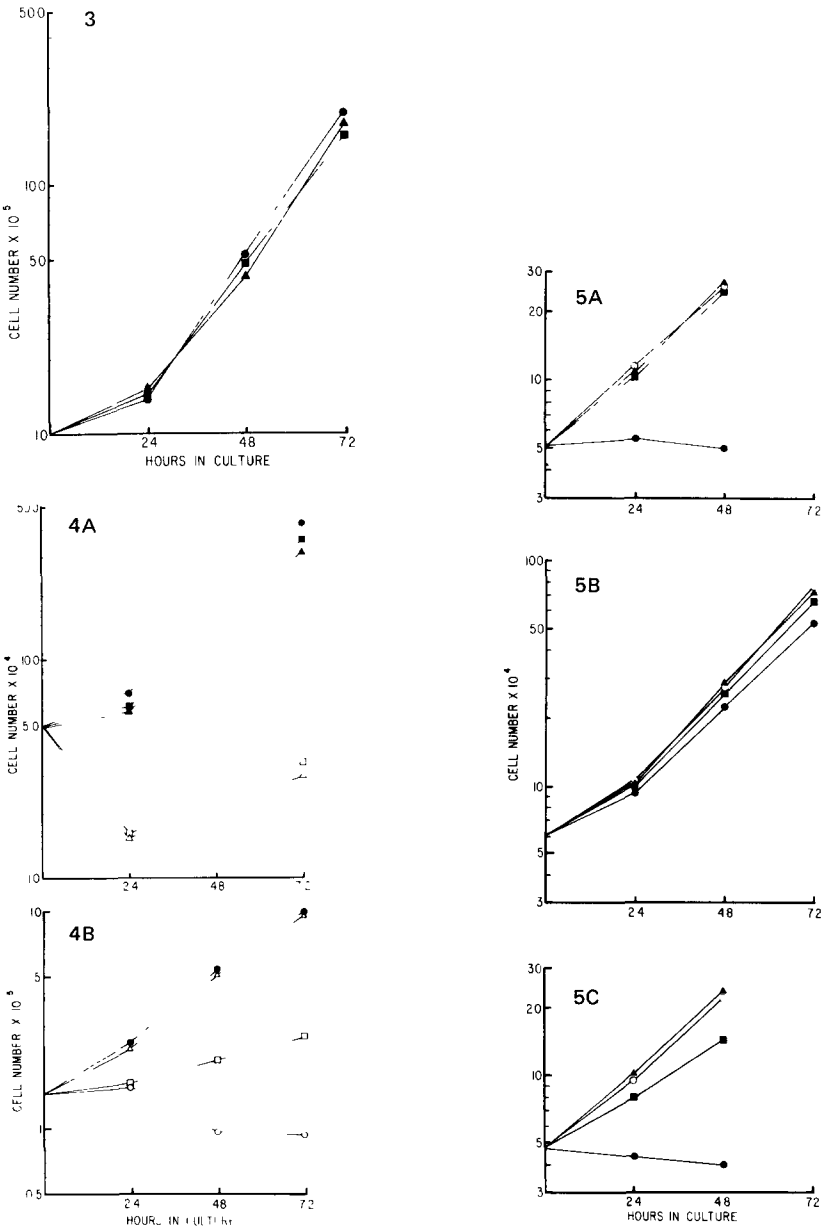

Fig. 3. Effect of liposome-encapsulated tubercidin and free tubercidin in EMT6 monolayer cultures. Control (●), free 0.1 μM tubercidin, 10 μM NBMPR (▲); tubercidin, final concentration 0.1 μM, entrapped in egg phosphatidylcholine/cholesterol/stearylamine (10 : 5 : 1) small unilamellar vesicles in the presence of 10 μM NBMPR (■).

Fig. 4. Toxicity of empty liposomes in S49 suspension cultures. Molarities are expressed in terms of total lipid (including cholesterol and stearylamine). A. Egg phosphatidylcholine/cholesterol (2 : 1) small unilamellar vesicles, 300 μM (□), 60 μM (■); egg phosphatidylcholine small unilamellar vesicles, 200 μM (△), 40 μM (▲); control (●). B. Egg phosphatidylcholine/cholesterol/stearylamine (2 : 1 : 1) small unilamellar vesicles, 200 μM (○), 20 μM (□), 2 μM (△), control (●).

