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Further Studies on the Charge-Related Alterations of Methotrexate Transport in Ehrlich Ascites Tumor Cells by Ionic Liposomes: Correlation with Liposome-Cell Association

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Summary. Interaction of positively (phosphatidylcholine/stearylamine 5:1) or negatively (phosphatidylcholine/stearic acid 5:1) charged liposomes with Ehrlich ascites tumor cells for 1–5 min increases or decreases, respectively, the bidirectional fluxes of the folic acid analog, methotrexate. These effects on influx and efflux appear to be symmetrical since the liposomes do not change the intracellular level of methotrexate at the steady state. Influx kinetics show that these alterations result from an increase or decrease in the V_{max} with no change in the K_m^{in}. These effects appear to be specific for the methotrexate-tetrahydrofolate carrier system since the transport of other compounds which utilize this carrier, aminopterin, 5-methyltetrahydrofolate, and 5-formyltetrahydrofolate, is affected similarly to methotrexate, whereas, the transport of folic acid, a compound similar in structure and charge but not significantly transported by this carrier is unaffected by liposomes. Once cells are exposed to charged liposomes, the effects on methotrexate transport cannot be reversed by washing the cells free of the extracellular liposomes. If, however, cells are exposed to liposomes of one charge, washed and then exposed to liposomes of the opposite charge, methotrexate influx is reversed to control rates. The effects of charged liposomes on methotrexate influx were not abolished by treating the cells with neuraminidase, metabolic inhibitors or lowering the temperature to 4 °C. Studies on the uptake of [^{14}C] liposomes show that these effects are not proportional to the total amount of lipid associated with the cell but result from an initial rapid liposome-cell association that is not dependent on temperature or energy metabolism nor related to cell surface charge.

Key words methotrexate · liposome · transport · Ehrlich · tumor · cell

Introduction

Liposomes have been proposed as nontoxic, biodegradable carriers for introducing drugs, enzymes or other agents into cells [24, 38]. Evidence, however, indicates that these lipid vesicles are not inert vehicles, but depending on composition, are capable themselves of alterating cell membrane composition [5, 9, 43], function [27, 39, 42], and a variety of cellular activities [10, 20, 30]. These observations become important when studying the effects of an encapsulated cytotoxic agent since activity may be obscured or complicated by effects of the liposome alone. Recently, methotrexate, a synthetic analog of folic acid, has been encapsulated within liposomes and the kinetics of plasma clearance [23, 25], cell association [12, 46] and antitumor effects [28, 49, 50] have been characterized for this way of administration of the drug. Previous studies from this laboratory, however, have shown that exposure of Ehrlich ascites tumor cells to positively charged liposomes stimulates the bidirectional fluxes of unencapsulated methotrexate, presumably by accelerating the rate-limiting step in the translocation process [13]. In order to explore further the relationship between this phenomenon and liposomal charge, this report describes the effects of negatively charged liposomes on methotrexate transport and contrasts these data with the effects of positively charged liposomes. Further, we have characterized the association of charged lipsomes with the cell to correlate the effects on methotrexate transport with liposomal uptake and to determine whether these effects are energy- or temperature-dependent. Finally, we investigate the role of cellular surface charge in the transport of methotrexate, the liposome-cell association and the effects of charged liposomes on methotrexate influx.

Materials and Methods

Chemicals

Egg phosphatidylcholine, stearylamine, stearic acid, sodium azide and 2-deoxyglucose were obtained from Sigma Chemical Co. (St. Louis, Mo.). Phosphatidylcholine was purified by column chromatography on alumina [44] and purity confirmed by thin-layer chromatography [8].[3′,5′9(N)-^{3}H] methotrexate, [3′,5′,9(N)-^{3}H] aminopterin, [3′,5′,9(n)-^{3}H] folic acid and 5-[^{3}H] methyltetrahydrofolic acid were obtained from Amersham/Searle Corporation (Arlington Heights, Ill.) and purified by diethylaminoethyl cellulose chromatography [17]. 5[^{3}H]-formyltetrahydrofolic acid was from Moravek Biochemicals (City of Industry, Calif.). [COOH-^{14}C] inulin and [^{14}C] methyl iodide were from New England Nuclear (Boston, Mass.). [^{14}C] phosphatidylcholine was synthesized from [^{14}C]

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methyl iodide and egg phosphatidylcholine [45]. Neuraminidase (vibro cholerae) was obtained from Calbiochem-Behring Corp. (La Jolla, Calif.).

