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Leakage and delivery of liposome-encapsulated methotrexate-γ-aspartate in a chemically defined medium

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A chemically defined medium was developed to study liposome-mediated delivery of methotrexate-γ-aspartate to cells under conditions where dilute suspensions of negatively charged liposomes to not leak extensively. The defined medium induced 14% leakage of methotrexate-γ-aspartate from egg phosphatidylglycerol / cholesterol (67:33) liposomes diluted to 53 nM lipid. In contrast, commercially available serum replacements induced up to 91% leakage from the same liposomes. The growth inhibitory properties of non-loaded phosphatidylglycerol liposomes were greater in the chemically defined medium that they were in medium supplemented with 10% serum. Egg phosphatidylglycerol, dioleoylphosphatidylglycerol and dilaurylphosphatidylglycerol liposomes inhibited cell growth more than dimyristoylphosphatidylglycerol and dipalmitoylphosphatidylglycerol liposomes. In 10% serum, phosphatidylglycerol liposomes with widely varying phase-transition temperatures were nearly equally effective to deliver drug to CV1-P and L929 cells, despite great differences in liposome stability. Liposome encapsulated methotrexate-γ-aspartate was more potent when the cells were grown in the defined medium, and the increase in drug delivery was observed from phosphatidylglycerol liposomes of different phase-transition temperatures. The minimum fraction of negatively charged phospholipid required for optimal liposome-mediated drug delivery varied between cell types and among growth media. The growth inhibitory effects of liposome-encapsulated methotrexate-γ-aspartate was also determined under conditions where the cells were exposed to drug for periods shorter than the entire growth assay. Reduction of the exposure time decreased the potency of both encapsulated and free drug in medium containing 10% serum, and decreased the potency of free drug in the defined medium. However, the potency of encapsulated drug in the defined medium was similar for all exposure lengths between 1 and 48 hours.

Introduction

Liposomes have been proposed as carriers for the delivery of molecules to cells [1]. They have proved particularly effective for promoting intracellular delivery of molecules that do not penetrate cells readily [2–4]. Studies of the delivery of such molecules have demonstrated that adsorptive endocytosis is the predominant mechanism of delivery, as shown by the inhibition of delivery by lysosomotropic agents [2]. This mechanism of delivery requires that liposomes retain their contents until endocytosis occurs. Consequently, the leakage of liposomes in serum would be expected to reduce drug delivery, and should be minimized in order to optimize liposome-mediated drug delivery.

In vitro experiments to study liposome-mediated drug delivery often employ lipid concentrations of about 60 nM [2], and we have recently studied the leakage of methotrexate-γ-aspartate from dilute liposome suspensions in two different ways [5,6]. In the first of these, a two compartment growth inhibition assay showed that EPG liposomes rapidly leak 94% of their contents, while DSPG liposomes leak only 42% of their contents [5]. Despite the extensive leakage of drug from EPG liposomes, the effects of encapsulated drug on target cells

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Abbreviations: Apo A-1, apolipoprotein A-1; apo B, apolipoprotein B; Apo E, apolipoprotein E; HDL, high density lipoproteins; LDL, low density lipoproteins; DLPG, dilauroylphosphatidylglycerol; DMPG, dimyristoylphosphatidylglycerol; DOPG, dioleoylphosphatidylglycerol; DPPG, dipalmitoylphosphatidylglycerol; DPtPG, dipentadecanoylphosphatidylglycerol; DSPG, distearoylphosphatidylglycerol; EPC, egg phosphatidylcholine; EPG, egg phosphatidylglycerol; PI, soybean phosphatidylinositol; PS, bovine brain phosphatidylserine.

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were caused by the delivery of the residual encapsulated material to the cells by the liposomes. In the second of these studies, a dihydrofolate reductase inhibition assay was used to monitor leakage of methotrexate-γ-aspartate directly [6]. Negatively charged EPG liposomes diluted to 53 nM lipid were observed to leak 94% of their contents after 2 min incubation in 10% newborn calf serum. The leakage of EPG liposomes was shown to increase with increasing protein to lipid ratios that are observed with lipid dilution. Leakage from liposomes in 10% serum was shown to depend on the negative charge of the liposomes, and on the acyl chain composition of phosphatidylglycerol liposomes. EPG liposomes leaked extensively in solutions of bovine serum albumin. However, treatment of the albumin with sonicated lipid suspensions, a treatment known to remove contaminating apolipoproteins, reduced EPG leakage to a nominal value, indicating that albumin itself deos not induce extensive leakage of EPG liposomes.

In this report we have used salt fractionated albumin to develop a chemically defined medium which does not induce extensive leakage of negatively charged liposomes. We have used this defined medium to determine liposome-mediated delivery of methotrexate-γ-aspartate to cells under conditions of increased liposome stability. In addition, the defined medium is useful for evaluating the effects of lipid charge and acyl chain composition on liposome-mediated drug delivery.

Materials and Methods

Lipids and other materials

Phospholipids (Avanti, Pelham, AL) were stored in chloroform solution under argon gas in sealed ampules at −20°C. Cholesterol (Sigma, St. Louis, MO) was recrystallized from methanol four times and stored under the same conditions as the phospholipids. Methotrexate-γ-aspartate was synthesized and provided by J.R. Piper, Southern Research Institute, Birmingham, AL [7]. Affinity-isolated dihydrofolate reductase from bovine liver as a suspension in ammonium sulfate was obtained from Sigma (St. Louis, MO) and used without further purification. Dihydrofolic acid and reduced nicotinamide adenine dinucleotide phosphate were obtained from Sigma. Fraction V powder of bovine serum albumin was obtained from Sigma (Purified by low temperature solvent precipitation followed by charcoal treatment and dialysis. 98–99% pure with globulins as the major contaminant as reported in Sigma technical information). Fraction V powder of bovine serum albumin initially purified by salt fractionation was obtained from Sigma (further purification by ion exchange and gel filtration chromatography according to Sigma technical information). Newborn calf serum and fetal bovine serum (Gibco, Long Island, NY) was stored frozen at −20°C until used.

Low protein serum replacement (LPSR-1) and controlled process serum replacement CPSR-4 were obtained from Sigma. LPSR-1 is a synthetic serum replacement prepared from albumin, transferrin, insulin and unidentified growth factors, attachment factors, binding proteins and other growth promoting agents (Sigma technical information). CPSR-4 is manufactured from whole blood collected from formula-fed calves. CPSR-4 contains growth factors extracted from clotted calves blood, and contains lower levels of lipid, cholesterol and triacylglycerol than serum (Sigma technical information).

