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Liposome-dependent delivery of pteridine antifolates: a two-compartment growth inhibition assay for evaluating drug leakage and metabolism

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We have developed a two-compartment growth inhibition assay that can provide information about leakage, metabolism and delivery of liposome-dependent drugs under cell culture conditions, and at drug concentrations that are relevant to drug delivery. Two cell lines are grown in separate compartments separated from each other by a 0.1 μm polycarbonate membrane. The membrane allows free drugs to diffuse rapidly from one compartment to another, and does not allow liposomes to diffuse through. Liposomes are added to the first compartment, which contains target cells. The extent of leakage caused by these cells is determined by the growth inhibition of non-target cells in the second compartment. We show that methotrexate and methotrexate-γ-aspartate leak rapidly and almost completely when encapsulated in phosphatidylglycerol/cholesterol (67:33) liposomes. In contrast, there is only 42% leakage when the drugs are encapsulated in distearoylphosphatidylglycerol/cholesterol (67:33) liposomes. We also demonstrate that the target cells (CV1-P) may partially degrade encapsulated methotrexate-γ-aspartate to methotrexate. Therefore, methotrexate-γ-aspartate may be a lysosomally cleaved pro-drug of methotrexate.

Introduction

The mechanism by which drug enters cells is one of the most important considerations for optimizing liposome-mediated drug delivery [1]. Drugs that can enter cells rapidly as free compounds will do so after leakage from liposomes, and are defined as liposome-independent agents [2]. Alternatively, drugs that cannot pass readily through the plasma membrane will enter cells through uptake of the liposomes, and have been defined as liposome-dependent drugs [3]. The potency of a liposome-dependent drug is increased by encapsulation in negatively charged [2] or neutral antibody-directed liposomes [4–6].

The potency of an encapsulated liposome-dependent drug depends on the uptake of intact liposomes by endocytosis [7]. Consequently, the stability of the liposome is critical to efficient drug delivery. Liposomes are known to lose their contents through adsorption to the cell membrane [8], and through serum-induced leakage [9,10]. However, there has not been a systematic evaluation of liposome leakage under the conditions that closely parallel those prevailing at the IC_{50} of encapsulated drug. This stems from the fact that in some cases the drug concentration at the IC_{50} is as low as 0.005 μM [11], and leakage studies with drugs are currently not feasible at such low concentrations if conventional analytical techniques are followed.

In this paper, we report a two-compartment system (Fig. 1) that can provide information about leakage, and about the effects of encapsulated drug on target and non-target cells. The chamber and well of the two compartment system are separated from each other by a semi-permeable membrane, which allows free drug to diffuse rapidly from one compartment to the other. Liposomes or free drugs are added to one compartment, which contains cell population 1, and the extent of leakage caused by cell population 1 and serum is determined by the growth inhibition of a separate cell population, cell population 2, in the second compartment. If we assume that liposomes leak their contents shortly after addition to the target cell population (cell population 1), then by comparing the IC_{50} values of both the free and encapsulated drugs on cell population 2, one can evaluate the extent of leakage according to the following equation:

$$\text{Apparent \% leakage} = \left(\frac{IC_{50}\text{ of free drug}}{IC_{50}\text{ of encapsulated drug}}\right)_{\text{Cell 2}} \times 100$$

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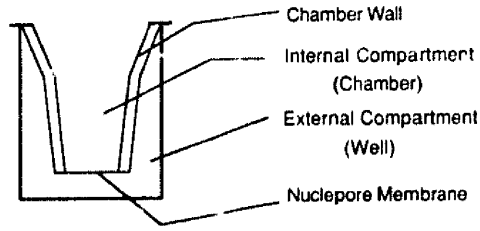

Fig. 1. The two-compartment growth inhibition assay. The two-compartment system is formed by suspending the Costar Transwell in the well of a Costar 24 well plate. The chamber and well of the two-compartment system are separated from each other by a 0.1 μm pore size polycarbonate membrane, which allows free drug to diffuse rapidly from one compartment to the other, and does not allow liposomes to diffuse through. CV1-P cells are grown on the bottom surface of the well, and EL4 cells are grown in suspension in the Transwell chamber.

We call this 'apparent leakage', because, in practice, a slower and more extensive loss of liposome contents would give similar results in this system.