Cells, Media, and Incubation Techniques

Ehrlich ascites tumor cells were grown in male CF1 mice (Sprague Dawley, Madison, Wis.) and passed weekly by intraperitoneal inoculation of 0.2 ml of undiluted ascites fluid. Cells were suspended in buffer composed of (in mM): 136 NaCl, 4.4 KCl, 16 $NaHCO_3$, 1.1 KH_2PO_4, 1.0 $MgCl_2$, and 1.9 $CaCl_2$. The pH was maintained at 7.4 by passing warmed and humidified 95% O_2-5% CO_2 over the cell suspension. The suspension was stirred by a Teflon paddle in specially designed flasks inserted in a 37 °C water bath. Unidirectional fluxes and net uptake of [^{3}H] methotrexate and other folates were measured as previously described [15, 17]. Transport fluxes were stopped by injection of the cell suspension into 10 volumes of 0 °C 0.85% NaCl solution (pH 7.4). Influx and efflux of methotrexate is highly temperature sensitive and is essentially abolished at 0 °C [17]. The cell fraction was separated by centrifugation ($500 \times g$ for 30–60 sec) and washed twice with the 0 °C NaCl solution. Washing procedure took no longer than 5 min. The washed pellet was aspirated into the tip of a Pasteur pipette, extruded onto a polyethylene tare, and dried overnight at 70 °C. The dried pellets were weighed on a Cahn 4700 electrobalance (Cahn Instruments, Paramount, Calif.), placed in a scintillation vial, and dissolved in 0.2 ml of 1 N KOH for 1 h at 70 °C. The digest was neutralized with 0.2 ml of 1 N HCl and 3 ml of Ready-Solv (Beckman, Irvine, Calif.) was added to the scintillation vial. Radioactivity was determined in a Beckman LS-230 scintillation spectrometer and counting efficiencies were determined employing [^{3}H]- or [^{14}C]-toluene internal standards. The uptake of radiolabeled liposomes was determined in an identical manner except that the cells were washed in 37 °C saline. Data was expressed as nmol of radiolabeled material per g dry weight of cells ($\sim 3 \times 10^9$ cells).

Intracellular water was determined from the differences between wet weight and dry weight of a cell pellet less the [^{14}C] inulin space as described in detail [3, 17]. The chloride distribution ratio was measured as an indication of changes in membrane potential [3, 17]. Trypan blue exclusion was determined as a measure of cell viability and was not significantly reduced by any of the experimental conditions employed in this study. The viability was greater than 95% when first isolated from the peritoneal cavity and was reduced to 85% after 4 hr at 37 °C in the transport buffer. Cells were treated with neuraminidase by incubating 5×10^7 cells with 10 units of enzyme per ml in 20 mM 2 N-morpholinoethanesulfonic acid (MES), pH 5.6, 135 mM NaCl and 1.0 mM $CaCl_2$. Release of sialic acid residues from the cells was determined by the thiobarbituric acid assay [48]. Maximum release (~ 1 nmol/10^6 cells) occurred after 30 min under these conditions.

Preparation of Liposomes

Positively charged liposomes were prepared by mixing 125 μmol phosphatidylcholine and 25 μmol stearylamine in chloroform and drying on a rotary evaporator at 35 °C. The flask was flushed with nitrogen and placed on a lyophilizer for several hours to remove residual chloroform. The lipid film was then suspended in 5 ml of buffer containing 140 mM NaCl, 5.5 mM KCl, and 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes) at pH 7.4 and agitated on a Vortex mixer for 5 min. The suspension was transferred to a screw cap vial and sealed under nitrogen with Teflon tape. The vial was partially submerged in a bath sonicator (Cole-Parmer model 8845-30, 80 watts), and subjected to sonication at 25 °C for 1–2 hr until no further clearing of the solution was observed. The liposome preparation was then centrifuged for 1 hr at $100{,}000 \times g$ and the supernatant used for these studies. Negatively charged liposomes were prepared in the same manner except 25 μmol of stearic acid was substituted for the stearylamine. Radiolabeled liposomes were prepared by including 0.1 μCi of [^{14}C] phosphatidylcholine per μmol of unlabeled phosphatidylcholine. Phospholipid content was assayed by measuring lipid phosphorous [1].