In some cases, bovine serum albumin which was initially purified by cold ethanol precipitation was treated as previously described with sonicated liposomes prepared from EPC, and is referred to as lipid-treated albumin [6]. Endotoxin levels were determined in treated albumin preparations with a *Limulus* Amebocyte lysate assay (Sigma).

Liposome preparation

Large unilamellar liposomes were prepared under sterile conditions by a small scale adaptation of the reverse phase evaporation method [8]. Briefly, 10 μmol of phospholipid and 5 μmol of cholesterol were dried down in a 13 × 100 mm screw-capped culture tube. The lipid was suspended in 1 ml of either diethyl ether (EPG, EPG, DOPG, DMPG) or diisopropyl ether (all higher phase transition lipids). 0.4 ml of drug solution was added, and the mixture was sonicated for three minutes in a bath type sonicator (Laboratory supply company, Hicksville, NY). Liposomes were formed by evaporation of the ether and under vacuum on a rotary evaporator. All drug containing liposomes were prepared from phospholipid/cholesterol (67 : 33) and will be referred to subsequently by the phospholipid content alone.

Methotrexate-γ-aspartate solutions were prepared for encapsulation at drug concentrations from 12.8 to 18.4 mM in 50 mM morpholineethanesulfonic acid (Mes)/50 mM morpholinepropanesulfonic acid (Mops) (Sigma) and adjusted to pH 7.2 with sodium hydroxide. The tonicity of the drug solutions was measured using an osmometer (Wescor, Logan, UT) and adjusted to 290 mmol/kg with NaCl. Liposomes were separated from unencapsulated drug by gel chromatography on a 1 × 15 cm sterile Sephadex G-50 (Pharmacia, Piscataway, NJ) column. The column was eluted with sterile 50 mM Mes/50 mM Mops/1 mM EDTA/NaCl (pH 7.3) at the same tonicity as the encapsulated drug solution. Drug concentrations in the liposomes were determined by solubilizing a portion of the liposome suspension in 1 : 3 : 1 chloroform/methanol/water, and measuring the absorbance at 370 nm using a molar absorption coefficient of 7943 l/mol per cm. Lipid content was measured by phosphorus analysis [9].

Electron microscopy of liposome preparations

Some liposome preparations were subjected to electron microscopy in order to establish their size distribution. Liposomes were allowed to adsorb onto a parlodion-coated grid, and were then stained with 2% ammonium molybdate. Liposomes were examined and photographed at 19000 × or 38000 × final magnification in a Jeol 100CX microscope. The resultant photographs were measured to obtain the diameters of 250 liposomes. The fraction of the volume in each size class and the mean diameter was then calculated from these measurements.

Enzyme inhibition assay for methotrexate-γ-aspartate

Methotrexate-γ-aspartate is a potent competitive inhibitor of dihydrofolate reductase [7], and was measured by its ability to inhibit the dihydrofolate-dependent oxidation of NADPH catalysed by dihydrofolate reductase. Oxidation of NADPH was monitored through the reduction of absorbance at 340 nm. Methotrexate-γ-aspartate that is encapsulated in liposomes is unable to bind to dihydrofolate reductase. Therefore, this assay determines the amount of drug that has leaked from liposomes without separating free drug from liposome encapsulated drug.

All reagents were dissolved in 10 mM phosphate-buffered saline (pH 7.4), 290 mmol/kg, which contained 10% v/v serum or 4 mg/ml albumin as appropriate. In all cases, samples were determined at the same time as a series of standards containing between 1 and 6 pmol of drug. Effective drug concentration was measured from the plot of absorbance at 340 nm vs. concentration of methotrexate-γ-aspartate, which was linear up to 6 nM drug (not shown). Incubation times were controlled by adding reagents to successive tubes in 15-s intervals. 10 μl of liposome suspension or drug standard containing 1–6 pmol of drug was diluted with 790 μl of buffer at 22° C and vortexed. 100 μl of buffer containing 2 mU of dihydrofolate reductase and 150 nmol NADPH was added to the liposome suspension and vortexed. The mixture was allowed to incubate for 2 min in order to allow the drug, which is a slow, tight-binding inhibitor, to bind to the enzyme. 100 μl of buffer containing 150 nmol dihydrofolic acid was added to the liposome suspension and the mixture was vortexed and placed in a 37° C water bath for 8 min. The reaction was stopped by adding 100 μl of 0.1 mM methotrexate (this reagent did not contain proteins). The asorbance of the samples was measured at 340 nm in a Beckman DU-64 spectrophotometer.

Growth inhibition experiments

The growth-inhibitory properties of the encapsulated drug were measured on L929, a murine fibroblast from the C3H mouse obtained from L.B. Epstein, UC San Francisco, CA, and on CV1-P, an African green monkey kidney cell line obtained from P. Berg, Stanford University, Palo Alto, CA. Cells were grown in Dubecco's modified Eagle's minimal essential medium (Gibco, Grand Island, NY) supplemented with either 10% newborn calf serum (Gibco), 10% fetal calf serum (Gibco), 5% CPSR-4 or the components of the chemically defined supplement. The chemically defined supplement contained 1 mg/ml salt-fractionated albumin, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite (Sigma), 7 μg/l biotin and 0.68 mg/l vitamin B-12 and 90 μg/l linoleic acid (Sigma) [10–12]. Cells were plated for growth inhibition at approximately $2 \cdot 10^4$ cells per well for CV1-P, and about $4 \cdot 10^4$ cells per well for L929 cells in 24-well plates (Costar). Cells were grown at 37° C in a humid incubator (NAPCO, Portland, OR) with 7% CO_2. Triplicate wells containing cells were treated with drug after overnight incubation. Drug solutions were prepared as a half-logarithmic dilution series at 100-times the final desired concentrations. In the case of liposome preparations, no attempt was made to maintain a constant lipid concentration, which varied in accordance with the drug concentration. Control wells were treated with buffer alone. Three wells were counted at the time of treatment to give the original cell concentration. The CV1-P cells were allowed to grow for 72 h and the L929 cells were allowed to grow for 48 h. After growth the cells were counted with a Coulter counter, model ZM. To count CV1-P cells, the medium was removed and the cells were resuspended by treatment with 1 ml of 0.05% trypsin in phosphate-buffered saline/1 mM EDTA solution at 37° C for 10 min. To count L929 cells, the medium was removed and the cells were resuspended by treatment with 1 ml of 10 mM phosphate-buffered saline with 1 mM EDTA solution for 10 min at 37° C. 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 graphically from the plots. For some growth inhibition studies the length of liposome exposure was less than the entire growth assay. In these studies, the medium containing the drug was aspirated at the end of the drug incubation period, the cells were gently washed with phosphate-buffered saline containing 0.8 mM calcium chloride and 0.8 mM magnesium chloride, and fresh medium at 37° C was added. The cells were returned to incubation at 37° C for the rest of the growth period.