We show here that there is close to 100% leakage when methotrexate is encapsulated in phosphatidylglycerol/cholesterol (67 : 33) liposomes, but only 42% when encapsulated in distearoylphosphatidylglycerol/cholesterol (67 : 33) liposomes, suggesting that distearoylphosphatidylglycerol/cholesterol (67 : 33) liposomes are more stable than phosphatidylglycerol/cholesterol (67 : 33) liposomes. Unexpectedly high leakage values are found when methotrexate-γ-aspartate is encapsulated in distearoylphosphatidylglycerol/cholesterol (67 : 33) liposomes. This unexpected result suggests that a small fraction of the encapsulated methotrexate-γ-aspartate may be metabolized to a more potent form, presumably methotrexate.

Materials and Methods

Egg yolk phosphatidylglycerol, and distearoylphosphatidylglycerol were obtained from Avanti Polar Lipids (Birmingham, AL), and were stored as a chloroform solution at −20 °C in glass ampoules under argon. Egg yolk phosphatidylglycerol will subsequently be referred to simply as phosphatidylglycerol. Cholesterol was obtained from Sigma Chemical Co. (St Louis, MO), and was purified by four recrystallizations from methanol. The product was stored in the same way as phospholipids [7]. 6.5 mm Transwell chambers with 0.1 μm pore size polycarbonate membranes (not treated for cell culture) were a custom purchase from Costar (Cambridge, MA). Methotrexate was purchased from Sigma. Methotrexate-γ-aspartate was synthesised and provided by J.R. Piper, Southern Research Institute (Birmingham, AL) [13]. Sterile solutions of free or encapsulated drug were stored at 4 °C, and used within one month of preparation. Carboxyfluorescein was obtained from Eastman Chemical Co. (Rochester, NY) and purified with Sephadex LH-20 [14]. The purified carboxyfluorescein was dissolved in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) to a final concentration of 1.5 or 2 mM at pH 7.2. The tonicity of this solution was adjusted to 290 mmol/kg with NaCl. This solution was used for the two-compartment system validation experiments. Methotrexate and methotrexate-γ-aspartate solutions were prepared for encapsulation at a concentration of 10 mM in 50 mM 4-morpholineethanesulfonic acid (Mes)/50 mM Hepes and adjusted to pH 7.2 with sodium hydoxide. The tonicity of the solutions were adjusted to 290 mmol/kg with NaCl. Large unilamellar liposomes were prepared under sterile conditions by reverse-phase evaporation [15]. All liposomes used were prepared from phospholipid/cholesterol (67 : 33), and will subsequently be referred to by phospholipid alone.

Liposomes were separated from unencapsulated drug by gel chromatography with a sterile 1 × 15 cm Sephadex G-50 (Pharmacia, Piscataway, NJ) column. The column was eluted with 50 mM Mes/50 mM Hepes-NaCl (pH 7.2), 290 mmol/kg. Drug concentration in the liposomes was determined from the absorbance at 370 nm after solubilization of a sample of the vesicles in chloroform/methanol/buffer 1 : 3 : 1 assuming a molar absorption coefficient of 7943 at 370 nm [16]. The phospholipid content of the vesicles was measured by phosphorus determination [17].

CV1-P, an African green monkey kidney cell line [18], was obtained from P. Berg, Stanford University, Palo Alto. EL4, a T-lymphoma was obtained from W. Ershler, University of Wisconsin, Madison, who originally purchased this cell line from American Type Culture Collection. Cells were grown in Dulbecco's modified Eagle's minimal essential medium (pyruvate, 1 g/l glucose), with 100 units/ml penicillin and streptomycin, and 10% fetal calf serum (K-C Biologicals).

Two dialysis experiments, the carboxyfluorescein flux experiment and the phospholipid flux experiment, were carried out as follows in order to validate the two-compartment system. For the carboxyfluorescein flux experiment, 0.7 ml of Hepes buffer was first added to three individual wells of a 24-well plate (Costar). Three Transwell chambers were suspended from the top of the wells, and 0.15 ml of 1.5 mM carboxyfluorescein solution was added to each of the three chambers. The plate was then incubated at 37 °C on an American Rotator V shaker (American Scientific Products, McGaw Park, IL) at a rate of 120 rpm. At separate time points, 10-μl samples were taken from the chambers and the wells. These samples were diluted to 1.0 ml with Hepes buffer. The amount of carboxyfluorescein that diffused across the polycarbonate membrane of the chambers was determined by the absorbance at 493 nm using a molar absorption coefficient of 70000. We also modified this experiment by adding 0.7 ml of 2.0 mM carboxyfluorescein in the well and 0.15 ml of Hepes buffer in

the chamber, and similarly followed the diffusion of carboxyfluorescein across the membrane with time. For the phospholipid flux experiment, unloaded liposomes at a concentration of 1.0 μmol/0.15 ml was added to nine individual chambers suspended from the top of wells of a 24-well plate which contained 0.7 ml of Hepes buffer. The plate was then incubated at 37°C with shaking at 120 rpm. At 1, 2 and 3 days, the contents of three chambers and wells were assayed for lipids by phosphorus determinations [17]. The percent of liposomes retained in the chamber was calculated using the following equation:

$$\% \text{ Retention} = \frac{\text{Lipid content of chamber}}{\text{Lipid content of chamber} + \text{lipid content of well}} \times 100$$