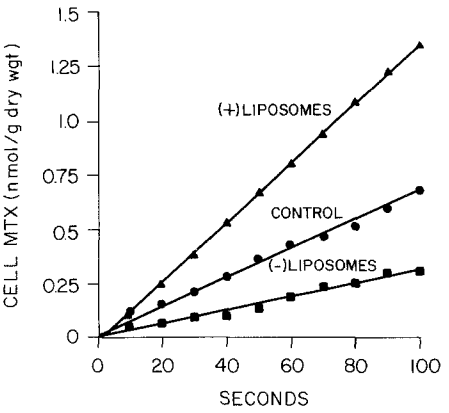

Fig. 1. Effect of positive and negative liposomes on methotrexate influx. Cells were exposed to positive (phosphatidylcholine/stearylamine 5:1) or negative (phosphatidylcholine/stearic acid 5:1) liposomes 5 min before addition of 1 μM methotrexate. The phospholipid concentration was 2.5 mM

Results

Effect of Charged Liposomes on the Influx of Methotrexate and other Folates

Influx of methotrexate was measured over 100-sec intervals. During this period the intracellular concentration of methotrexate does not exceed the binding capacity of dihydrofolate reductase (methotrexate transport is rate-limiting to the binding of this enzyme) thus insuring that uptake of [^{3}H] methotrexate was unidirectional and any alterations in uptake reflected changes in the influx process, unaffected by the intracellular disposition of this agent. Whereas cells exposed to positively charged liposomes for 5 min exhibited an increase in methotrexate influx [13], negatively charged liposomes caused inhibition (Fig. 1). Although positively and negatively charged liposomes produced opposite effects, certain similarities (*not shown*) were observed in the characteristics of these alterations. Thus, the alterations in methotrexate influx produced by either liposome (*i*) exhibited a lag period between the time that the cells were exposed to liposomes and the time that maximal effects on MTX transport were attained which varied from 1–5 min (*ii*) did not nonspecifically alter the permeability of the cell membrane since methotrexate influx in the presence of both lipsomes was completely abolished by the competitive inhibitor 5-CHO-H_4folate or the sulfhydryl inhibitor *p*-mercuriphenylsul-

Table 1. Effect of removing extracellular liposomes or exposure to oppositely charged liposomes on methotrexate influx

	Identically charged liposomes	Liposome-free buffer	Oppositely charged liposomes
(+) Liposomes	186.5±12.0	177.6±7.1	87.2±6.9
(−) Liposomes	68.4± 4.0	62.7±3.5	97.2±3.6

Cells were exposed to positively or negatively charged liposomes (2.5 mM phospholipid) for 5 min, washed twice and resuspended into liposome-free buffer or buffer containing liposomes of identical or opposite charge and methotrexate influx determined in the presence of 1 μM [^{3}H] methotrexate. The data are expressed as a percentage of the methotrexate influx in cells not exposed to liposomes and represent the mean ±SE of four separate experiments.

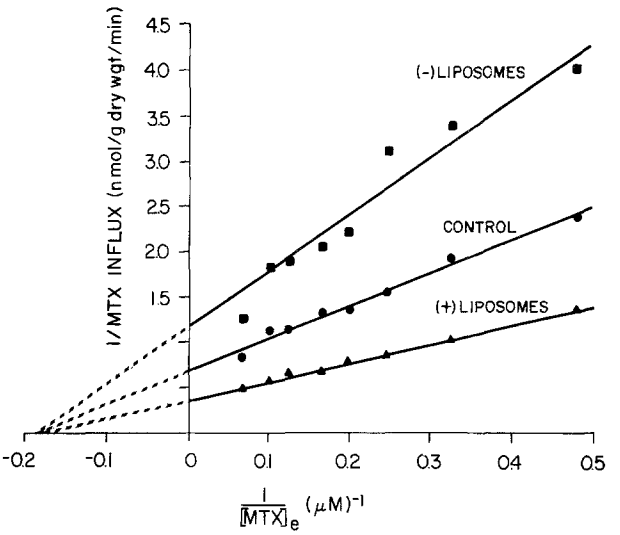

Fig. 2. Double reciprocal plot of methotrexate (MTX) influx kinetics in control cells and cells preincubated for at least 5 min with positive (phosphatidycholine/stearylamine 5:1) or negative (phosphatidylcholine/stearic acid 5:1) liposomes. The phospholipid concentration was 2.5 mM. Lines were fitted to the measured values by the method of least squares

fonic acid, agents that interact with the high affinity methotrexate-tetrahydrofolate carrier, (*iii*) did not alter the Q_{10} (the ratio of the influx rate at 37 °C over the rate at 27 °C and ranges between 5–6 for MTX influx in the Ehrlich ascites tumor cell), and (*iv*) were not produced by the amphiphile (stearic acid or stearylamine) alone when added in comparable amounts as in the liposomes as an ethanol solution or a sonicated emulsion.