Results

Liposome leakage in serum replacements

The leakage of liposomes in commercially available serum replacements that contain less protein than serum are shown in Table I, EPG and PI liposomes leaked over 90% of their contents in 2% LPSR-1, which contains approximately one third the protein concentration found in 10% serum. The extensive leakage of EPG liposomes in 2% LPSR-1 may be due to apolipoprotein contaminants, which have been found in highly purified albumins [13,14]. Liposome leakage was also determined in solutions containing CPSR-4. Table I shows that in 10% CPSR-4, EPG liposomes leaked 76%, PS liposomes leaked 31%, and PI liposomes leaked 66% of their contents. When the concentration of CPSR-4 was reduced to 5%, the leakage from EPG liposomes was reduced to 58%.

Bovine serum albumin which was initially purified by cold-ethanol precipitation was shown to induce 67% leakage from EPG liposomes. Treatment of the albumin with lipid was previously shown to reduce EPG leakage to 7% [6]. Table I shows that the addition of insulin and transferrin to the lipid-treated ialbumin did not increase EPG-leakage above 7%. Therefore, the low levels of insulin and transferrin used in the defined medium were not disruptive towards dilute suspensions of negatively charged liposomes. As an alternative to treating albumin with lipid, we determined the stability of EPG liposomes in a solution of bovine serum albumin which was initially purified by salt fractionation. Table I shows that EPG liposomes leaked 14% of their contents in a 1 mg/ml solution of salt fractionated albumin which was endotoxin free (detection limit was 0.0123 ng endotoxin per ml). Therefore, salt-fractionated albumin, insulin and transferrin were used to prepare a medium, which does not induce extensive leakage of phosphatidylglycerol liposomes. This chemically-defined medium was used in subsequent growth inhibition studies.

TABLE I

Methotrexate-γ-aspartate leakage from liposomes in serum replacements

Medium[a]	% Leakage (mean ± s[b])		
	EPG	PS	PI
10% Serum	94 ± 13	78 ± 9	90 ± 11
2% LPSR-1	91 ± 7	70 ± 9	92 ± 5
10% CPSR-4	76 ± 4	31 ± 3	66 ± 4
5% CPSR-4	58 ± 2		
Lipid-treated albumin[c]	7 ± 2		
Salt-fractionated albumin[d]	14 ± 1		

[a] EPG, PS and PI liposomes were diluted in 10 mM phosphate-buffered saline adjusted to 290 mmol/kg with NaCl and supplemented with newborn calf serum or serum replacements.
[b] Standard deviations of four measurements.
[c] 1 mg/ml lipid-treated albumin with 5 μg/ml insulin, 5 μg/ml transferrin and 10 μg/ml epidermal growth factor.
[d] 1 mg/ml albumin initially purified by salt fractionation.

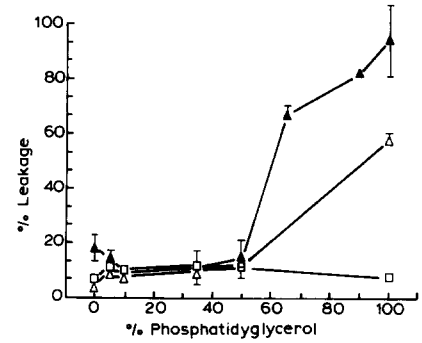

Fig. 1. Liposome leakage in serum replacements as a function of EPG content. Liposomes were prepared with varying amounts of EPG and EPC. The total lipid concentration was from 53 to 110 nM phospholipid. Liposomes were diluted in 10 mM phosphate-buffered saline, 290 mmol/kg, supplemented with either, 10% (v/v) newborn calf serum (▲), 5% (v/v) CPSR-4 (△) or 1 mg/ml lipid-treated albumin with 5 μg/ml insulin and 5 μg/ml transferrin (□). The means of three measurements are plotted, and error bars show the standard deviation.

Effect of charge on liposome leakage in serum replacements

Fig. 1 shows the effect of charge on liposome leakage in phosphate-buffered saline supplemented with serum, or serum replacements. Liposomes prepared with EPC, or mixtures of EPG and EPC that contain up to 50% EPG, leaked less than 10% of their contents in the presence of all supplements. With both 10% serum and 5% CPSR-4, leakage is much higher for liposomes prepared from EPC/EPG mixtures containing more than 50% EPG. In solutions of lipid-treated albumin, leakage of EPG liposomes was very low for all liposome preparations. Therefore, a medium supplemented with 1 mg/ml salt-fractionated albumin, insulin and transferrin will cause minimal liposome leakage regardless of the amount of negative charge on the liposomes.

The effect of lipid composition on liposome leakage in serum replacements

Fig. 2 shows the leakage of phosphatidylglycerol liposomes, prepared from lipids of varying phase transition temperatures in solutions containing either 10% serum, 5% CPSR-4, or 1 mg/ml lipid-treated albumin. In the presence of serum, liposome leakage is inversely related to the phase transition temperature of the phosphatidylglycerol. In the presence of 5% CPSR-4, a similar relationship is seen, although leakage is overall lower. In the presence of a lipid-treated albumin, leakage is minimal for all liposome preparations.