One compartment growth inhibition studies were carried out as previously described [2]. Briefly, cells were suspended for EL4 cells for growth inhibition at $3 \cdot 10^4$ cells per well in a 24-well plate (Costar). Triplicate wells were treated with drug immediately after EL4 cells were added to the wells. 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 72 h before counting with a Coulter counter, model ZM. EL4 cells were resuspended directly in medium, diluted 1/50 in isotonic counting fluid (Diagnostic Technology, Inc., Hayward, CA) and counted. Percent growth were 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 for the cell line. The concentration of drug required to produce 50% inhibition of growth (IC_{50}), was determined from the plots.

In the two-compartment growth inhibition studies, two cell lines, CV1-P and EL4, were used. EL4 cells were chosen because they are suspension cells, which, when placed in the chamber of the two compartment system, will not block the polycarbonate pores by adhering to the membrane. CV1-P cells were chosen because earlier evidence shows that this cell line is very sensitive to liposome-mediated drug delivery [2]. The two-compartment growth inhibition studies were carried out in the following way. 0.7 ml of CV1-P cells at a concentration of $2 \cdot 10^4$ per ml were first added to each well of a 24-well plate. The cells were allowed to adhere to the surface of the plate for 6 h under normal growth conditions. A chamber was then suspended from the top of each well. To each chamber, 0.15 ml of EL4 cells at a concentration of $3.3 \cdot 10^4$ per ml was added. Triplicate wells were treated with drug as soon as EL4 cells were added to the chambers. Control wells were treated with buffer alone. Three wells and three chambers were counted at the time of treatment to give the original cell concentration. The cells were allowed to grow for 72 h at 37°C with shaking, and were then counted with a Coulter Counter, model ZM. The whole content of each chamber was transferred to a counting vial containing 9.8 ml diluent, and was counted to determine EL4 cells. CV1-P cells were freed of medium, and resuspended by treatment with 1 ml of 0.1% trypsin in phosphate buffered saline, 1 mM EDTA solution at 37°C for 20 min. The cell suspension was diluted 1/50 with isotonic counting fluid and counted. The mean percent growth was determined, plotted against the $\log_{10}$ of the drug concentration for each cell line, and the IC_{50} was determined from the plots as described above for the one-compartment system.

Results

Validation of the two-compartment system

A two-compartment system for growth inhibition must fulfill a number of criteria in order to be successful in evaluating liposome leakage. The membrane, which separates the two compartments, must only allow free drugs to pass through while retaining the liposomes in the compartment where they are originally placed. In addition, free drug must reach equilibrium rapidly across the membrane, and the system must be easily used in a standard cell culture environment. The Costar Transwell is a plastic chamber that may be placed in the wells of a 24 well plate. The lower surface is a polycarbonate membrane that can allow the passage of molecules, while retaining particles such as liposomes to an extent that depends on the pore size of the polycarbonate membrane and the liposome size.

Figs. 2 and 3 show the results of a carboxyfluorescein flux study, in which 0.1 μm pore size Transwells were used in a two compartment system. When 0.15 ml of 1.5 mM carboxyfluorescein solution was added to the chambers, it took the carboxyfluorescein approximately

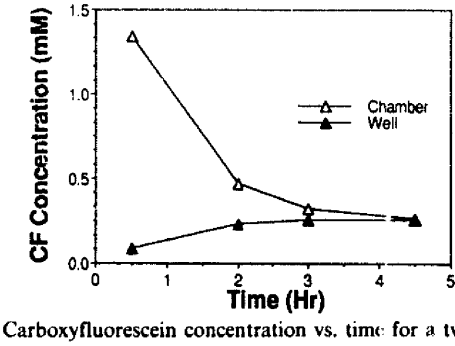

Fig. 2. Carboxyfluorescein concentration vs. time for a two-compartment system. 0.15 ml of 1.5 mM carboxyfluorescein (CF) was added to the chambers. The amount of carboxyfluorescein that diffused across the membrane of the chambers into the wells that contained 0.7 ml Hepes buffer was determined by the absorbance at 493 nm using a molar absorption coefficient of 70000.