Fig. 5. Toxicity of phosphatidylserine-containing liposomes in S49 suspension cultures. Molarities are expressed in terms of total lipid. A. Phosphatidylcholine/phosphatidylserine (2 : 1) small unilamellar vesicles, 200 μM (●), 20 μM (■), 2 μM (▲), control (○). B. Phosphatidylcholine/phosphatidylserine/cholesterol (1 : 1 : 1) small unilamellar vesicles, 200 μM (●), 20 μM (■), 2 μM (▲), control (○). C. Phosphatidylserine/cholesterol (2 : 1) small unilamellar vesicles, 300 μM (●), 30 μM (■), 3 μM (▲), control (○).

and 10 μM NBMPR in the medium (Fig. 3). Cells cultured in the presence of 0.1 μM free tubercidin or liposome-encapsulated tubercidin in the absence of NBMPR showed no growth over the time period of the experiment (results not shown). This was again highly suggestive that cell death was being mediated via a leakage mechanism.

S49 and AE_1 cells

We have attempted to confirm and extend our observations with the EMT6 cell line using the S49 and mutant AE_1 cell lines in suspension culture. We found that S49 cells could be completely protected against the cytotoxic effect of 0.1 μM tubercidin in the presence of 10 μM NBMPR and that no inhibition of cell growth could be observed in the AE_1 cells in the presence of 0.1 μM tubercidin, confirming the transport deficiency for this compound in the AE_1 cell line (results not shown).

The S49 and AE_1 cell lines proved to be much more sensitive than the EMT6 cell line to cell death by empty liposomes (Figs. 4A and B and 5A—C). It became necessary to keep our lipid concentration of phosphatidylcholine/cholesterol liposomes to levels below 60 μM (0.06 μmol/10^5 cells) (Fig. 4A) in order to avoid cytotoxicity from this source. Liposomes containing stearylamine were even more toxic and the level of stearylamine in the culture medium had to be kept below 1 μM (0.001 μmol/10^5 cells) to avoid cytotoxicity from this source (Fig. 4B). Phosphatidylserine/cholesterol (2 : 1) liposomes also proved to be very cytotoxic to this cell line (Fig. 5C), but inclusion of 50 mol% phosphatidylcholine and 33 mol% cholesterol reduced the toxicity considerably (Fig. 5B). Inclusion of 66 mol% phosphatidylcholine in the absence of cholesterol also reduced the toxicity (Fig. 5A).

A typical experiment carried out in the presence of 0.1 μM tubercidin, free or encapsulated in phosphatidylcholine/cholesterol (2 : 1) sonicated liposomes, had the following results. Toxicity of free tubercidin could be eliminated in the presence of 10 μM NBMPR. Empty liposomes (200 μM phospholipid) showed considerable toxicity; cell growth was almost completely eliminated over a 72 h period. Tubercidin-containing liposomes were only slightly more toxic than empty liposomes and this slight increase in toxicity could be elminated in the presence of 10 μM NBMPR (results not shown). All subsequent experiments were performed with non-toxic liposome concentrations.

Because of the low solubility of tubercidin (a saturated solution is approx. 10 mM), its high leakage rate and the low capture volume of sonicated liposomes (approx. 0.1—0.2 μl/μM phospholipid) it was impossible to entrap tubercidin in sufficient quantities at low lipid concentrations to perform these experiments with no liposome toxicity. We therefore began to use tubercidin 5′-phosphate, a more soluble form of tubercidin, which showed cytotoxicity identical to that of tubercidin against S49 and AE_1 cells, and the S49 cells could similarly be protected against the effects of free tubercidin 5′-phosphate by the use of 10 μM NBMPR (results not shown). Tubercidin 5′-phosphate had a much higher latency period in liposomes at 37°C both in the presence and absence of 10% horse serum than did tubercidin (Table I). The results of a typical experiment are shown in Fig. 6A. When the experiment was repeated in the presence of NBMPR, the results were identical, except that the response of free