The effect of time on liposome leakage

All liposome leakage measurements up to this point were determined after 2 min of incubation. Fig. 3 shows

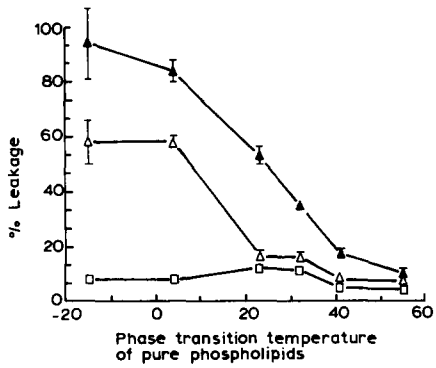

Fig. 2. Leakage of phosphatidylglycerol liposomes in serum and serum replacements. The phase transition temperatures of the pure phospholipids are: EPG, −18°C; DLPG, 4°C; DMPG, 23°C; DPtPG, 33°C; DPPG, 41°C; DSPG, 55°C. Liposomes were diluted to between 53 and 234 nM phospholipid in 10 mM phosphate-buffered saline (pH 7.4), 290 mmol/kg containing either 10% (v/v) newborn calf serum (▲), 5% (v/v) CPSR-4 (△) or 1 mg/ml lipid-treated albumin with 5 μg/ml insulin and 5 μg/ml transferrin (□). The means of three measurements are plotted, and error bars show the standard deviation.

liposome leakage in 10% serum during incubation for up to 48 h. Leakage from EPG liposomes is rapid and approaches 100%, and does not change as incubation time is increased from 2 min to 48 h. Leakage from DMPG increased from 42% to about 85% in 24 h of incubation. Leakage from DPPG liposomes increased only slightly after the prolonged incubation in the presence of serum. In the chemically defined medium, leakage from EPG, DMPG and DPPG liposomes were similar after 2 min of incubation. However, after 24 h in the defined medium, the leakage from EPG liposomes increased to 75%, and continued incubation resulted in an increase of leakage to 85%. Therefore, EPG liposomes are more stable in the defined medium than in 10% serum, but do leak over longer periods. Similarly, leakage from DMPG liposomes increased to 45% after 24 h of incubation, but longer incubation time did not further increase liposome leakage. DPPG liposomes were stable in the defined medium for up to 48 h.

Cell growth in serum replacements

The potency of liposome encapsulated methotrexate-γ-aspartate was evaluated by determining the concentration of drug necessary to inhibit cell growth by 50%. Therefore, the ability of serum replacements to support cell division is required to evaluate liposome drug delivery. The growth of the cell lines was determined in medium supplemented with either 10% fetal calf serum, 10% newborn calf serum, 5% CPSR-4, or in the chemically defined supplement. Fig. 4 shows that the growth of CV1-P was similar in medium containing 10% serum, serum replacements, or in the defined supplement. To date, CV1-P cells have survived continuously in the defined medium for 7 months. However, in

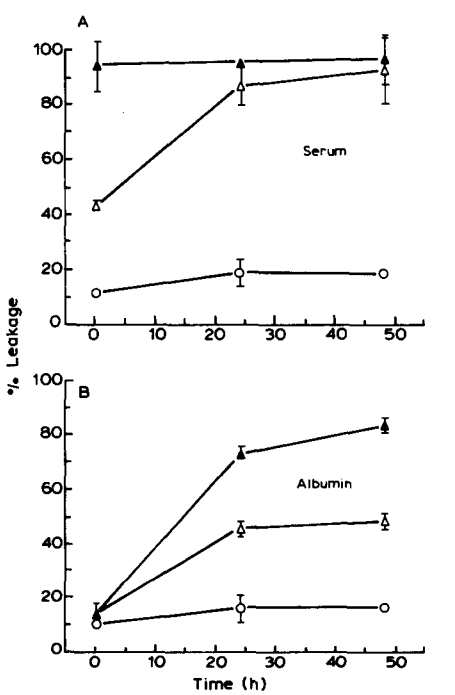

Fig. 3. Liposome leakage as a function of incubation time. Liposomes containing methotrexate-γ-aspartate were diluted in phosphate-buffered saline containing either 10% newborn calf serum (A) or 1 mg/ml lipid-treated albumin (B). ▲, EPG; △, DMPG; ○, DPPG. The means of three measurements are plotted, and error bars show the standard deviation.

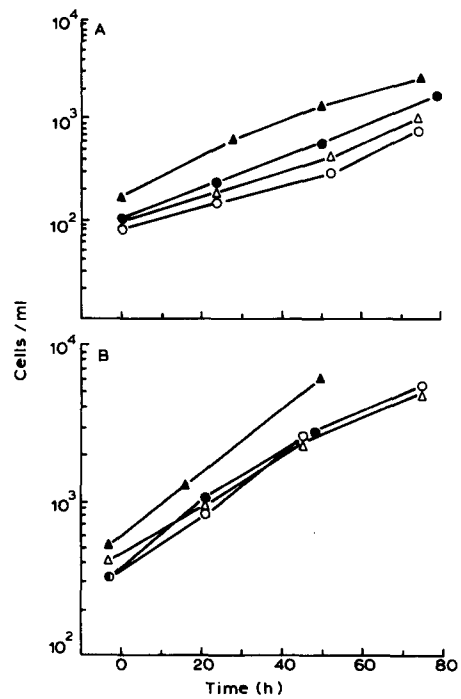

Fig. 4. Cell growth in serum and in serum replacements. (A) CV1-P; (B) L929. Dulbecco's modified Eagle's minimal essential medium was supplemented with either 10% fetal bovine serum (▲), 10% newborn calf serum (△), 5% CPSR-4 (●) or the chemically defined supplement (○). The means of three measurements are plotted, and the standard deviation is less than the size of the points.

a defined medium supplemented with lipid-treated albumin (instead of salt-fractionated albumin), the doubling time for CV1-P cells was increased from 22 to 31 h (data not shown). Lipid-treated albumin was found to contain 5 ng/ml endotoxin, which may explain the lower growth rate in its presence.

In earlier experiments L929 cells were grown in medium supplemented with 5 or 10% fetal calf serum [2,3,15,16]. Fig. 4 shows that the growth of L929 cells in medium supplemented with 10% newborn calf serum was similar to the cell growth in the presence of 10% fetal calf serum. L929 growth in medium containing 10% newborn calf serum was similar to the cell growth in medium containing 5% CPSR-4 or in the chemically defined medium. To date, L929 cells have grown continuously in the chemically-defined medium for 7 months.

