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The effects of liposome size and surface charge on liposome-mediated delivery of methotrexate-γ-aspartate to cells in vitro

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We have studied the liposome-mediated delivery of methotrexate-γ-aspartate to five cell lines. The sensitivity of the cells to encapsulated drug varies widely in accordance with their ability to take up the liposomes. CV1-P cells can be 150-times more sensitive to encapsulated methotrexate-γ-aspartate than to free drug, while AKR/J SL2 cells are only twice as sensitive to the encapsulated drug. Negatively-charged liposomes are much more efficient for delivery than are neutral liposomes, and cholesterol is an essential component of the liposome membrane for optimal drug delivery. The optimal liposome size for drug delivery is 0.1 μm, although the amount of cell-associated lipid is the same for all liposome sizes. The effect of the encapsulated drug is inhibited by NH_4Cl, suggesting an endocytic mechanism for delivery. The potency of the encapsulated drug is not affected by wide variations in the drug:lipid ratio.

Introduction

The mechanism by which drug enters cells is one of the most important considerations for optimizing liposome-mediated drug delivery [1]. When in contact with cells [2] and plasma components [3], liposomes release their encapsulated contents at rates that depend on their phospholipid composition and cholesterol content. Alternatively, cells can take up liposomes by endocytosis, process them in the lysosomal compartment, and transfer their aqueous contents to the cytoplasm [4]. The relative importance of these two processes for drug delivery depends on the stability of the liposomes, the efficiency with which cells take up the liposomes, the transport properties of the drug, and the stability of the drug in the lysosomal milieu. Drugs that can enter cells rapidly as free compounds will do so after leakage from liposomes, and may be defined as liposome-independent agents. Drugs that cannot pass readily through the plasma membrane will enter cells through uptake of the liposomes, and have been defined as liposome-dependent agents [5].

The in vivo potency of drugs may be improved by either delivery mechanism. Liposome-independent drugs may exhibit enhanced effects through controlled release [6,7], or through increased localisation in target organs [8,9]. Liposome-dependent drugs are potentially capable of highly specific effects, especially if used with ligand-directed liposomes. However, they are less well studied, and their potency depends on the efficient uptake of the liposomes by the target cells.

In vitro studies can readily distinguish between liposome-dependent and liposome-independent drugs. Liposome-dependent drugs can be delivered selectively by antibody-directed liposomes [10,11,12]. In some cases, encapsulated liposome-

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dependent drugs can be more effective than the free drug [11]. In contrast, an encapsulated liposome-independent drug will show potency that is equal or less than that of the free drug. A liposome-independent drug is unlikely to show specific in vitro effects when delivered to target cells in ligand-directed liposomes. Cytosine arabinoside is a typical example of a drug that acts solely through its leakage from liposomes [13] and shows no specific effects in targeted liposomes [12]. Indeed, most known drugs are liposome-independent, because their activity relies on rapid flux through the cell membrane.

The identification of liposome-dependent drugs is possible for the pteridine antifolates, because detailed information is available about their transport and dihydrofolate reductase-inhibitory properties. Methotrexate-γ-aspartate is an effective dihydrofolate reductase inhibitor [14] that enters cells 100-times less effectively, and is 160-times less toxic, than methotrexate. When encapsulated in antibody-directed liposomes [11], methotrexate-γ-aspartate was 10-times more effective than the free drug. The discovery of the liposome-dependency of methotrexate-γ-aspartate shows the potential of selecting compounds by evaluating their transport properties.

Negatively-charged liposomes have proved able to interact with cells, and to deliver polio RNA [15], carboxyfluorescein [4], SV40 DNA [16] or gelonin [17] more effectively than neutral liposomes. The efficiency for delivery, ease of preparation, and versatility of negatively-charged liposomes prompted us to evaluate their use for the in vitro discovery of liposome-dependent drugs. In this report we describe the delivery of methotrexate-γ-aspartate in phosphatidylserine liposomes, showing that these vesicles are effective for delivery to a wide range of cell types. We also describe the effects of liposome size, and drug : lipid ratio on the potency of drug delivery.