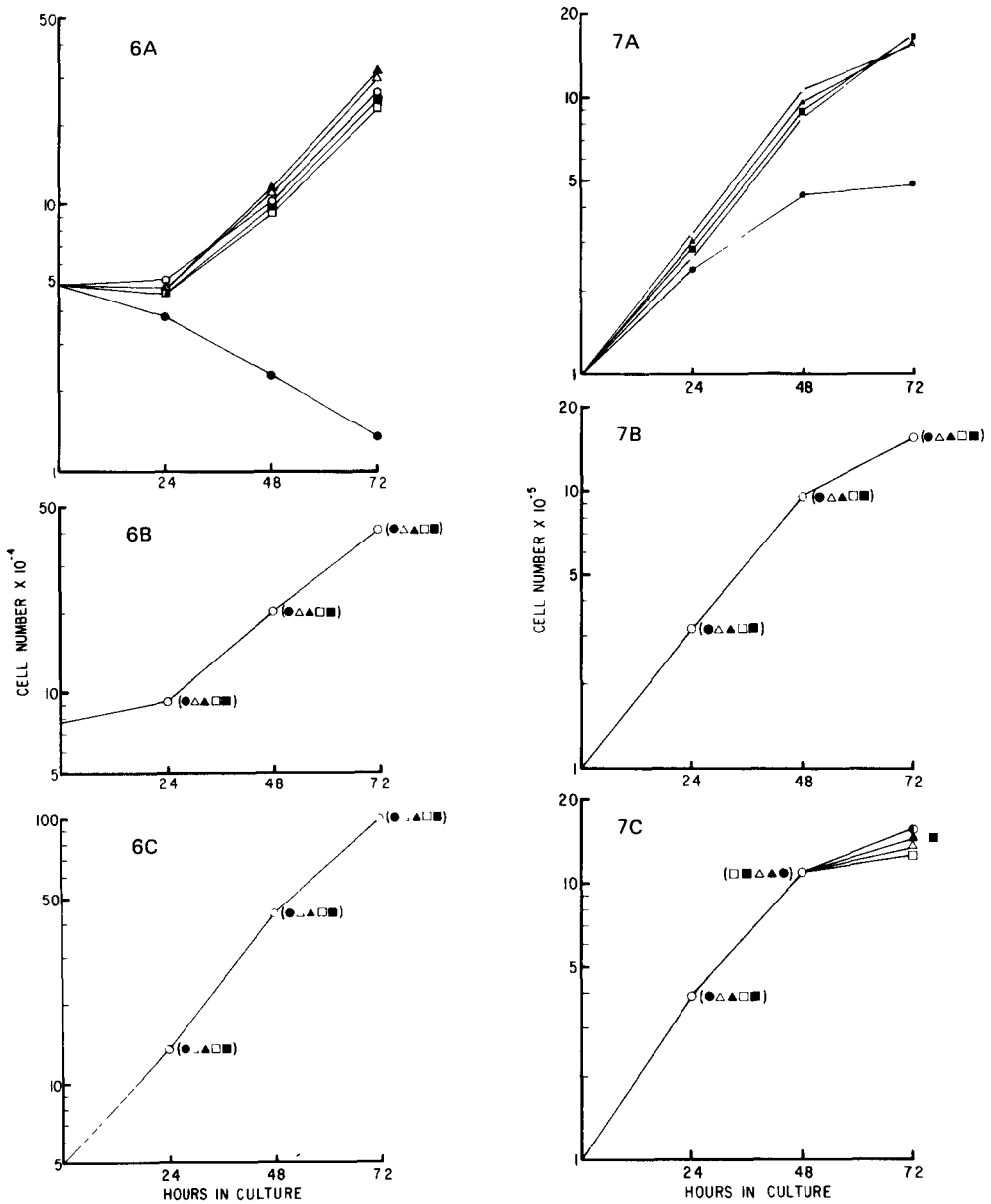

Fig. 6. Toxicity of free or liposome-entrapped tubercidin 5′-phosphate in S49 or AE_1 suspension cultures in the presence or absence of 10 μM NBMPR. A. S49 cells control (○); 0.1 μM tubercidin 5′-phosphate (●); tubercidin 5′-phosphate, 0.1 μM final concentration entrapped in phosphatidylcholine/cholesterol (2 : 1) small unilamellar vesicles (■); empty phosphatidylcholine/cholesterol (2 : 1) small unilamellar vesicles (□); tubercidin 5′-phosphate, 0.1 μM final concentration entrapped in phosphatidylcholine/cholesterol (2 : 1) reverse-phase evaporation liposomes (▲); empty phosphatidylcholine/cholesterol (2 : 1) reverse-phase evaporation liposomes (△). B. All experiments done with S49 cells in presence of 10 μM NBMPR, same symbols as in A. C. Experiments done with AE_1 cells, same symbols as in A.