Effect of lipid on cell growth

Previous work demonstrated that negatively charged phospholipids can inhibit cell growth, although the concentration required in most cases was greater than the amount of lipid used for liposome-mediated delivery of methotrexate-γ-aspartate to CV1-P, L929 or RAW 254 cells in 10% fetal bovine serum [2]. Of these three cells lines, CV1-P cells were the most sensitive to growth inhibition by EPG liposomes. In order to verify that lipid effects were not contributing to the growth inhibition of liposome encapsulated methotrexate-γ-aspartate, the growth inhibitory effects of empty phosphatidylglycerol liposomes were determined with cells grown in 10% newborn calf serum or in the chemically defined medium. Table II shows that in 10% newborn calf serum, liposomes prepared with DOPG, EPG and DLPG were more inhibitory to CV1-P growth than liposomes prepared with DMPG or DPPG. The growth inhibitory effects of liposomes prepared from various phosphatidylglycerols were as much as 10-fold greater in the defined medium than in 10% serum, suggesting that serum may modulate the effects of these liposomes. In the defined medium, liposomes prepared from DOPG, EPG and DLPG were again more inhibitory to CV1-P cells than liposomes prepared with DMPG, DPPG or DSPG. Despite the increased effects of the phosphatidylglycerols in the defined medium, the amount of lipid used for drug delivery experiments is not sufficient to impair growth of CV1-P cells, and the growth inhibition of liposome encapsulated methotrexate-γ-aspartate must be entirely caused by the effects of the drug.

TABLE II

The effect of lipid on cell growth

Liposome[c]	IC_{50} (mM) (mean ± s[d])	
	defined medium[a]	10% serum[b]
1. CV1-P		
DOPG	0.025	0.24
EPG	0.021 ±0.007	0.14±0.04
DLPG	0.019 ±0.004	0.14±0.04
DMPG	0.198 ±0.144	>1
DPPG	0.181 ±0.127	>1
DSPG	0.089 ±0.058	0.80
2. L929		
EPG	0.0086±0.0004	>1

[a,b] Dulbecco's modified Eagle's minimal essential medium supplemented with: [a] chemically defined supplement, or [b] 10% newborn calf serum.

[c] Liposomes were prepared with phospholipid/cholesterol (67:33).

[d] Standard deviations of three measurements.

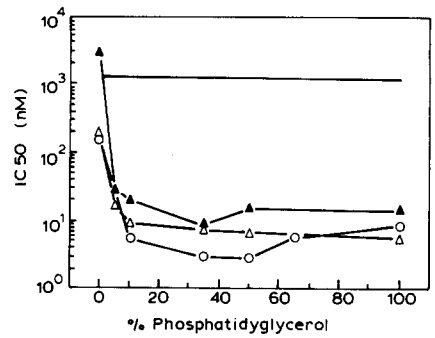

Fig. 5. The potency of liposome-encapsulated methotrexate-γ-aspartate for CV1-P cells as a function of EPG content. Methotrexate-γ-aspartate was encapsulated in liposomes prepared with EPG and EPC, phospholipid:cholesterol ratio of 67:33. ▲, 10% newborn calf serum; △, 5% CPSR-4; ○, defined medium. The solid line denotes the IC_{50} of free drug.

Table II shows that the IC_{50} for EPG towards L929 cells was greater than 1 mM when the cells were grown in medium supplemented with 10% serum. In contrast, the IC_{50} for EPG against L929 cells grown in the chemically defined medium was 8.6 μM, indicating that the effects of EPG towards L929 cells are over 100-fold greater in the chemically defined medium. Despite the increase in the growth inhibitory properties of EPG, the amount of lipid used for drug delivery to L929 cells in the chemically defined medium does not impair growth of L929 cells.

The effect of charge on delivery on liposome-encapsulated methotrexate-γ-aspartate

In earlier studies it was shown that negatively charged PS liposomes associate with CV1-P to a greater extent than neutral EPC liposomes, and methotrexate-γ-aspartate encapsulated in PS liposomes was more effective than methotrexate-γ-aspartate in EPC lipsoomes to inhibit CV1-P cell growth [2]. However, no information has previously been obtained to establish how much negatively charge phospholipid is required to achieve optimal delivery of this drug. Fig. 5 shows the efficiency of liposome-mediated delivery of methotrexate-γ-aspartate delivery to CV1-P cells as a function of the EPG

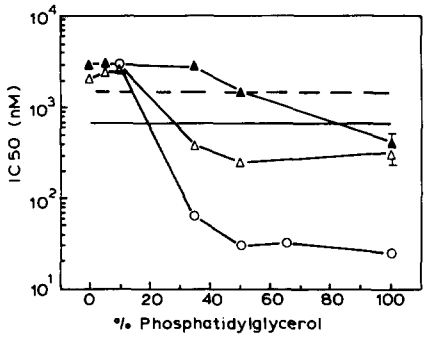

Fig. 6. The potency of liposome-encapsulated methotrexate-γ-aspartate for L929 cells as a function of EPG content. Methotrexate-γ-aspartate was encapsulated in liposomes prepared with EPG and EPC, phospholipid:cholesterol ratio, 67:33. ▲, 10% newborn calf serum; △, 5% CPSR-4; ○, defined medium. The solid line denotes the IC_{50} of the free drug in 10% serum and in 5% CPSR-4. The dotted line denotes the IC_{50} of free drug in the defined medium. The IC_{50} values for 100% EPG in 10% calf serum and in 5% CPSR-4 are the means of three measurements and the error bars show the standard deviation.

content. In 10% serum, free methotrexate-γ-aspartate (IC_{50} = 1.0 μM) is more potent than drug encapsulated in EPC liposomes (IC_{50} > 3 μM). This indicates that EPC liposomes retain their contents during the growth assay and do not deliver drug to CV1-P cells. However, liposomes containing only 5% negatively charged EPG (IC_{50} = 30 nM) are 33-times more effective than free drug. Inclusion of 10, 50 or 100% negative charge further increased the potency of the encapsulated drug (IC_{50} ≈ 15 nM) to optimal levels. The effect of liposome charge on drug delivery to CV1-P in 5% CPSR-4 was similar to the results seen in 10% serum with one notable exception. Methotrexate-γ-aspartate encapsulated in EPC liposomes (IC_{50} = 0.19 μM) is more potent than the free drug when CV1-P cells are grown in 5% CPSR-4. This indicates that CV1-P cells take up neutral liposomes to some extent under these conditions. Liposomes prepared with 10, 35, 50 or 100% EPG were 300-times more potent than the free drug in 5% CPSR-4. In the chemically defined medium EPC liposomes (IC_{50} = 0.16 μM) are more effective than the free drug. Drug encapsulated in liposomes with 10, 35, 50 or 100% EPG were nearly equally effective and were about 415-times more potent than the free drug.