Materials and Methods

Phosphatidylcholine, phosphatidylserine and cholesterol were purified and stored as previously described [7]. [^{3}H]Dipalmitoylphosphatidylcholine was prepared by New England Nuclear and purified by thin-layer chromatography within 1 month of use. Methotrexate-γ-aspartate was synthesised and provided by J.R. Piper, Southern Research Institute, Birmingham, AL [14]. Sterile solutions of free or encapsulated drug were stored at 4°C, and used within 1 month of preparation. Carboxyfluorescein was obtained from Eastman Chemical Co. (Rochester) and purified with Sephadex LH-20 [18]. Methotrexate-γ-aspartate solutions were prepared for encapsulation at 0.5 mM, 5 mM or 50 mM drug in 50 mM morpholinoethanesulfonic acid/50 mM morpholinopropanesulfonic acid and adjusted to pH 6.7 with sodium hydroxide. The tonicity of the solutions were adjusted to 290 mosmol/kg with NaCl. 0.5 mM methotrexate-γ-aspartate solutions also contained 20 mM carboxyfluorescein. Liposomes were prepared under sterile conditions. Large unilamellar liposomes were prepared in 0.5–50 mM drug by reverse-phase evaporation [19], and were extruded through polycarbonate filters [20]. Small unilamellar liposomes were prepared in a 12×120 mm tube (Kimax) from 20 μmol lipid and 0.4 ml 50 mM methotrexate-γ-aspartate. The tube was purged with argon, and sonicated for 1 h in a bath-type sonicator (Laboratory Supply Co., Hicksville, NY).

Liposomes were separated from unencapsulated drug by gel chromatography with a sterile 1×15 cm Sephadex G-75 (Pharmacia) column. The column was eluted with sterile 50 mM morpholinoethanesulfonic acid/50 mM morpholinopropanesulfonic acid/NaCl (pH 6.7), 290 mosmol/kg. Drug concentration in the liposomes was determined after extracting a portion of liposomes at pH 7.5 [21]. The drug partitioned into the upper phase, and was measured assuming a molar extinction coefficient of 7943 at 370 nm. For liposomes prepared in 0.5 mM drug, the concentration was estimated from the carboxyfluorescein concentration, assuming that both drug and dye were comparably captured. The carboxyfluorescein concentration was determined after extraction assuming a molar extinction coefficient of 70000 at 493 nm. Lipid content was measured by phosphorus analysis [22]. For cell association experiments, liposomes were prepared with $4 \cdot 10^6$ cpm [^{3}H]dipalmitoylphosphatidylcholine per μmol lipid. ^{3}H content was measured by scintillation counting using Liquiscint scintillant (National Diagnostics).

AKR/J SL2, a T-lymphoma derived from

AKR/J mice [23], was provided by R.C. Nowinsky, Seattle. Cl 18, a myeloma derived from the C3H mouse, and RAW 264, a macrophage tumor derived from an Abelson leukemia virus-treated Balb/c mouse [24], were obtained from M. Cohn, San Diego. L929, a murine fibroblast from the C3H mouse, was obtained from L.B. Epstein, San Francisco. CV1-P, an African green monkey kidney cell line [25], was obtained from P. Berg, Stanford University (Palo Alto). Cells were grown in Dulbecco's modified Eagle's minimal essential medium, with 100 units/ml penicillin and streptomycin. The medium was supplemented with 10% fetal calf serum (K-C Biologicals) for AKR/J SL2, Cl 18, RAW 264, 5% fetal calf serum for L929 and 5% newborn calf serum (Gibco) for CV1-P.

Cells were plated for growth inhibition at $3 \cdot 10^4$ cells per well in 24-well plates (Costar). Triplicate wells were treated with drug after overnight incubation. Control wells were treated with buffer alone. Three wells were counted at the time of treatment to give the original cell concentration. The cells were allowed to grow for 48 h and exhibited varying growth rates. AKR/J SL2 control wells grew from $1 \cdot 10^5$ cells/ml to $1 \cdot 10^6$ cells/ml. L929, RAW 264 and Cl 18 cells grew from $8 \cdot 10^4$ cells/ml to $6 \cdot 10^5$ cells/ml. CV1-P cells grew from $5 \cdot 10^4$ cells/ml to $3 \cdot 10^5$ cells/ml. After growth the cells were counted with a Coulter counter, model Fn. AKR/J SL2, Cl 18 and RAW 264 were resuspended directly in medium, diluted 1/50 in isotonic counting fluid (American Scientific Products) and counted. L929 and CV1-P cells were freed of medium and resuspended by treatment with 1 ml of 0.05% trypsin in phosphate-buffered saline/1 mM EDTA solution at 37°C for 10 min. The cell suspension was diluted 1 : 50 with isotonic counting fluid and counted. Percent growth was determined according to the equation:

$$\% \text{ growth} = \frac{[\text{sample count} - \text{original count}]}{[\text{control count} - \text{original count}]} \times 100$$

The mean percent growth was plotted against the $\log_{10}$ of the drug concentration. The concentration of drug required to produce 50% inhibition of growth (IC_{50}), was determined from the plots.