Fig. 7. Toxicity of free or liposome-entrapped tubercidin 5′-phosphate in S49 or AE_1 suspension cultures in the presence or absence of 10 μM NBMPR. A. S49 cells control (○); 0.1 μM tubercidin 5′-phosphate (●); tubercidin 5′-phosphate, 0.1 μM final concentration entrapped in phosphatidylcholine/cholesterol/stearylamine (10 : 5 : 1) reverse-phase evaporation liposomes (■); empty phosphatidylcholine/cholesterol/stearylamine (10 : 5 : 1) reverse-phase evaporation liposomes (□); tubercidin 5′-phosphate, 0.1 μM final concentration entrapped in phosphatidylserine/phosphatidylcholine/cholesterol (8 : 2 : 5) reverse-phase evaporation liposomes (▲); empty phosphatidylserine/phosphatidylcholine/cholesterol (8 : 2 : 5) reverse-phase evaporation liposomes (△). B. Same as A but in the presence of 10 μM NBMPR. C. Same as A but using AE_1 cells instead of S49 cells.

tubercidin could be eliminated (Fig. 6B). AE_1 cells in the absence of NBMPR had a response identical to that observed with S49 cells in the presence of 10 μM NBMPR (Fig. 6C). The results of all experiments were virtually superimposable, with no evidence for liposome-mediated uptake of tubercidin 5′-phosphate from small or large unilamellar vesicles into AE_1 cells or into S49 cells over a 72 h period. The cells in Fig. 6B and C were protected against uptake of free tubercidin 5′-phosphate either by NBMPR or by a transport deficiency so cytotoxicity could only result from liposome-mediated uptake of drug. The fact that no cytotoxicity is seen is convincing evidence that significant liposome uptake is not occurring.

Experiments were run in the presence of liposomes composed of phosphatidylserine/phosphatidylcholine/cholesterol (8 : 2 : 5) to test the hypothesis that the 2 mM $CaCl_2$ present in the culture medium may promote fusion between the negatively charged cell surface and negatively charged liposomes. Liposomes were also formed from phosphatidylcholine/cholesterol/stearylamine (10 : 5 : 1) in order to test the hypothesis that positively charged liposomes may be able to interact directly with the negatively charged cell surface and fusion or endocytosis of liposomes may be triggered. The results are shown in Fig. 7A—C. With the exception of free 0.1 μM tubercidin 5′-phosphate in S49 cells in the absence of NBMPR no cell death was evident. Again, no evidence for liposome-mediated uptake of liposome content could be demonstrated for these lipid compositions in the S49 and AE_1 cell lines.

Discussion

Our preliminary experiments with EMT6 cells in monolayer culture (Fig. 1A—C) indicated that liposome-entrapped cytosine-1-β-D-arabinofuranoside, actinomycin D and methotrexate were less toxic in the ED_{50} range of drug concentration than was free drug. At higher drug concentrations liposome-entrapped drug and free drug were equally effective. A multitude of explanations are possible and these include: leakage of drug from the liposomes over a period of time with uptake of leaked drug by the cells (unless the liposomes were leaking very rapidly, leaked drug would initially be present in less total concentration than free drug and cell death would lag behind that of free drug); uptake of liposome-entrapped drug via fusion or endocytosis; toxicity of liposomes themselves; any combination of the above. The amount of cytotoxicity observed appeared to be roughly equivalent for liposomes of different size, charge or phase transition. This would suggest that fusion and endocytosis may not be the major mechanism involved here. Other experiments we have done with this cell line do not give evidence for substantial cytotoxicity of liposomes themselves at the concentrations used. (eg. Table II and Fig. 3), so the major portion of the cytotoxicity was probably due to drug leaked out of liposomes under the influence of serum proteins and cell-surface proteins. Leakage experiments confirm that a large percentage of contents have leaked out of liposomes after a 24 h incubation at 37°C in culture media (Table I).