The efficiency of liposome-mediated drug delivery to L929 cells as function of EPG content is presented in Fig. 6. In 10% newborn calf serum, drug encapsulated in EPC lipsoomes (IC_{50} = 3 μM) was less potent than the free drug (IC_{50} = 0.62 μM), indicating that EPC liposomes are stable in 10% serum for the entire growth assay, and do not deliver drug to L929 cells. This is consistent with earlier results for L929 cells grown in 10% fetal bovine serum [2]. Inclusion of 5 or 10% negative charge in the liposomes did not increase the potency of encapsulated drug (IC_{50} = 3 μM). When the negative charge of the liposomes was increased to 50%, the IC_{50} of the encapsulated drug was 1.5 μM. This value is greater than the IC_{50} of the free drug, suggesting that liposomes with 50% negative charge are both stable in 10% serum and minimally taken up by L929 cells. Methotrexate-γ-aspartate encapsulated in EPG liposomes (IC_{50} = 0.35 μM) is 1.6-times more potent than the free drug. This is consistent with the earlier reports for increased delivery of PS encapsulated methotrexate-γ-aspartate to L929 cells in 10% fetal calf serum [2]. In contrast, when L929 cells were grown in 5% CPSR 4, drug encapsulated in 35% EPG liposomes (IC_{50} = 0.4 μM) was more effective than the free drug (IC_{50} = 0.73 μM). Liposomes prepared with 50 or 100% EPG were the most potent in 5% CPSR-4. In the defined medium, drug in liposomes prepared with 35% EPG (IC_{50} = 63 nM) was 22-times more potent than the free drug, and was nearly as effective as drug in liposomes prepared from 50–100% EPG. Therefore, the optimum liposome charge for drug delivery to L929 cells is affected by the choice of the growth supplement.

The effect of lipid composition on delivery of liposome encapsulated methotrexate-γ-aspartate

Fig. 7 shows the effect of lipid phase transition temperature on the IC_{50} of drug encapsulated in phos-

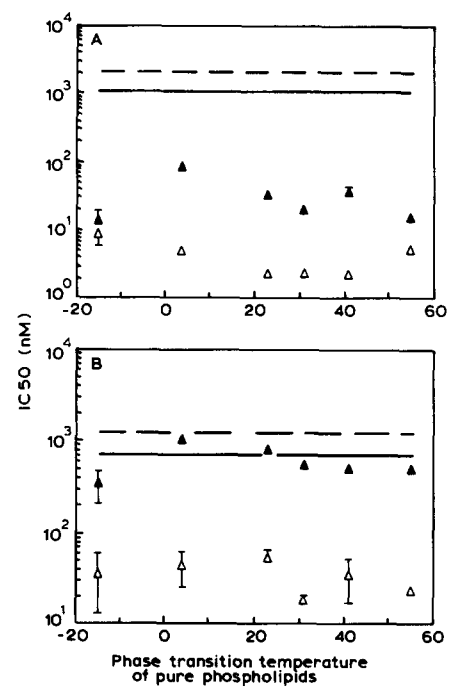

Fig. 7. The effect of the acyl chain of phosphatidylglycerol liposomes on liposome-mediated delivery of methotrexate-γ-aspartate to CV1-P and L929 cells. Methotrexate-γ-aspartate was encapsulated in phosphatidylglycerol liposomes. The phase transition temperatures of the pure phospholipids are: EPG, −18°C; DLPG, 4°C; DMPG, 23°C; DPtPG, 33°C; DPPG, 41°C; DSPG, 55°C. (A) CV1-P cells; (B) L929 cells. ▲, medium supplemented with 10% newborn calf serum; △, defined medium. The dotted line denotes the IC_{50} of the free drug in the defined medium. The solid line denotes the IC_{50} for free drug in 10% serum. The means of three measurements are plotted, and error bars are the standard deviation. Cells were exposed to drug for either 48 h (L929) or 72 h (CV1-P).

phatidylglycerol liposomes for CV1-P and L929 cells. In 10% serum, phosphatidylglycerol liposomes with widely varying phase transition temperatures were effective in delivering drug to CV1-P cells. Drug encapsulated in EPG liposomes (IC_{50} = 14 nM) was the most effective and was 75-times more potent than the free drug (IC_{50} = 1.05 μM). Drug loaded in DLPG liposomes (IC_{50} = 88 nM) was the least effective in 10% serum. In the chemically defined medium, liposomes with varying phase transition temperatures were nearly equally effective for drug delivery to CV1-P cells. Drug encapsulated in DMPG, DPtPG and DPPG liposomes (IC_{50} = 2.3 nM) was 935 times more effective than the free drug (IC_{50} = 2.15 μM).

The effect of the phase transition temperature of phosphatidylglycerol liposomes on the delivery of methotrexate-γ-aspartate to L929 cells is shown in Fig. 7. After continuous exposure to liposomes in 10% serum, drug encapsulated in EPG liposomes (IC_{50} = 0.35 μM) was the most effective against L929 cells and was twice as potent as the free drug (IC_{50} = 0.7 μM). Drug encapsulated in DLPG and DMPG liposomes was less potent, and drug in DPPG and DSPG liposomes (IC_{50} = 0.54 μM) was slightly more potent than the free drug (IC_{50} = 0.7 μM). In the chemically defined medium, drug encapsulated in liposomes of widely varying phase transition temperature was equally effective for growth inhibition of L929 cells. Further, the potency of liposome-encapsulated drug was much greater for all liposome preparations than it was in 10% serum. In the defined medium, drug encapsulated in EPG liposomes (IC_{50} = 36 nM) was 40-times more potent than the free drug, and drug in DSPG liposomes (IC_{50} = 25 nM) was 57-times more effective than the free drug (IC_{50} = 1.4 μM).