Once cells were exposed to the charged liposomes and maximal stimulation or inhibition of methotrexate influx attained, the effects were not reversed by washing the cell free of extracellular lipid (Table 1). If, however, cells were exposed to liposomes of one charge, washed and then exposed to liposomes of the opposite charge, methotrexate influx was reversed to the control rate (Table 1).

The effects of charged liposomes on influx kinetics was determined by measuring methotrexate influx over 100-sec intervals at varying concentrations of methotrexate in control cells or cells treated with liposomes. To maintain a maximum and constant effect, cells were exposed to liposomes 5–15 min prior to exposure to methotrexate. Figure 2 is a double reciprocal plot of a representative experiment. Neither liposome affected the K_m of influx; however, positively charged liposomes stimulated the V_{max} as previously reported [13], whereas negatively charged liposomes reduced the V_{max}. In three such experiments, the V_{max} for control, positive or negative liposome-treated cells were 2.04±0.38, 3.38±0.55, and 1.30±0.31 nmol methotrexate per g dry wt per min, respectively ($p<0.01$ for both liposomes). The K_m^{in}'s were 5.16±0.16, 5.01±0.51, and 5.18±0.19 μM, respectively ($p>0.2$).

Table 2 shows the effects of positively or negatively charged liposomes on the influx of several naturally occurring folates or synthetic analogs. Although the magnitude of the effects varied with each compound,

Table 2. Effect of charged liposomes on influx of folates and their analogs

	n	(+) Liposomes	(−) Liposomes
[^{3}H] methotrexate	8	172.4± 3.3 ($p<0.001$)	64.5±2.6 ($p<0.001$)
[^{3}H] aminopterin	3	167.8±18.6 ($p<0.01$)	65.0±8.3 ($p<0.01$)
[^{14}C] 5-CH_3-H_4-folate	3	114.1± 2.4 ($p<0.05$)	75.8±8.7 ($p<0.05$)
[^{3}H] 5-CHO-H_4-folate	3	115.8± 4.8 ($p<0.05$)	76.7±6.1 ($p<0.05$)
[^{3}H] folic acid	5	96.8±13.3 ($p>0.2$)	95.7±6.7 ($p>0.2$)

Cells were exposed to positively or negatively charged liposomes (2.5 mM phospholipid) for 5 min and then influx of tritiated substrate measured over 100 sec. The concentration of folic acid was 10 μM and all other folates 1 μM. The data are expressed as a percentage of the methotrexate influx in cells not exposed to liposomes and represent the mean ±SE of *n* separate experiments.

positively charged liposomes consistently stimulated and negatively charged liposomes inhibited the influx of aminopterin, 5-CHO-H_4folate and 5-CH_3-H_4folate, compounds that utilize the same carrier system as methotrexate. Liposomes did not, however, affect the influx of the folic acid, a compound structurally similar to methotrexate with a comparable charge but not transported appreciably by the methotrexate-tetrahydrofolate cofactor carrier at low extracellular concentrations of folate [21, 41].