Association of liposomes with adherent cells was studied by plating cells in 60-mm dishes containing 3 ml medium. The cells were grown to 10^6 cells per dish (confluent monolayers contain $5 \cdot 10^6$ cells). The medium was aspirated and 0.5 ml of fresh medium with serum was added. Radiolabelled liposomes were then added in 0.025 ml phosphate-buffered saline and the cells were incubated for 1 h. The plates were aspirated and washed four times with phosphate-buffered saline containing 0.36 mM $CaCl_2$ and 0.42 mM $MgCl_2$. The cells were removed with 0.25 ml 0.25% trypsin and transferred to scintillation vials. The dishes were washed once and the wash was added to the cell suspension. The combined solution was counted (20 min counting or 10000 accumulated counts per sample) after addition of scintillant. Control experiments were done to establish whether liposomes bound to the dishes and eluted with trypsin. Plates containing medium alone were incubated, washed and trypsinised as described.

Non-adherent cells were harvested by centrifugation from medium, and were resuspended in fresh medium with serum at 10^7 cells per ml. Cells were dispensed in 0.2 ml portions into 5 ml disposable tubes. Liposomes were added in 0.01 ml phosphate-buffered saline, and the mixture was incubated for 1 h at 37°C. Cells were layered over 2 ml 10% (w/v) Dextran T40 in phosphate-buffered saline with 1% bovine serum albumin in a fresh tube. The cells were pelleted through the dextran by centrifugation at $1000 \times g$ for 10 min. The supernatant was aspirated and the cells were transferred to scintillation vials after resuspension in buffer. Association data were analysed to determine the amount of lipid associated per cell as a function of lipid concentration. The data was fitted by regression analysis. The slope, r^2, and two coordinates are presented in the tables.

Results and Discussion

Growth inhibitory effects of non-loaded liposomes

Tables I–III show the growth inhibitory properties of various types of liposome that do not contain drug. Growth of AKR/J SL2 cells is inhibited by all liposomes that contain phosphatidylserine. Neither the size of the liposomes nor the presence of cholesterol appreciably changes the growth inhibitory effects on this cell line. The growth of all other cell lines tested was inhibited to a lesser extent by phosphatidylserine-cholesterol

(67 : 33) liposomes of all sizes (small unilamellar vesicles, SUV, 0.1 μm, REV). Phosphatidylserine liposomes were more growth inhibitory than phosphatidylserine-cholesterol (67 : 33) liposomes. Phosphatidylserine-cholesterol (50 : 50) liposomes were highly growth inhibitory, particularly for Cl 18, CV1-P and RAW 264. Phosphatidylglycerol-cholesterol (67 : 33) liposomes had little growth inhibitory effect on AKR/J SL2 or L929, but were quite growth inhibitory for Cl 18, CV1-P and RAW 264. Phosphatidylcholine-cholesterol (50 : 50) liposomes had no detectable growth inhibitory effects on any cell line.

The growth inhibitory effects of liposomes are probably due to an effect of the lipid after cellular uptake. The mechanism of growth inhibition by phosphatidylserine and phosphatidylglycerol is unknown. Cholesterol is known to transfer rapidly to cells from liposomes that contain more cholesterol than the plasma membrane of the cell [26]. Cholesterol loading may modulate cell growth, and may explain the growth inhibitory effects of phosphatidylserine-cholesterol (50 : 50) liposomes. Conversely, the growth inhibitory effects of phosphatidylserine liposomes may be caused by depletion of cellular cholesterol. The failure of phosphatidylcholine-cholesterol (50 : 50) to inhibit growth at the concentrations used may be due to lesser efficiency with which these liposomes bind to cells (see below). The extent of binding may affect the rate or extent of cholesterol exchange.

TABLE I

GROWTH INHIBITORY EFFECTS OF NON-LOADED LIPOSOMES: EFFECT OF LIPOSOME SIZE

The IC_{50} is the concentration of lipid which inhibits growth by 50%. All liposomes were prepared from phosphatidylserine-cholesterol (67 : 33).