The effect of exposure length on liposome-mediated delivery of methotrexate-γ-aspartate

Fig. 8 shows the change of IC_{50} as a function of exposure length for free and encapsulated methotrexate-γ-aspartate on CV1-P cells. In 10% newborn calf serum, the IC_{50} for free drug and liposome-encapsulated drug decreases as the length of exposure is increased. Similar results were reported earlier for methotrexate-γ-aspartate encapsulated in PS liposomes when CV1-P cells were grown in medium supplemented with 5% newborn calf serum [15]. EPG and DSPG liposomes were equally effective for delivery of encapsulated drug to CV1-P cells at all exposure times. In contrast to drug delivery in 10% serum, liposome-mediated drug delivery to CV1-P cells in the chemically defined medium was nearly as effective after 1 h (IC_{50} for EPG liposomes = 23 nM) or 72 h of exposure (IC_{50} for EPG liposomes = 9 nM). As a result, drug encapsulated in EPG liposomes is nearly 10^4-times more potent than the free drug when the exposure length is 1 h. Drug encapsulated in DSPG liposomes was equally effective as drug encapsulated in EPG liposomes at all exposure times.

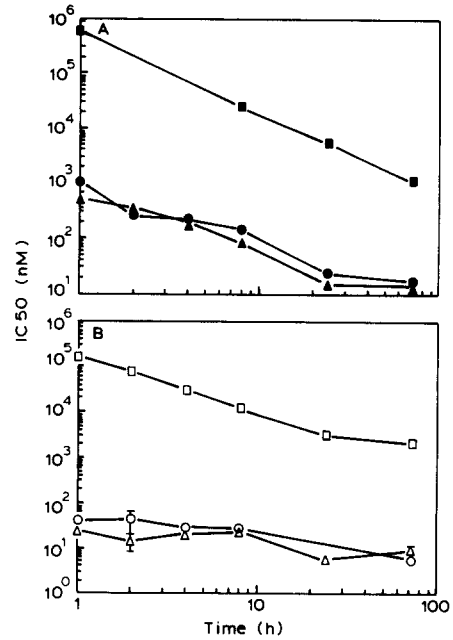

Fig. 8. The effect of length of exposure on the potency of free and encapsulated methotrexate-γ-aspartate for inhibition of CV1-P growth. The cells were exposed to the drug and were washed free of the drug at the time indicated as described in Methods. (A) Cells grown in medium supplemented with 10% newborn calf serum. ▲, drug encapsulated in EPG; ●, drug encapsulated in DSPG; ■, free drug. (B) Cells grown in the defined medium. △, drug encapsulated in EPG; ○, drug encapsulated in DSPG; □, free drug. The means of three measurements are plotted, and the error bars show the standard deviation.

The effect of the length of exposure on methotrexate-γ-aspartate delivery to L929 cells is shown in Fig. 9. In 10% serum, the potency of liposome-encapsulated drug and free drug increases with increasing exposure time. In 10% serum, drug encapsulated in DSPG liposomes was less potent than drug encapsulated in EPG liposomes for all exposure times. However, in the defined

TABLE III

Size distribution of liposomes

All liposomes were prepared from the stated phospholipid or phospholipid mixture in a 67:33 ratio with cholesterol. The fraction of the liposome volume found in particles of the specified size range was computed as described under Methods. The mean diameter is skewed towards smaller diameters, because of the relationship between particle diameter and particle volume.

Phospholipid composition	Fraction of total volume in size range				Mean diameter (μm)
	0–0.1 μm	0.1–0.2 μm	0.2–0.3 μm	0.3–0.4 μm	
EPG	0.21	0.73	0.07	0	0.063
EPG/EPC (1:9)	0.28	0.41	0.30	0	0.060
DSPG	0.13	0.52	0.11	0.25	0.061

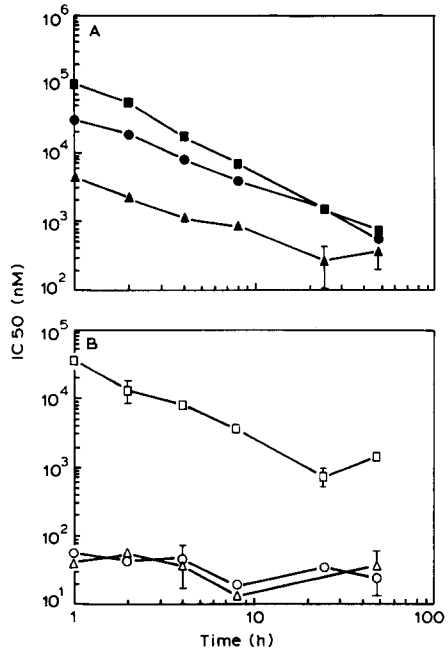

Fig. 9. The effect of length of exposure on the potency of free and encapsulated methotrexate-γ-aspartate for inhibition of L929 growth. The cells were exposed to the drug and were washed free of the drug at the time indicated as described in Materials and Methods. (A) Cells grown in medium supplemented with 10% newborn calf serum. ▲, drug encapsulated in EPG; ●, drug encapsulated in DSPG; ■, free drug. (B) Cells grown in the defined medium. △, drug encapsulated in EPG; ○, drug encapsulated in DSPG; □, free drug. The means of three measurements are plotted, and the error bars show the standard deviation.

medium drug encapsulated in DSPG liposomes or EPG liposomes was nearly as effective after 1 h of exposure to cells as was liposome encapsulated drug after continuous exposure to cells. As a result, encapsulated methotrexate-γ-aspartate is 10^3-times more potent than the free drug when the cells are exposed to drug for 1 h.

Electron microscopy of liposomes

Methotrexate-γ-aspartate delivery is known to be affected by liposome size [2]. In order to ascertain if any of our observations might partly be caused by differences between different preparations in their size distribution, we have examined the size distribution of three of the liposome preparations (Table III). The preparations appear to be quite similar in size distribution. The mean diameters are very similar. There are some differences in the distribution of liposome volume, DSPG having the lowest fraction in the 0–0.1-μm size range, and being the only preparation to contain liposomes in the 0.3–0.4-μm size range. Overall, these liposomes are smaller than has usually been reported for liposomes prepared by reverse phase evaporation. This is probably caused by the use of the small scale preparation procedure. In general, it does not appear that differences in the size of the liposomes between different preparations are a factor in these drug delivery studies.