The results obtained for EMT6 cells in the presence of cytosine-1-β-D-arabinofuranoside and NBMPR are somewhat difficult to interpret as we were not able to obtain complete protection in the presence of 10 μM NBMPR against either

2.2 or 0.22 μM cytosine-1-β-D-arabinofuranoside. Obviously a major component of cell death was due to cytosine-1-β-D-arabinofuranoside which had escaped from liposomes and had been transported into the cells, as liposome-mediated cell death was in every case less in the presence of NBMPR than in its absence (Table II). However, since the protection obtained against cytosine-1-β-D-arabinofuranoside was dependent on the concentration of free cytosine-1-β-D-arabinofuranoside in solution, and we could not be absolutely sure of the rate at which cytosine-1-β-D-arabinofuranoside was leaving liposomes under the influence of serum proteins and cell surface proteins, then some of the cytotoxicity could conceivably be due to fusion or endocytosis of liposome-entrapped cytosine-1-β-D-arabinofuranoside. However, experiments in which cytosine-1-β-D-arabinofuranoside was entrapped in liposomes at 0.22 μM final concentration showed no significant difference from control in the presence of 10 μM NBMPR. This would suggest that a major protein of cell death in this system was mediated via transport of leaked drug into the cell.

We were able to protect completely EMT6 cells in monolayer culture against the effects of 0.1 μM tubercidin by culturing in the presence of 10 μM NBMPR. When the cells were completely protected against free drug we did not observe any cytotoxicity when tubercidin entrapped in phosphatidylcholine/cholesterol/stearylamine (10 : 5 : 1) was included in the culture medium. This provides confirmatory evidence that the cytotoxicity observed in EMT6 cells in the presence of liposome-entrapped drug was due to uptake of leaked drug by the cells.

As can be seen from Table I, except in the case of tubercidin 5′-phosphate, there was a considerable loss of vesicle contents over a 24 h incubation period in culture medium containing serum. The effects of serum proteins on leakage of liposome contents are well documented [3—5]. However, most investigators in the area of liposome-cell interactions have conducted leakage experiments in the presence of buffer, and not in the presence of medium which generally contains 10—15% serum. The presence of cells may also increase the leakage of contents from liposomes as has been demonstrated in the absence of serum by Szoka et al. [15]. It is possible to manipulate liposome composition to produce liposomes which are resistant to serum-induced leakage [29], but it remains to be seen whether these liposomes are capable of fusing with or being endocytosed by cells.

Considerable lipid toxicity from empty liposomes was observed in the S49 cell line, at higher lipid concentrations (Figs. 4 and 5). For most of our experiments, it was possible to keep the level of lipid below 0.002 μmol lipid phosphate/10^5 cells (2 μM) which represents approx. $4 \cdot 10^6$ small unilamellar vesicles/cell [16,20]. This is a large excess of liposomes as it takes approx. 10^5 small unilamellar vesicles to cover the surface area of a 10 μm cell. Or put in other terms, 0.002 μmol phospholipid/10^5 cells represents about a 25-fold excess of lipid phospholipid over cell plasma membrane phospholipid. At these levels of phospholipid, neither we nor others [16—19] have noted any toxic effects due to liposomes alone for a variety of liposome compositions. However, in experiments with tubercidin, we had to use higher lipid concentrations in order to expose cells to a final concentration of 0.1 μM tubercidin. This was a result of the relatively low solubility of tubercidin (10 mM) and the low cap-

ture efficiency of sonicated liposomes for this compound (0.01—0.02%/μmol phospholipid). Concentrations of lipid used were therefore 10—100-fold higher than that needed for more soluble compounds, or for more hydrophobic compounds such as actinomycin D which can associated with the lipid bilayer. At these higher lipid levels we observed lipid toxicity in the S49 cell line. All liposomes tested were toxic to S49 cells at concentrations of 200 μM (0.2 μmol lipid/10^5 cells). Liposomes containing stearylamine or those composed of phosphatidylserine/cholesterol (2 : 1) were toxic to S49 cells at concentrations above 2—3 μM (0.002—0.003 μmol total lipid/10^5 cells or 0.5 nmol stearylamine/10^5 cells). The observation that both stearylamine and phosphatidylserine are toxic to EMT6 cells at a level of 0.2—0.3 μmol phospholipid/10^5 cells has been made by Dunnick et al. [20].