Cell type	IC_{50} (mM)		
	REV [a]	0.1 μm [b]	SUV [c]
AKR/J SL2	0.05	0.06	0.05
L929	> 0.40	> 0.40	0.24
Cl 18	0.19	0.20	0.14
RAW 264	0.30	> 0.40	0.23
CV1-P	n.d.	> 0.40	> 0.40

[a] Prepared by reverse phase evaporation, not extruded.
[b] Extruded to 0.1 μm.
[c] Sonicated vesicles.

TABLE II

GROWTH INHIBITORY EFFECTS OF NON-LOADED LIPOSOMES: EFFECT OF CHOLESTEROL (chol) CONTENT

The IC_{50} is the concentration of lipid which inhibits growth by 50%. All liposomes were prepared from phosphatidylserine by reverse-phase evaporation and were not extruded.

Cell type	IC_{50} (mM)		
Mol chol/ mol lipid:	0	33	50
AKR/J SL2	0.05	0.05	0.04
L929	0.20	> 0.40	0.18
CL 18	0.11	0.19	0.03
RAW 264	0.21	0.30	0.07
CV1-P	0.16	n.d.	0.08

The growth inhibition produced by non-loaded liposomes necessitates care in interpreting growth inhibition by drug-loaded liposomes. We have calculated the drug : lipid ratio for all of our liposome preparations. If the lipid concentration at the drug IC_{50} exceeds the IC_{10} for empty liposomes of the same composition and size, a '+' has been placed next to the value to indicate some involvement of lipid in the growth inhibition. If the lipid concentration at the IC_{50} equals or exceeds the IC_{50} for comparable empty liposomes, the value has been marked '++' to indicate that most or all growth inhibition may be due to the lipid.

TABLE III

THE GROWTH INHIBITORY EFFECTS OF NON-LOADED LIPOSOMES: EFFECT OF PHOSPHOLIPID COMPOSITION

The IC_{50} is the concentration of lipid which inhibits growth by 50%. All liposomes were prepared by reverse-phase evaporation and were not extruded. The vesicles contained 33 mol per 100 mol cholesterol. PS, phosphatidylserine; PG, phosphatidylglycerol; PC, phosphatidylcholine.

Cell type	IC_{50} (mM)		
	PS	PG	PC
AKR/J SL2	0.05	0.37	> 0.40
L929	> 0.40	> 0.40	> 0.40
Cl 18	0.19	0.08	> 0.40
RAW 264	0.30	0.10	> 0.40
CV1-P	n.d.	0.04	> 0.40

Growth inhibitory effects of encapsulated methotrexate-γ-aspartate

Table IV shows the IC_{50} of free or encapsulated methotrexate-γ-aspartate for the five cell lines. The free drug has a similar growth inhibitory potency for all cell lines. Encapsulation in phosphatidylserine-cholesterol liposomes increases the potency of the drug by 2–38-fold, depending on the cell type. AKR/J SL2 show a limited sensitivity to drug in phosphatidylserine-cholesterol liposomes, and there is a partial involvement of the lipid in the growth inhibition. Phosphatidylserine-cholesterol liposomes are much more effective than phosphatidylcholine-cholesterol liposomes for drug delivery to all cell lines except RAW 264, which is affected equally by the two preparations. Previous studies of SV40 DNA [16] and carboxyfluorescein [4] delivery to CV1-P cells have demonstrated that negatively-charged liposomes are more effective for delivery than neutral liposomes.

Encapsulation in pure phosphatidylserine liposomes produces only a small increase in drug potency. This result suggests that the presence of cholesterol is important for efficient drug delivery. Cholesterol also improves the delivery of SV40 DNA by liposomes of certain compositions [27], and reduces leakage of liposome contents that occurs when liposomes are incubated with serum [3,13] or cells [2]. These results emphasize that minimizing such leakage is important for effective drug delivery.