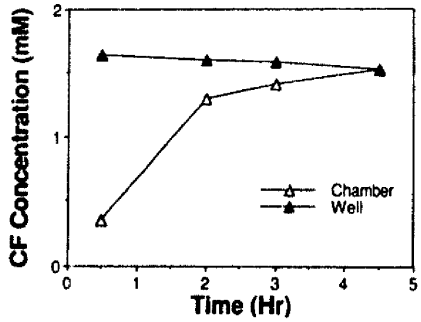

Fig. 3. Carboxyfluorescein concentration vs. time for a two-compartment system. 0.70 ml of 2.0 mM carboxyfluorescein (CF) was added to the wells. The amount of carboxyfluorescein that diffused across the membrane from the wells into the chambers that contained 0.15 ml Hepes buffer was determined by the absorbance at 493 nm using a molar absorption coefficient of 70000.

4.5 hours to reach the same concentration in the two compartments (Fig. 2). Similar results were obtained when 0.7 ml of 2.0 mM carboxyfluorescein was added to the wells (Fig. 3).

Table I shows the results of a phospholipid flux experiment using either phosphatidylglycerol or distearoylphosphatidylglycerol empty liposomes prepared by the REV method. Less than 5% of the phospholipid diffused into the well in 72 h for both liposome preparations. This amount of phosphate is negligible, and close to the limits of the assay used. Therefore, we can conclude that a negligible amount of liposomal lipid diffused into the wells in 3 days.

Two-compartment growth inhibition assay

Free and phosphatidylglycerol-encapsulated methotrexate and methotrexate-γ-aspartate. Table II shows the results of one and two compartment studies using free and phosphatidylglycerol-encapsulated methotrexate. Free methotrexate is a more potent inhibitor of EL4 growth than it is an inhibitor of CV1-P growth. Moreover, methotrexate is a more potent inhibitor of EL4

TABLE I

The retention of liposomes in 0.1 μm Transwell chambers

Nonloaded phosphatidylglycerol/cholesterol (67:33) and distearoylphosphatidylglycerol/cholesterol (67:33) liposomes were prepared by reverse-phase evaporation. Liposomes at a concentration of 1.0 μmol phospholipid/0.15 ml were added to the chambers suspended in the wells, which contained 0.7 ml of Hepes buffer. The two compartment system was incubated at 37°C with shaking. The percent of liposomes retained in the chamber by the membrane with respect to time was determined by phosphorus analysis.

Time (h)	% Phospholipid retained in chamber	
	Phosphatidylglycerol	Distearoylphosphatidylglycerol
24	95.9	100
48	94.4	96.9
72	94.6	94.8

TABLE II

Growth inhibitory potency of free and liposome-encapsulated methotrexate

Cell type	CV1-P		EL4	
	IC_{50} (μM)	n [a]	IC_{50} (μM)	n [a]
One-compartment system				
Free drug	–		0.019±0	3
PG [b]	–		0.026±0.004	2
DSPG [b]	–		0.026±0.004	2
Two-compartment system				
Free drug	0.06 ±0.02	5	0.011±0.001	5
PG [b]	0.013±0.004	2	0.011±0	2
DSPG [b]	0.020±0.009	3	0.026±0.006	5

[a] n represents the number of determinations. The IC_{50} value given is the mean of all determinations ± S.D.

[b] Methotrexate was encapsulated in phosphatidylglycerol/cholesterol (67:33) (PG) and distearoylphosphatidylglycerol/cholesterol (67:33) (DSPG) liposomes prepared by reverse-phase evaporation. The final drug/lipid ratio for PG- and DSPG-encapsulated methotrexate was 122 mmol/mol and 30 mmol/mol, respectively. Free or encapsulated drug was added to the wells, and cells were allowed to grow for 3 days before counting.

growth in a two compartment system than it is in a one compartment system. The reasons for this are unclear, but this observation suggests that the value obtained for the free drug in a two-compartment system will be the most appropriate for comparison with values obtained for encapsulated drug. Encapsulated methotrexate is 4.6 times more potent that free methotrexate for growth inhibition of CV1-P cells. Encapsulated methotrexate has an IC_{50} identical to that of free methotrexate for EL4 cells. This is consistent with the hypothesis that the contents of the liposomes undergo virtually complete leakage within a short time of addition to the well.