The increased toxicity when cytosine-1-β-D-arabinofuranoside was trapped in small unilamellar vesicles containing stearylamine which we observed in the experiments reported in Fig. 2 may be due to this mechanism, as we needed to use higher concentrations of lipid in this experiment in order to expose the cells to 44 μM cytosine-1-β-D-arabinofuranoside. Lipid concentration in this instance was approx. 1.0 μmol phospholipid/10^5 cells, of which 10% was stearylamine (i.e., 0.1 μmol stearylamine/10^5 cells).

The mechanism for liposome toxicity at high lipid concentrations is not understood, but it is not surprising that a cell which may have a large proportion of its surface area covered with liposomes may have problems reproducing and growing. Huang and Pagano [21] have reported that in Chinese hamster V79 cells approx. 0.1% of the total lipid (initially 10^8 vesicles/cell) becomes associated with the cells. This would represent 10^5 vesicles/cell which would be enough to cover most of the surface area of a 10 μm cell. Dunnick et al. [20] have also reported that approx. 10^5 small unilamellar vesicles are taken up by EMT6 cells. Adams et al. [22] have reported that liposomes containing phosphatidylcholine/cholesterol/stearylamine (5 : 5 : 1) (5—10 mg/mouse) were toxic in vivo when injected intracerebrally. While liposome toxicity does not appear to be a major problem to cells in culture, at low concentrations, the above observations suggest the need for precautions in interpreting experimental results when higher concentrations of liposomes are used, particularly those containing stearylamine or high percentages of phosphatidylserine.

Evidence of liposome toxicity was seen in the experiments in which S49 cells were exposed to liposome-entrapped tubercidin. Liposomes containing entrapped tubercidin were more toxic than empty liposomes (approx. 200 μM phospholipid). In all these experiments toxicity due to liposome-entrapped tubercidin could be eliminated in the presence of NBMPR, but liposome toxicity still remained. Interpretation of these results is difficult because of the complicating factor of liposome toxicity.

We therefore conducted a series of experiments using liposome-entrapped tubercidin 5′-phosphate which is less permeable than tubercidin and has a longer latency period in liposomes. This compound was equitoxic with tubercidin in causing cell death. It is probably dephosphorylated by cellular ecto-5′-nucleotidase and enters the cell as free tubercidin. In the cell, it is rephosphorylated and is metabolically active as the triphosphate [23]. Direct liposome-mediated introduction of tubercidin 5′-phosphate into the cell may there-

fore allow the cell to bypass one of the intracellular activating steps. Tubercidin, an adenosine analog, inhibits DNA, RNA and protein synthesis and is toxic in all phases of the cell cycle [23]. NBMPR was equally effective in blocking toxicity of 0.1 μM tubercidin 5'-phosphate as it was against 0.1 μM tubercidin, arguing that the toxic effects are attributed to the entry of free tubercidin rather than tubercidin 5'-phosphate. Little or no toxicity was found in S49 cells or AE_1 cells when experiments were performed with liposome-entrapped tubercidin 5'-phosphate, although free tubercidin 5'-phosphate showed substantial toxicity (Figs. 6 and 7). Because of its high latency, even in the presence of medium, it appears that not enough free tubercidin 5'-phosphate was released into the medium to cause any significant increase in cell death. No toxicity due to empty liposomes was evident in these experiments and no direct liposome-mediated toxicity was apparent. Changing the size and charge of the liposomes and changing liposome composition did not affect these negative results. Tubercidin 5'-phosphate when entrapped in reverse-phase liposomes or small unilamellar liposomes by the procedures described in Materials and Methods was active at the normal concentration when released from liposomes by extensive resonication and exposed to cells in culture. Therefore, none of the procedures used to entrap tubercidin 5'-phosphate interfered with its toxicity or degraded it. We conclude that there is no direct liposome-mediated uptake of drug by the cells and any toxicity seen in earlier experiments in this laboratory was due to uptake of leaked drug by the cells.

However, lack of pharmacological action of the drug in the presence of transport inhibitor could also result from a second, less likely mechanism. If the liposome-entrapped drug were taken up by the cell by endocytosis and the drug were subsequently degraded in the lysosomal apparatus, then similar results would be seen. We do not favor this explanation for the following reasons: the cell lines used in our experiments have not been reported to be actively endocytotic; in our experiments we used liposomes of many sizes and types which would probably not be taken up by endocytosis to the same extent; and finally, many of our liposomes preparations, which were pharmacologically active in the absence of transport inhibitor, became inactive in the presence of transport inhibitor. If endocytosis of liposomes with subsequent degradation of drug were the major pathway of uptake, no pharmacological activity would have been seen.