Liposome size and drug delivery

Unextruded reverse phase evaporation vesicles have diameters between 0.08 and 1 μm [20]. In order to define the optimal liposome size for drug delivery, we prepared reverse-phase evaporation vesicles extruded through 0.2 or 0.1 μm polycarbonate membranes, and small vesicles prepared by extensive sonication. The results in Table V show that the reduction of vesicle size by extrusion to 0.2 and 0.1 μm increases the growth inhibitory potency of the drug for all cell lines tested. Sonicated vesicles are less effective than vesicles extruded to 0.1 or 0.2 μm, but more effective than unextruded vesicles. The lesser potency of sonicated vesicles may be due to a high leakage rate for these liposomes at the cell surface, or to the larger number of vesicles which must be taken up for drug delivery. It is interesting that RAW 264, a phagocytic tumor cell line, should show the same size dependence for optimal drug delivery as all other cell lines. This suggests either that phagocytosis is not the mechanism for liposome uptake by RAW 264 cells, or that the efficiency of phagocytosis and adsorptive pinocytosis are comparably dependent on the size of the vesicles.

Our data suggests that the optimal size of nega-

TABLE IV

GROWTH INHIBITORY POTENCY OF ENCAPSULATED METHOTREXATE-γ-ASPARTATE

Cell type	IC_{50} (μM)			
	free drug	PS [a]	PS-chol. [b]	PC-chol [c]
AKR/J SL2	0.59	0.29	0.31	1.0
		(−)	(+)	(−)
L929	0.56	0.47	0.05	1.8
		(−)	(−)	(−)
Cl 18	1.10	0.48	0.07	1.0
		(−)	(−)	(−)
RAW 264	0.76	0.25	0.08	0.07
		(−)	(−)	(−)
CV1-P	0.64	n.d.	0.02	n.d.
			(−)	

Methotrexate-γ-aspartate was encapsulated using an original concentration of 5 mM. Drug:lipid ratios (mmol/mol) were [a] 43; [b] 10; [c] 9. Liposome compositions were [a] phosphatidylserine; [b] phosphatidylserine-cholesterol (67:33); [c] phosphatidylcholine-cholesterol (67:33). The liposomes were prepared by reverse-phase evaporation and extruded to 0.1 μm. Growth inhibition by lipid is indicated by (−), no lipid effects; or (+), some lipid effect.

tively charged vesicles for delivery is 0.05–0.1 μm. Liposome uptake may be mediated by coated pits, which have a diameter of 0.15 μm [28]. This observation is consistent with previous reports by Matthay et al. [29] and Machy and Leserman [30] on drug delivery by antibody-targeted liposomes of different sizes. In both of these studies, lymphoma cells were sensitive to drug delivery by sonicated (0.05–0.07 μm) liposomes, but not by larger (0.4 μm) vesicles. Machy and Leserman [30] also showed that L929 cells were more sensitive to drug delivery by small antibody-directed liposomes. Our results are also consistent with recent electron microscopic observations on the uptake of liposomes by CV1-P cells [4], where the liposomes found in intracellular vesicles had a mean diameter of 0.1 μm, while those adsorbed to the surface had a larger mean diameter.

Effect of drug : lipid ratio

It is clear from several reports that the most likely mechanism of drug delivery involves the endocytosis of the liposomes [4,10–12,29,30]. Machy and Leserman [30] have concluded that smaller liposomes are more effective for drug delivery, because they are more readily endocytosed by cells. However, the amount of drug in each liposome may also be an important determinant for optimal delivery, and is closely interrelated with the liposome size. At a fixed initial drug concentration, the amount of drug captured within a liposome is proportional to the cube of its radius. Consequently, a liposome whose diameter is 0.1 μm will contain 64-times less drug than a liposome whose diameter is 0.4 μm.

Drug : lipid ratio may be examined independently by preparing vesicles of defined size in initial drug solutions of different concentration. Table VI shows that the potency of methotrexate-γ-aspartate encapsulated in 0.1 μm extruded vesicles is very similar at the three different drug : lipid ratios of 0.23, 8.5 and 80 mmol/mol. The result is most striking for CV1-P cells where the 300-fold variation in drug : lipid ratio is accompanied by only a 1.5-fold variation in the IC_{50}. The failure of potency to change with drug : lipid ratio confirms that liposome size is the major determinant of optimal drug delivery.