Table III shows the results of one and two compartment studies using free and phosphatidylglycerol-encapsulated methotrexate-γ-aspartate. The potency of free methotrexate-γ-aspartate is significantly higher on EL4 cells than it is on CV1-P cells. Moreover, free methotrexate-γ-aspartate is more potent for EL4 cells in the two-compartment system than it is in the one compartment system. These observations are very similar to comparable observations with methotrexate. Again, the reasons are unclear, but it is obviously necessary to use values obtained in the two-compartment system for comparison with the encapsulated drug. Encapsulated methotrexate-γ-aspartate is 109-times more potent than free methotrexate-γ-aspartate for growth inhibition of CV1-P cells. This is consistent with all previous observations that have demonstrated this drug to be liposome-dependent [2,11]. Encapsulated methotrexate-γ-aspartate is slightly more potent than free methotrexate-γ-aspartate for growth inhibition of EL4 cells, although statistical analysis demonstrates that these values are not significantly different. The similarity of

TABLE III

Growth inhibitory potency of free and liposome-encapsulated methotrexate-γ-aspartate

Cell type	CV1-P		EL4	
	IC_{50} (μM)	n [a]	IC_{50} (μM)	n [a]
One-compartment system				
Free drug	–		0.45 ± 0.25	5
PG [b]	–		0.28 ± 0	2
DSPG [b]	–		0.28 ± 0	2
Two-compartment system				
Free drug	1.41 ± 0.58	9	0.33 ± 0.08	5
PG [b]	0.013 ± 0.007	7	0.28 ± 0.02	3
DSPG [b]	0.015 ± 0.008	3	0.28 ± 0.06	3

[a] n represents the number of determinations. The IC_{50} value given is the mean of all determinations ± S.D.

[b] Methotrexate-γ-aspartate was encapsulated in phosphatidylglycerol/cholesterol (67:33) (PG) and distearoylphosphatidylglycerol/cholesterol (67:33) (DSPG) liposomes prepared by reverse-phase evaporation. The final drug/lipid ratio for PG- and DSPG-encapsulated methotrexate was 125 mmol/mol and 56 mmol/mol, respectively. Free or encapsulated was added to the wells, and cells were allowed to grow for 3 days before counting.

these values suggests that a rapid and extensive loss of liposome contents occurs shortly after addition to the medium.

Distearoylphosphatidylglycerol-encapsulated methotrexate and methotrexate-γ-aspartate. Table II shows the results of one- and two-compartment studies using free and distearoylphosphatidylglycerol-encapsulated methotrexate. Encapsulated methotrexate is 3-times more potent than free methotrexate for growth inhibition of CV1-P cells. However, encapsulated methotrexate is about 2.4-times less potent than free methotrexate for growth inhibition of EL4 cells. This value is consistent with the hypothesis that approx. 42% of the liposome contents leak shortly after addition to the medium. This is in contrast to the apparently complete leakage that occurred when phosphatidylglycerol was used to encapsulate the drug.

Table III shows the results of one- and two-compartment studies using free and distearoylphosphatidylglycerol-encapsulated methotrexate-γ-aspartate. Encapsulated methotrexate-γ-aspartate is 94-times more potent than free methotrexate-γ-aspartate for growth inhibition of CV1-P cells. This is consistent with all previous observations that have demonstrated this drug to be liposome-dependent [2,11].

Distearoylphosphatidylglycerol-encapsulated methotrexate-γ-aspartate is slightly more potent than free methotrexate-γ-aspartate for growth inhibition of EL4 cells. Statistical analysis demonstrates that the growth inhibitory potency of distearoylphosphatidylglycerol-encapsulated methotrexate-γ-aspartate and free methotrexate-γ-aspartate are not significantly different. The similarity of these values suggests either a rapid and extensive loss of liposome contents shortly after addition to the medium, or a small extent of conversion of the drug to a more potent form, presumably methotrexate.

Serum induction versus cellular induction: two-compartment system in the absence of CV1-P cells

Table IV shows the results of two compartment studies in the absence of CV1-P cells using free and phosphatidylglycerol-encapsulated methotrexate and methotrexate-γ-aspartate. Both free methotrexate and methotrexate-γ-aspartate are slightly more potent than encapsulated drugs for growth inhibition of EL4 cells. The similarity of these values demonstrates a rapid and extensive loss of liposome contents (95% and 97% leakage) shortly after addition to the medium. This implies that the serum and growth medium may be responsible for most of the leakage seen in our growth inhibition studies.