Conclusion

Extensive literature on the interactions of liposomes with cells has been accumulating over the past several years. However, due to the complex nature of liposome-cell interactions, interpretation of experimental results in terms of mechanisms of liposome-cell interactions has proved to be difficult or impossible. None of the mechanisms such as endocytosis, fusion or absorption of liposomes to cells which are involved in liposome-cell interactions are mutually exclusive. The experimental design of many previous experiments does not allow for unambiguous interpretation of the data (for an excellent recent review and criticism of the literature see Ref. 2). It is particularly difficult to distinguish between vesicle fusion with cells and absorption of liposomes to

cells using many of the methodologies in current use. If we want to be able to maximize the therapeutic benefits of liposome-associated drugs, an understanding of the principal mechanisms of liposome-cell interactions becomes important. We described here an experimental system which should be useful in answering some of these questions. We have shown that the cytotoxicity observed in the EMT6 and S49 cells appears to be due to active compound which has leaked out of liposomes and has been subsequently taken up by cells by a transport mechanism. We could not demonstrate any directly mediated uptake of liposome-entrapped drugs for the cell lines examined for a variety of liposome sizes and compositions.

What does this mean for the future of liposome-entrapped drugs? Many authors are in agreement that substantial numbers of liposomes will be absorbed to the surface of cells (for reviews, see Refs. 1 and 2). Since this appears to be a major mechanism of liposome-cell interaction we should perhaps try to take advantage of this. Absorbed liposomes as they become leaky under the influence of serum proteins or cell surface proteins can lead to a locally high concentration of drug near the cell surface. When this drug is taken up into cells by passive diffusion or transport the resulting effect could be equal to or greater than that observed for a similar final concentration of free drug, uniformly distributed. Some of the observations of increased toxicity of liposome-entrapped antineoplastic drugs could be due to this mechanism. We can try to increase the numbers of liposomes absorbed to cell surfaces by taking advantage of charge-charge interactions. Inclusion of stearylamine in small percentages in liposomes may increase the chances of their being absorbed to negative cell surfaces. We can also try to increase the absorption to the cell surface by incorporating specific targeting agents on the liposome surface. This may not only increase the number of locally absorbed liposomes, but also may have the advantage of keeping them away from cells where pharmacological effects are not wanted. Weinstein et al. [24] have reported that significantly greater numbers of antibody-labelled liposomes bound to target cells as compared to controls, but they reported no increase in the amount of vesicle contents delivered to the cell interior. This problem could be overcome by designing liposomes with specific leakage properties, for example, those described by Weinstein et al. [25,26] composed of dipalmitoyl phosphatidylcholine/distearoyl phosphatidylcholine (7 : 3) which go through a phase transition and release their contents when exposed to local hyperthermia (42°C). Charged liposomes may, in some cases, by changing cell surface pH, for example, be able to increase the transport of drugs across the cell membrane. Fry et al. [27] have reported that positively charged liposomes increase the influx of methotrexate into Ehrlich ascites tumor cells, whereas negative liposomes reduce methotrexate influx.

Cells which are actively endocytotic present less of a problem. A recent experiment by Post et al. [28] has shown that liposomes containing entrapped macrophage-activating factor were able to activate mouse macrophages at concentrations 20 000-times lower than free macrophage-activating factor. These experiments were carried out the presence of a potent inhibitor of free macrophage-activating factor which prevented activation caused by leaky liposomes. However, the problem of degradation of compounds in the liposomal apparatus

of the cell may still prevent the introduction of some classes of compounds into cells by this route.

One of the major problems to be overcome is how to kill cells which have developed drug resistance, for example, the transport-resistant cell line, AE_1, used in our experiments. Neither free drug nor liposome-entrapped drug was effective against these cells. More work needs to be done in the area of developing methodologies, e.g., fusing liposomes with cells, in order to overcome resistance in a case such as this.