Discussion

The mechanism of growth inhibition of cells by phosphatidylglycerol is unknown. In the presence of serum, the concentration of EPG which inhibits CV1-P growth is 6.7-times greater than the concentration of EPG which inhibits cell growth in the defined medium. For L929 cells, the growth inhibitory properties of EPG are at least 116-times greater in the defined medium than in 10% serum. This suggests that serum components, possibly lipoproteins, modulate the growth inhibitory effects of EPG. Liposomes prepared with DOPG and DLPG inhibited cell growth at concentrations similar to EPG. Therefore, the growth inhibitory effects of phosphatidylglycerol liposomes appears not to depend on the presence of any particular fatty acyl chains. Liposomes prepared with DMPG, DPtPG, DPPG and DSPG were not as growth inhibitory to cells as EPG liposomes. This suggests that phosphatidylglycerol liposomes with phase transition temperatures less than 23° C are more growth inhibitory than phosphatidylglycerol liposomes with higher phase transition temperatures.

EPG-mediated delivery of methotrexate-γ-aspartate to L929 cells was considerably increased when the cells were grown in the defined medium compared to drug delivery in 10% serum. This appears to indicate that the increase in EPG liposome stability increases drug delivery. However, liposome-mediated drug delivery in the defined medium was increased for phosphatidylglycerol liposomes of various acyl chain compositions. We conclude that the increase in drug delivery with various phosphatidylglycerol liposomes is not a result of an increase in liposome stability, because no increase in stability occurs for the higher phase transition phosphatidylglycerols. Further evidence that liposome stability does not affect drug delivery was observed with liposome-mediated drug delivery to CV1-P cells in the defined medium and in 10% serum. Methotrexate-γ-aspartate encapsulated in liposomes of varying stability was about equally effective for growth inhibition of CV1-P cells in 10% serum. In the defined medium, IC_{50} values were similar for the various preparations, and were not appreciably different from the values obtained in 10% serum.

The minimum negative charge of liposomes that was required for optimal drug delivery was different for CV1-P and L929 cells. For CV1-P cells in 10% serum, optimal liposome-mediated drug delivery was achieved with liposomes with between 5% and 100% negatively charged phospholipids. This indicates that a low negative charge density on the liposomes is sufficient to cause efficient drug delivery. However, the stability of liposomes in 10% serum decreased considerably as the

EPG content of the liposomes was increased from 50 to 100%. Therefore, the stability of liposomes prepared with egg phospholipids does not appear to affect drug delivery to CV1-P cells. For L929 cells in 10% serum, drug encapsulated in liposomes with 50% or less EPG was less effective than the free drug. The minimum EPG content of liposomes required for optimal drug delivery was less when the cells were grown in medium supplemented with CPSR-4, or in the defined medium, as compared to cells grown in 10% serum. These results with L929 cells suggest that some component or components of serum inhibit effective drug delivery by liposomes, and that this competition is greatest for liposomes containing less than 50% EPG.

It is interesting to speculate on the differences observed in the potency of drug encapsulated in negatively charge liposomes in the chemically defined medium. In our previous study, we showed that the leakage of negatively charged liposomes is induced by a factor or factors associated with low density lipoproteins, possibly its apolipoproteins [6]. The interaction of liposomes with apo E and apo B could also provide the mechanism of their uptake by cultured cells via the LDL receptor. Although apo E is not present in the chemically defined medium, apo E is synthesized by a wide variety of tissues and cells, including the cell types used in these experiments [17,18] and would be present to interact with the liposomes. One consequence of fibroblast growth in lipoprotein deficient medium is an increase in the expression of LDL receptors [19]. If liposome uptake occurs via the LDL receptors, their increased number might promote liposome uptake, which might cause an increase in potency of the encapsulated drug. Moreover, if liposome uptake down regulates the LDL receptors, uptake may be initially quite rapid and subsequently slower. This phenomenon would result in a rapid delivery of a large amount of drug, and might explain why the potency of encapsulated drug is independent of exposure time in the chemically defined medium. There is some evidence that supports this possibility. Neutral vesicles of Intralipid (an emulsion of phosphatidylcholines and triacylglycerols) have been shown to acquire apo E from human plasma, and these particles are taken up by cultured fibroblasts via the LDL receptor [20]. Studies of liposome interaction with cells may help to determine if the binding of negatively charged liposomes to L929 cells is increased in a chemically defined medium. However, it will be necessary to devise methods that will allow binding studies to be done at lipid concentrations comparable to those present at the IC_{50} of the encapsulated drug. We hope to devise such methods in future studies.

Liposome binding to the surface of cultured cells is known to be rapid and energy independent [2], while endocytosis and lysosomal processing to release encapsulated contents may be slower processes. Consequently, the liposomes that associate with the cells may release their drug contents to the cell over a long period of time. The IC_{50} of encapsulated drug is dependent on exposure length in serum-containing medium. This observation confirms that liposome uptake can occur over a prolonged period, and that the potency of the encapsulated drug is thereby increased. In contrast, the IC_{50} of encapsulated drug appears largely independent of exposure length in the chemically defined medium. Moreover, potency of encapsulated drug is at least as great in the defined medium as it is in serum supplemented medium. These observations taken together suggest that liposome-cell association is initially very rapid in the defined medium, and that subsequent processing of the liposomes makes drug available to the cells continuously over a prolonged period.

The observation that liposome-mediated drug delivery can be fully effective after only a short exposure period could be particularly important for the in vivo development of targeted drug delivery. Exposure of target cells to liposomes in vivo is likely to be transient, as competing processes will eliminate them from the extracellular milieu. It is currently unreasonable to expect that the interaction of liposomes with cells in a chemically defined medium will parallel the in vivo interaction of liposomes with cells, where plasma or some of its components will be present. However, we hope in future studies to establish whether similar interactions might occur in serum for liposomes directed to the cell surface by other ligands.

The results described in this and our previous paper [6] have interesting possible consequences for the in vivo use of liposomes. The leakage mechanism that we have observed appears only to affect liposomes at very low lipid concentrations. Therefore, the initially high plasma concentrations produced by intravenous injection would afford protection from this leakage mechanism. As liposomes are cleared from the circulation, the concentration will fall to levels that may allow an accelerated leakage by this mechanism. The importance of this phenomenon would depend upon the lipid concentration necessary for the activity of the encapsulated agent. In any event, the potential problem may be avoided by using liposomes containing less than 50 mol negatively charged lipid per 100 mol phospholipid. Liposomes of this composition deliver drug with equal efficiency in at least one cell line that we have studied. Therefore, they are likely to be of equal potency in vivo.

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