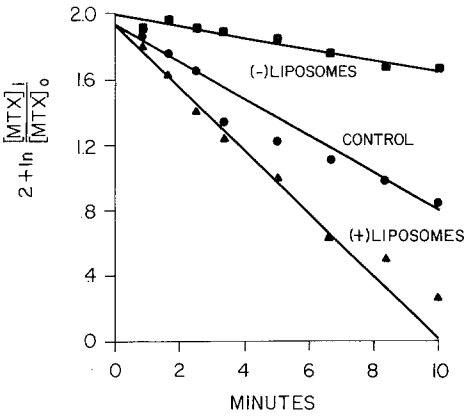

Fig. 3. Effect of positive or negative liposomes on methotrexate (MTX) efflux. Cells were incubated with 2 μM methotrexate at 37 °C for 50 min, washed twice in 0 °C buffer and then portions resuspended into methotrexate-free buffer with or without positive (phosphatidylcholine/stearylamine 5:1) or negative (phosphatidylcholine/stearic acid 5:1) liposomes, 2.5 mM phospholipid. The plot represents a semilogarithmic analysis of the fraction of exchangeable intracellular methotrexate remaining as a function of time after the cells were resuspended into methotrexate-free buffer. $[MTX]_o$ and $[MTX]_i$ refer to the exchangeable intracellular methotrexate concentration before and after resuspension. Exchangeable methotrexate was determined from the net methotrexate level in the cell less that component tightly bound to dihydrofolate reductase established from the washout analysis. A factor of 2 was added to each logarithmic function to eliminate negative values. Lines were fitted to the measured values by the method of least squares

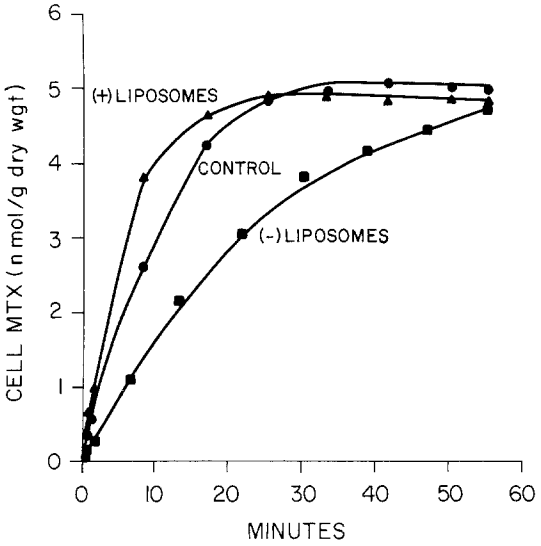

Fig. 4. Effect of positive or negative liposomes on the level of intracellular methotrexate at the steady state. Cells were incubated with 1 μM methotrexate with or without positive (phosphatidylcholine/stearylamine 5:1) or negative (phosphatidylcholine/stearic acid 5:1) liposomes, 2.5 mM phospholipid

Effect of Charged Liposomes on Methotrexate Efflux and Net Transport

Intracellular methotrexate consists of an exchangeable fraction which rapidly leaves the cells and a fraction tightly bound to dihydrofolate reductase. Efflux of free methotrexate conforms to a first-order process and a linear plot can be obtained from the ln of the free intracellular methotrexate versus time. The slope of this plot is the efflux rate constant. Cells were incubated for 50 min with 2 μM methotrexate at 37 °C, washed twice in 0 °C buffer by centrifugation and resuspended into methotrexate-free buffer with or without liposomes. Figure 3 shows a semilogarithmic plot of a representative experiment. In three experiments the rate constant was increased 56.7±8.9% by positively charged liposomes but decreased 51.5±13.0% by negatively charged liposomes. Thus, efflux of methotrexate was affected by liposomes in a manner similar to influx with the specific effect dependent on net liposomal charge.

The symmetrical effects on bidirectional fluxes of methotrexate were confirmed by the absence of any change in the level of intracellular methotrexate at the steady state (Fig. 4). Further, in four separate experiments charged liposomes had no effect on the intracellular water or the chloride distribution indicating that the electrochemical potential for intracellular methotrexate at the steady state remained the same. Thus, although methotrexate influx was increased or decreased by positively or negatively charged liposomes, respectively, these differences in net uptake of methotrexate declined as uptake deviated from the initial rates so that the steady-state levels achieved were identical.

Characteristics of Liposome-Cell Association and their Relationship to Alterations of Methotrexate Transport

The time-course of liposome-cell association was determined in order to establish whether the magnitude of the alterations of methotrexate transport were related to the amount of lipid that became incorporated into the cell. Liposomes were prepared in the usual manner except that trace amounts of [^{14}C] phosphatidylcholine were incorporated into each preparation. Association of radiolabel with cells was followed as described in Materials and Methods. Figure 5 shows a representative time course for the uptake of positively or negatively charged liposomes. Both profiles suggest a rapid initial association with the cells (within 10 sec) followed by slower components of uptake which gradually approaches a plateau after longer incubations. Positively charged liposomes initially associated with the cell more rapidly and attained a much higher level than negatively charged liposomes. The arrow indicates the approximate time that lipo-