Acknowledgements

I would like to thank Dr. A.R.P. Paterson for his generous gifts of chemicals, for a critical reading of the manuscript, and for his invaluable help and encouragement through this project. I would like to thank Dr. D. Chapman for help and advice in culturing EMT6 cells and Dr. C.E. Cass for her help and advice with the S49 and AE_1 cell lines. Dr. H. Baer is thanked for his original suggestion for using NBMPR as a transport inhibitor and thanks to Dr. M. Poznansky for helpful criticisms of the manuscript. E. Gyorffy and S. Musolf were recipients of Summer Research Awards from the Faculty of Medicine, University of Alberta. This work has been supported by a grant from the National Cancer Institute of Canada.

References

1 Pagano, R.E. (1978) Annu. Rev. Biophys. Bioeng. 7, 435—468
2 Poste, G. (1980) in Liposomes in Biological Systems (Gregoriadis, G. and Allison, A.C., eds.), pp. 101—151, John Wiley and Sons, New York
3 Allen, T.M. and Cleland, L. (1980) Biochim. Biophys. Acta 597, 418—426
4 Kirby, C., Clarke, J. and Gregoriadis, G. (1980) FEBS Lett. 111, 324—328
5 Scherphof, G., Morselt, H., Regts, J. and Wilschut, J. (1979) Biochim. Biophys. Acta 556, 196—207
6 Cass, C.E., Dahlig, E., Lau, E.Y., Lynch, T.P. and Paterson, A.R.P. (1979) Cancer Res. 39, 1245—1252
7 Paterson, A.R.P. (1981) in Molecular Actions and Targets for Chemotherapy (Sartorelli, A.C., ed.), Academic Press, New York, in the press
8 Paterson, A.R.P., Kolassa, N. and Cass, C.E. (1981) Pharmacol. Ther., in the press
9 Cohen, A., Ullman, B. and Martin, D.W., Jr. (1979) J. Biol. Chem. 254, 112—116
10 Rockwell, S.C., Kallman, R.F. and Fajardo, L.F. (1972) J. Natl. Cancer Inst. 49, 735—747
11 Horibata, K. and Harris, A.W. (1970) Exp. Cell Res. 60, 61—77
12 Paterson, A.R.P., Yang, S.E., Lau, E.Y. and Cass, C.E. (1979) Mol. Pharmacol. 16, 900—908
13 Szoka, F., Jr. and Papahadjopoulos, D. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 4194—4198
14 Dittmer, J.C. and Wells, M.A. (1969) Methods Enzymol. 14, 489
15 Szoka, F.C., Jacobson, K. and Papahadjopoulos, D. (1979) Biochim. Biophys. Acta 551, 295—303
16 Papahadjopoulos, D., Poste, G. and Mayhew, E. (1974) Biochim. Biophys. Acta 363, 404—418
17 Papahadjopoulos, D., Poste, G., Vail, J.W. and Biedler, J. (1976) Cancer Res. 36, 2988—2994
18 Podhajcer, O.L., Friedlander, M. and Gaziani, Y. (1980) Cancer Res. 40, 1344—1350
19 Todd, J.A., Levine, A.M. and Tokes, Z.A. (1980) J. Natl. Cancer Inst. 64, 715—719
20 Dunnick, J.K., Kallman, R.F. and Kress, J.P. (1976) Biochem. Biophys. Res. Commun. 73, 619—624
21 Huang, L. and Pagano, R.E. (1975) J. Cell Biol. 67, 38—48
22 Adams, D.H., Joyce, G., Richardson, V.J., Ryman, B.E. and Wisniewski, H.M. (1977) J. Neurol. Sci. 31, 173—179
23 Suhadolnik, R.J. (1979) Nucleosides as Biological Probes, pp. 158—169, John Wiley and Sons, New York
24 Weinstein, J.N., Blumenthal, R., Sharrow, S.O. and Henkart, P. (1978) Biochim. Biophys. Acta 509, 272—288
25 Weinstein, J.N., Magin, R.L., Yatvin, M.B. and Zaharko, D.S. (1979) Science 204, 188—191
26 Weinstein, J.N., Magin, R.L., Cysyk, R.L. and Zaharko, D.S. (1980) Cancer Res. 40, 1388—1395
27 Fry, D.W., White, J.C. and Goldman, I.D. (1979) J. Membrane Biol. 50, 123—140
28 Poste, G., Kirsh, R., Fogler, W.E. and Fidler, I.J. (1979) Cancer Res. 39, 881—892
29 Allen, T.M. (1981) Biochim. Biophys. Acta 640, 385—397