Table IV also shows the results of two-compartment studies in the absence of CV1-P cells using free and distearoylphosphatidylglycerol-encapsulated methotrexate and methotrexate-γ-aspartate. Both free methotrexate and methotrexate-γ-aspartate are more potent than encapsulated drugs for growth inhibition of EL4 cells, suggesting that most of the contents are still retained in the liposomes. If the IC_{50} of free drug is compared with that of encapsulated drug for both methotrexate and methotrexate-γ-aspartate, we can infer that leakage is 35% for both methotrexate and methotrexate-γ-aspartate. When we compare this apparent leakage value for methotrexate with the apparent leakage value obtained for methotrexate in the presence of CV1-P cells, we see a 7% difference between them, suggesting that serum in the growth medium may be responsible for 35% of the leakage, while CV1-P cells cause the residual 7% leakage. However, statistical analysis demonstrates that the dif-

TABLE IV

Drug potency in the two-compartment system without CV1-P cells

	Methotrexate		Methotrexate-γ-aspartate	
	IC_{50} (μM)	n [a]	IC_{50} (μM)	n [a]
Free drug	0.011 ± 0.006	5	0.32 ± 0.12	5
PG [b]	0.012 ± 0.005	4	0.33 ± 0.14	4
DSPG [b]	0.031 ± 0.007	3	0.91 ± 0.13	4

[a] n represents the number of determinations. The IC_{50} value given is the mean of all determinations ± S.D.

[b] The drugs were encapsulated in phosphatidylglycerol/cholesterol (67:33) (PG) and distearoylphosphatidylglycerol/cholesterol (67:33) (DSPG) liposomes prepared by reverse-phase evaporation. The final drug/lipid ratio was 122 mmol/mol for PG-encapsulated methotrexate, 125 mmol/mol for PG-encapsulated methotrexate-γ-aspartate, 30 mmol/mol for DSPG-encapsulated methotrexate, and 56 mmol/mol for DSPG-encapsulated methotrexate-γ-aspartate, respectively. Free or encapsulated drug was added to the wells, which contained no CV1-P cells. EL4 cells in the chambers were allowed to grow for 3 days before counting.

ference caused by the presence of CV1-P cells is not significant. Similar comparison of the leakage value for methotrexate-γ-aspartate with the leakage value of methotrexate-γ-aspartate obtained in the presence of CV1-P cells reveals that the leakage value for this drug is apparently increased by 83% to 118% in the presence of CV1-P cells. Statistical analysis demonstrates that this apparent increase in leakage induced by the presence of CV1-P cells is significant ($P < 0.0$). Since methotrexate-γ-aspartate should leak no more than methotrexate, it is more reasonable to suggest that methotrexate-γ-aspartate is partially metabolized to a more potent form, presumably methotrexate.

Growth inhibitory effects of non-loaded liposomes

It has been demonstrated that empty liposomes may inhibit the growth of some cell lines [2]. In order to exclude the possibility that lipid metabolites produced by CV1-P cells may inhibit EL4 cell growth in a two compartment system, non-loaded liposomes, at the lipid concentrations used when cells were treated with encapsulated methotrexate and methotrexate-γ-aspartate, were added to the wells where CV1-P cells were located. The growth inhibition of both CV1-P and EL4 cells was then followed. Fig. 4 shows that neither cell line is affected by empty distearoylphosphatidylglycerol liposomes. Fig. 5 shows that there is no inhibition on the growth of EL4 cells caused by the addition of empty phosphatidylglycerol liposomes to the wells. However, consistent with previous observations [2], there was inhibition of CV1-P cell growth at the highest phosphatidylglycerol concentrations used. From these observations, we can say that the effects of encapsulated drug on CV1-P cells are not caused by phospholipid. However, these experiments do raise the possibility that lipid metabolites released by CV1-P cells might act synergistically with drug to inhibit EL4 cells. Consequently, we have examined the effects of free

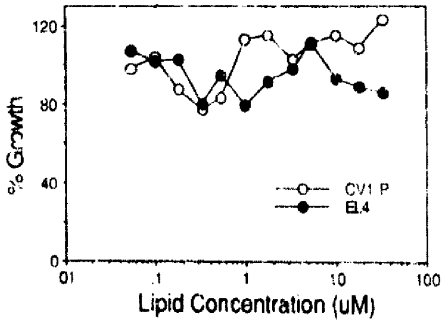

Fig. 4. The effect of nonloaded distearoylphosphatidylglycerol liposomes on cell growth. Nonloaded distearoylphosphatidylglycerol liposomes, at the lipid concentrations used when cells were treated with encapsulated methotrexate and methotrexate-γ-aspartate, were added to the wells where CV1-P cells were located. EL4 cells in the chamber and CV1-P cells in the wells were allowed to grow for 3 days before counting.