The data in Table VI also emphasize the importance of knowing the drug : lipid ratio for interpreting the cause of growth inhibition. On all cell lines except CV1-P, the preparation that contained 0.23 mmol/mol had growth inhibitory effects that could be attributed to lipid. For AKR/J SL2 cells,

TABLE V

GROWTH INHIBITORY POTENCY OF METHOTREXATE-γ-ASPARTATE IN LIPOSOMES OF VARIOUS SIZES

Cell type	IC_{50} (μM)				
	free drug	REV [a]	0.2 μm [b]	0.1 μm [c]	SUV [d]
AKR/J SL2	0.62	0.52	0.37	0.28	0.29
		(+)	(++)	(+)	(+)
L929	0.86	0.17	0.07	0.04	0.13
		(−)	(−)	(−)	(−)
CL 18	1.10	0.43	0.09	0.06	0.16
		(−)	(−)	(−)	(−)
RAW 264	0.76	0.19	0.09	0.05	0.07
		(−)	(−)	(−)	(−)
CV1-P	0.64	0.02	0.005	0.004	n.d.
		(−)	(−)	(−)	

[a] Prepared by reverse phase evaporation, no extrusion, from phosphatidylserine-cholesterol (50 : 50), drug : lipid ratio = 26 mmol/mol. [b] As in [a], except extruded to 0.2 μm and drug : lipid ratio = 9 mmol/mol. [c] As in [a], except extruded to 0.1 μm and drug : lipid ratio = 6 mmol/mol. [d] Prepared by sonication of hand-shaken phosphatidylserine-cholesterol (67 : 33) liposomes, drug : lipid ratio = 10 mmol/mol. Liposomes were prepared in 5 mM drug solution except for SUV, which were prepared in 50 mM drug solution. Growth inhibition by lipid is indicated by (−), no lipid effect; (+), some lipid effect; (++), growth inhibition totally due to lipid.

TABLE VI

THE EFFECT OF DRUG : LIPID RATIO ON THE GROWTH INHIBITORY POTENCY OF ENCAPSULATED METHOTREXATE-γ-ASPARTATE

Liposomes were prepared from phosphatidylserine-cholesterol (50 : 50) by reverse-phase evaporation and extruded to 0.1 μm. The original drug concentrations for the liposome preparations were 0.23 mmol/mol: 0.5 mM (with 30 mM carboxyfluorescein); 8.5 mmol/mol: 5 mM; 80 mmol/mol: 50 mM. After separation of non-encapsulated drug, the drug : lipid ratio was as indicated. Growth inhibition by lipid is indicated by (−), no lipid effects; (+), some lipid effects; (++), growth inhibition totally due to lipid.

Cell type	IC_{50} (μM)			
	free drug	0.23	8.5	80
AKR/J SL2	0.7	0.017 (++)	0.46 (++)	0.28 (−)
L929	1.3	0.049 (+)	0.047 (−)	0.032 (−)
RAW 264	0.74	0.023 (+)	0.11 (−)	0.19 (−)
CV1-P	0.75	0.005 (−)	0.007 (−)	0.005 (−)

the apparently low IC_{50} is not due to the drug, because the lipid concentration at the IC_{50} (0.074 mM) is twice the IC_{50} for empty liposomes.

Effect of ammonium chloride

In previous studies [11] the effects of methotrexate-γ-aspartate in antibody-conjugated liposomes was inhibited by NH_4Cl. Table VII shows similar results for negatively charged liposomes. For CV1-P cells and L929 cells, 5 mM NH_4Cl inhibited the potency of encapsulated drug, but not of the free drug. NH_4Cl is known to elevate the lysosomal pH, and inhibition of drug delivery suggests that endocytosis is involved in the effective delivery of the drug. For AKR/J SL2, Cl 18 and RAW 264 cells, NH_4Cl had no effect on either encapsulated or free drug potency. This may have occurred because it was necessary to use concentrations that may be only minimally effective in elevating lysosomal pH. These low concentrations were necessary because some cell lines were sensitive to growth inhibition by NH_4Cl. For AKR/J SL2 cells, control growth even in the presence of 5 mM NH_4Cl was only 15% of normal levels, and interpretation of the resultant drug growth inhibition curves is very difficult.

TABLE VII

THE EFFECT OF NH_4Cl ON THE GROWTH INHIBITORY POTENCY OF FREE AND ENCAPSULATED METHOTREXATE-γ-ASPARTATE

Methotrexate-γ-aspartate was encapsulated at 5 mM original concentration in phosphatidylserine-cholesterol (67 : 33) REV extruded to 0.2 μm, final drug : lipid ratio = 10 mmol/mol. Cells were incubated with (+) or without (−) 5 mM NH_4Cl, which was added 30 min before drug addition.

Cell type	IC_{50} (μM)				Growth inhibition by NH_4Cl (% control)
	free drug		encapsulated		
	−	+	−	+	
AKR/J SL2	0.45	1.7	0.22	0.42	87
L929	1.4	1.1	0.20	0.84	24
Cl 18	0.53	0.64	0.19	0.20	15
RAW 264	0.4	0.54	0.09	0.07	24
CV1-P	1.3	1.3	0.03	1.1	57

TABLE VIII

THE ASSOCIATION OF UNEXTRUDED LIPOSOMES WITH CELLS

Liposomes were prepared by reverse phase evaporation, and were not extruded. Duplicate samples of cells were incubated with 15, 50, 150, and 500 μM lipid as described in Materials and Methods. A regression analysis of the logarithmically transformed data was fitted to a straight line by the method of least squares in order to obtain the slope of the line, correlation coefficient (r^2), and level of cell association at 15 and 500 μM lipid.

Cell type	Lipid [a]	Slope	r^2	amol/cell	
				15 μM	500 μM
AKR/J SL2	PC	0.846	0.897	13	243
	PS	0.967	0.993	39	1168
L929	PC	0.618	0.960	115	1002
	PS	0.784	0.980	905	14158
Cl 18	PC	0.916	0.981	62	1548
	PS	1.009	0.995	84	2910
RAW 264	PC	0.843	0.977	216	4152
	PS	1.141	0.995	97	5346
CV1-P	PC	0.850	0.956	29	571
	PS	0.987	0.986	1061	33778
Plate [b]	PC	0.499	0.963	65	374
	PS	0.974	0.942	254	7722

[a] The liposomes were prepared from phosphatidylserine-cholesterol (50:50) (PS) or phosphatidylcholine-cholesterol (50:50) (PC) with $2 \cdot 10^6$ cpm [^{3}H]dipalmitoylphosphatidylcholine.

[b] Empty plate incubations were done as controls for L929 and CV1-P, and the 'amol/cell' was calculated assuming the presence of 10^6 cells per dish.

Association of liposomes with cells

Further insight into the mechanism of drug delivery may be gained by examining the association of liposomes with cells. Tables VIII and IX summarize cell uptake of liposomes in 1 h of incubation at 37°C. In all cases, the extent of liposome association with cells appears dependent on lipid concentration, and saturation of uptake

TABLE IX

THE ASSOCIATION OF EXTRUDED OR SONICATED PHOSPHATIDYLSERINE LIPOSOMES WITH CELLS

Liposomes were prepared either by reverse phase evaporation from phosphatidylserine-cholesterol (50:50) with $2 \cdot 10^6$ cpm [^{3}H]dipalmitoylphosphatidylcholine with extrusion to 0.1 μm (0.1), or by sonication of hand-shaken vesicles of the same composition (SUV). Experimental procedures and data analysis are described in Materials and Methods and Table VIII.

Cell type	Size of liposomes	Slope	r^2	amol/cell	
				15 μM	500 μM
AKR/J SL2	0.1	0.960	0.983	36	1035
	SUV	1.063	0.991	42	1754
L929	0.1	0.783	0.921	532	7080
	SUV	0.785	0.993	471	7380
Cl 18	0.1	0.933	0.982	74	1938
	SUV	1.011	0.981	122	4246
RAW 264	0.1	0.906	0.993	133	3191
	SUV	0.929	0.997	126	3268
CV1-P	0.1	0.667	0.984	2626	27231
	SUV	0.559	0.969	1315	9354

TABLE X

A COMPARISON OF LIPOSOME-CELL ASSOCIATION AND EFFICIENCY OF DRUG DELIVERY

The regressed association curves for 0.1 μm phosphatidylserine-cholesterol (50:50) vesicles were used to calculate the relative cell uptake at 15 and 500 μM lipid. The values are expressed relative to the comparable value for AKR/J SL2 cells. The values for drug delivery are derived from Tables V for 0.1 μm phosphatidylserine-cholesterol (67:33) vesicles; drug:lipid ratio = 80 mmol/mol. The values of relative drug delivery are expressed relative to that of AKR/J SL2 by calculating the ratio of the IC_{50} for AKR/J SL2 to that of the other cell line.