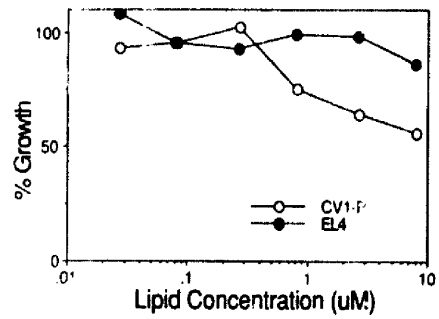

Fig. 5. The effect of nonloaded phosphatidylglycerol liposomes on cell growth. Nonloaded phosphatidylglycerol liposomes, at the lipid concentrations used when cells were treated with encapsulated methotrexate and methotrexate-γ-aspartate, were added to the wells where CV1-P cells were located. EL4 cells in the chambers and CV1-P cells in the wells were allowed to grow for 3 days before counting.

methotrexate-γ-aspartate mixed with phosphatidylglycerol liposomes at the same drug/lipid ratio as was present in our encapsulated preparations. The IC_{50} of methotrexate-γ-aspartate on EL4 cells in the two-compartment system was the same in the presence and absence of the phospholipid (data not shown). Therefore, we can say that the effects of the encapsulated drugs are entirely caused by the drug.

Discussion

We have described a simple method to study the leakage, metabolism and delivery of liposome dependent drugs under cell culture conditions, and at drug concentrations that are relevant to drug delivery. Methotrexate and methotrexate-γ-aspartate encapsulated in phosphatidylglycerol liposomes appear to leak rapidly and almost completely. In contrast, these two drugs encapsulated in distearoylphosphatidylglycerol liposomes leak far less extensively. This observation is consistent with the observation of Senior et al., who demonstrated that distearoylphosphatidylcholine/cholesterol (1 : 1) liposomes leak less than phosphatidylcholine/cholesterol (1 : 1) liposomes in the presence of serum [19].

One unusual feature of our experiments is the increased sensitivity of EL4 cells to the effects of free antifolates in the two compartment system. This effect is in no way linked to CV1-P cells as it occurs whether or not they are present in the well. Therefore, it must occur as a result of the physical environment created by the two-compartment system. Perhaps the major feature of this is that a small number of cells are grown in 0.15 ml of medium that is diffused against 0.7 ml of medium. From original and final counts of the controls, we know that EL4 cells divide about 16% faster in the two-compartment system than they do in the one-compartment system. This is likely to make them more sensitive to the effect of antifolates.

Some of the data that we have obtained, suggests that the target cells (CV1-P) may partially degrade encapsulated methotrexate-γ-aspartate to methotrexate. The strongest evidence for this phenomenon was obtained with distearoylphosphatidylglycerol liposomes. When distearoylphosphatidylglycerol liposomes were used, the IC_{50} on EL4 cells for encapsulated methotrexate and for encapsulated methotrexate-γ-aspartate in the absence of CV1-P cells suggest that approx. 35% leakage occurs from those liposomes. Encapsulated methotrexate-γ-aspartate in the presence of CV1-P cells gives an IC_{50} on EL4 cells that is slightly lower than that of free methotrexate-γ-aspartate. Although this value is not significantly different from that of free methotrexate-γ-aspartate, it is significantly lower than the two-compartment IC_{50} of encapsulated methotrexate-γ-aspartate on EL4 cells in the absence of CV1-P cells. Moreover, there is no evidence from the companion studies with distearoylphosphatidylglycerol-encapsulated methotrexate to suggest that processing by CV1-P cells can increase leakage from 35% to 118%. Consequently, we conclude that distearoylphosphatidylglycerol-encapsulated methotrexate-γ-aspartate is partly metabolised and released into the medium as methotrexate.

We described above a simple way of comparing IC_{50} values in order to estimate leakage. If we assume that methotrexate-γ-aspartate is converted in the CV1-P cells to methotrexate, that it is released into the medium by the CV1-P cells, that the conversion is rapid, and that sufficient methotrexate is produced to be the sole cause of EL4 growth inhibition, then the following equation can be used to estimate the extent of metabolism:

$$\text{Apparent \% metabolism} = \frac{IC_{50} \text{ of free methotrexate on EL4}}{IC_{50} \text{ of encapsulated methotrexate-γ-aspartate on EL4}} \times 100$$

Using this equation, we calculate that 4% of the encapsulated methotrexate-γ-aspartate is metabolised by the CV1-P cells to methotrexate, and released into the medium.

A number of pieces of evidence support the possibility that methotrexate-γ-aspartate is metabolised to methotrexate. We have previously demonstrated that liposome-mediated drug delivery involves lysosomal processing of the liposomes and their contents [2,11]. Therefore, the drug is likely to be exposed to the lysosomal degradative enzymes. It has been found that hydrolysis of pteroylpolyglutamates is catalyzed by enzymes that possess peptidase like activity and are referred to as γ-glutamyl hydrolases [20]. The majority of this enzyme activity is found associated with the lysosomal fraction after differential centrifugation of cell homogenates [21–25]. The hydrolase is nonspecific with regard to the pteridine or pteridine like moiety [26–30], although linkage through the γ- rather than α-carboxyl of glutamate is essential [29,31–33]. Baugh et al. [33] have demonstrated that this enzyme can remove any amino acid from the γ-carboxyl of pteridines, because non-glutamate C-terminal amino acids can all serve as substrates. Consequently, we can say that this enzyme is capable of converting methotrexate-γ-aspartate to methotrexate.

In spite of the extensive leakage and partial metabolism of phosphatidylglycerol-encapsulated methotrexate-γ-aspartate, this drug is nonetheless liposome-dependent in this form. On CV1-P cells, phosphatidylglycerol-encapsulated methotrexate-γ-aspartate is 109-times more potent than free methotrexate-γ-aspartate. It seems remarkable that the potency of the encapsulated drug should be so much increased despite the apparent loss of all of the encapsulated contents. However, the situation is quite complex, and we know very little about how much drug must reach cells and at what rate it must be delivered for optimal effects. We have previously speculated that optimal effects may require delivery of an initial large bolus of drug to cells, followed by a much lower rate of continuous drug delivery [11]. It seems possible that CV1-P cells may take up liposomes so rapidly, that many are internalised before extensive leakage can occur. This would provide an initial bolus, and would mean that CV1-P cells were exposed to a large fraction of the drug in the encapsulated form, even though the two-compartment system shows leakage to be quantitative.

Distearoylphosphatidylglycerol liposomes leak far less than phosphatidylglycerol liposomes. If we compare them in terms of how much drug they retain, distearoylphosphatidylglycerol retains about 58% of encapsulated contents, and phosphatidylglycerol no more than 1–2%. If drug delivery efficiency were directly related to retention, we might expect drug in distearoylphosphatidylglycerol liposomes to be approx. 30–60-times more potent than drug in phosphatidylglycerol liposomes. For delivery of fluoroorotic acid [34], this is indeed what we have observed, suggesting that liposome stability can affect the efficiency with which fluoroorotic acid is delivered. However, the potency of methotrexate-γ-aspartate is quite comparable when encapsulated in liposomes of either composition. The reasons for this are unclear, but it seems possible that liposomes resistant to serum induced leakage may also be resistant to lysosomal degradation. Consequently, they may release their contents more slowly. How this may affect drug potency, and why it affects one drug and not another will require further investigation.

We have demonstrated the metabolism of methotrexate-γ-asparate by CV1-P cells, and discussed the effects that this will have on non-target cell populations. It is also appropriate to consider how the metabolism of

methotrexate-γ-aspartate may be involved in its delivery to CV1-P cells themselves. We have previously speculated on the mechanism, by which methotrexate-γ-aspartate is transferred from the lysosomal compartment to the cytoplasm of the cell [5]. One possibility suggested was that the methotrexate-γ-aspartate might be degraded to methotrexate, and transported across the lysosomal membrane by the folate transport system. Our present observations suggesting that metabolism occurs, confirm that this mechanism may be important. There is a second reason why conversion of methotrexate-γ-aspartate may be important for full expression of its potency. The retention of antifolates in cells is known to occur through their polyglutamylation in the cytoplasm [35,36]. Methotrexate-γ-aspartate cannot be polyglutamylated [37,38], and its retention and potency may depend on it first being converted to the methotrexate. We currently do not know what fraction of the lysosomally processed methotrexate-γ-asparate is converted to methotrexate. However, it currently appears possible that methotrexate-γ-asparate may be a lysosomally cleaved pro-drug of methotrexate.

In conclusion, we have developed a simple two compartment growth inhibition assay, and have demonstrated how it may be used to obtain useful information about drug leakage, metabolism, and delivery. We hope in future work to study these parameters in more detail, and to examine other liposome-dependent drugs.

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