Cell type	Relative cell uptake		Relative drug delivery
	15 μM	500 μM	
AKR/J SL2	1.0	1.0	1.0
L929	14.8	6.8	8.8
RAW 264	3.7	3.1	3.3
CV1-P	73.0	26.3	53.8

does not appear to occur at the highest lipid concentrations studied. Some association curves showed a slope of 1 when plotted on a log-log scale, indicating that the same fraction of the liposomes associates with the cells at high and low lipid concentrations. In some cases, however, the slope of the log-log plot was less than 1, indicating that a larger proportion of liposomes associated with the cells at lower concentrations than at higher concentrations. The inclusion of metabolic inhibitors has very little effect on the extent of liposome-cell association (data not shown). This suggests that the majority of liposomes that associate with the cells in 1 h are adsorbed to the plasma membrane.

CV1-P, L929, Cl 18 and AKR/J SL2 cells take up neutral vesicles to a much lesser extent than negatively-charged liposomes (Table VIII). However, RAW 264 take up neutral and negatively charged vesicles with equal efficiency. These results correlate well with the relative potency of neutral and negatively charged vesicles for drug delivery to this cell line. The association of unextruded, 0.1 μm, or sonicated phosphatidylserine-cholesterol liposomes with cells appears comparable (cf. Tables VIII and IX). Others [29,30] have also observed that small and large antibody-conjugated liposomes associate with cells to a similar extent. Presumably, the initial adsorption of the liposomes to the cell membrane is not size-dependent, while the endocytosis of small vesicles is more effective.

In contrast to the observations on liposomes of different size, a correlation of uptake and delivery among the various cell lines can be established with 0.1 μm vesicles and is summarized in Table X. The relative cell uptake of lipid varies with the lipid concentration. This arises because the slope of the association curves are different for the various cell lines. The relative sensitivity of the cell lines to drug delivery is in all cases greater than the relative cell uptake at 500 μM lipid, and smaller than relative cell uptake at 15 μM lipid. Therefore, despite the discrepancies which may arise from the leakage of liposome contents, and the loss of lipid markers through exchange, there appears to be a correlation of uptake and drug delivery.

Concluding remarks

The potency of a liposome-dependent drug is affected by liposome charge and size, and varies among different cell lines. Negatively charged liposomes associate more effectively and deliver their contents more effectively than neutral liposomes. Smaller liposomes deliver drug more effectively than larger liposomes, presumably because they are internalised more efficiency. Optimal delivery is observed with negatively charged liposomes of 0.05–0.1 μm diameter.

Retention of liposome contents is an important factor in effective drug delivery. The loss of contents from vesicles adsorbed to the cell membrane [2] reduces considerably the efficiency of delivery. In addition, serum induced leakage from liposomes [3,13] will reduce the efficiency of the liposomes if growth inhibition requires continued drug deliver over prolonged periods. We have not made

any evaluation of leakage, which should be performed under conditions that closely parallel those prevailing at the IC_{50} of encapsulated drug. In some cases the lipid concentration at the IC_{50} is as low as 0.06 μM, and leakage studies with drugs are currently not feasible at such low concentrations. Nonetheless, the use of a drug whose potency is increased by encapsulation provides information on liposome-mediated drug delivery without the need for information on leakage.

The above experiments suggest some criteria by which liposome-dependent drug delivery may be evaluated. In at least one situation, the encapsulated drug should show greater potency than the free drug. This eliminates the ambiguity produced by the leakage of liposome contents, because the drug must have been delivered to the cells by the liposomes. If all the drug leaks within minutes following liposome addition to the medium, the IC_{50} will be the same as that of the free drug. Liposome-dependent delivery should also depend on the extent to which the cells take up the liposomes. We would expect all liposome-dependent drugs to be more effective in negatively charged liposomes than in neutral liposomes, to show the same relative potency on the various cell lines as methotrexate-γ-aspartate, and to be optimally effective in 0.1 μm liposomes. If a drug is to be tested for liposome-dependency, it should first be incorporated in phosphatidylserine-cholesterol liposomes, preferably extruded to 0.1 μm. The encapsulated drug should first be tested on CV1-P cells. The minimal drug : lipid ratio used should ensure that lipid concentrations do not exceed the lipid IC_{50}.

We have recently used the above approach with negatively charged liposomes to demonstrate the liposome dependence of 5-fluoroorotic acid, a fluorouracil derivative [31]. In future studies, we will further explore liposome-dependent drug delivery, examining other drugs and the effects of shorter times of cell exposure on the potency of free and encapsulated drugs.

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