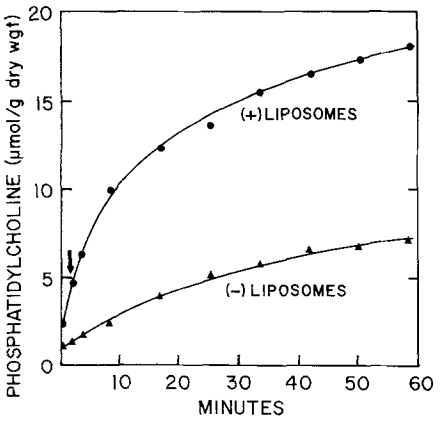

Fig. 5. Time course of the association of positive (phosphatidylcholine/stearylamine 5:1) or negative (phosphatidylcholine/stearic acid 5:1) liposomes with Ehrlich cells. The phospholipid concentration was 2.5 mM and had a specific activity of 0.1 μCi [^{14}C] phosphatidylcholine per μmol. The arrow indicates the average time at which liposomes have maximum effects on methotrexate transport

somes have maximal effects on methotrexate influx and suggests that the alterations in methotrexate transport are the consequence of a component of liposome-cell association that is complete within the first 5 min. Consequently, the major portion of the lipid which is taken up by the cells has no additional effect.

Relationship between Cell Surface Charge and the Effects of Charged Liposomes on Methotrexate Transport

Since methotrexate is a bivalent anion at physiological pH [36], and liposome effects on the transport of this agent are dependent on the ionic nature of the liposome, the possibility existed that the liposome effects may be related to a neutralization or potentiation of cell surface charge. The Ehrlich ascites tumor cell has a negative zeta potential as determined by cell electrophoresis and the cell membrane contains negative charges related in part to the carboxyl group of sialic acid [4, 51]. Accordingly, cells were incubated with or without 10 units of neuraminidase per ml as described in Materials and Methods for 30 min and washed twice in saline. This procedure has been shown to reduce the electrophoretic mobility of the Ehrlich cell suggesting that the surface potential has been reduced [51]. The cells were then exposed to positively or negatively charged liposomes for 5 min and methotrexate influx measured. No significant difference in the effects of liposomes on influx was observed between control or neuraminidase-treated cells (Table 3). Further, neuraminidase treatment has no significant effect on methotrexate uptake or liposome-cell association at early (5 min) or later (50 min) times in the time course (Table 3). Hence, changes in surface charge as contributed by sialic acid residues do not appear to play a role in the alterations of MTX transport by charged liposomes nor in the transport process itself.

Effect of Metabolic Inhibitors and Temperature on Liposome-Cell Association and Liposome-Induced Alterations of Methotrexate Transport

Since the mode of liposomal uptake into the cell may be an important factor governing the alterations of MTX transport, the effect of azide and 2-deoxyglucose or lowering the temperature to 4 °C on liposome-cell association was studied to determine the energy and temperature requirements of these interactions. Figure 6 shows a time course of liposome-cell association at 4 and 37 °C or after cells were incubated 30 min with 10 mM azide and 50 mM 2-deoxyglucose.

Table 3. Effect of pretreating cells with neuraminidase on the liposome-induced alterations in methotrexate transport, liposome-cell association and methotrexate influx[a]

	Methotrexate influx[b]	Positive liposomes[c]	Negative liposomes[c]	Liposome-cell association[d]			
				Positive liposomes		Negative liposomes	
				5 min	50 min	5 min	50 min
Control	100	175.0±12.1	61.3±2.6	7.8±0.4	19.4±4.0	2.0±0.4	8.5±1.0
Neuraminidase	94.7±6.7	181.6± 3.7	59.7±2.7	8.9±1.0	20.9±4.7	2.1±0.3	8.1±1.4

[a] Cells were incubated for 30 min with or without 10 units of neuraminidase per ml as described in Materials and Methods. The data are expressed as the mean ±SE of three separate experiments.

[b] Influx of 1 μM methotrexate in control and neuraminidase-treated cells. The data are expressed as a percentage of the control rate.

[c] Cells were exposed to positively or negatively charged liposomes for 5 min before measuring methotrexate influx. The data are expressed as a percentage of the methotrexate influx in cells not exposed to liposomes.

[d] Association of radiolabeled liposomes with control and neuraminidase-treated cells was determined at 5 and 50 min as described in Materials and Methods. The data are expressed as μmol of phosphatidylcholine per g of cell dry wt.

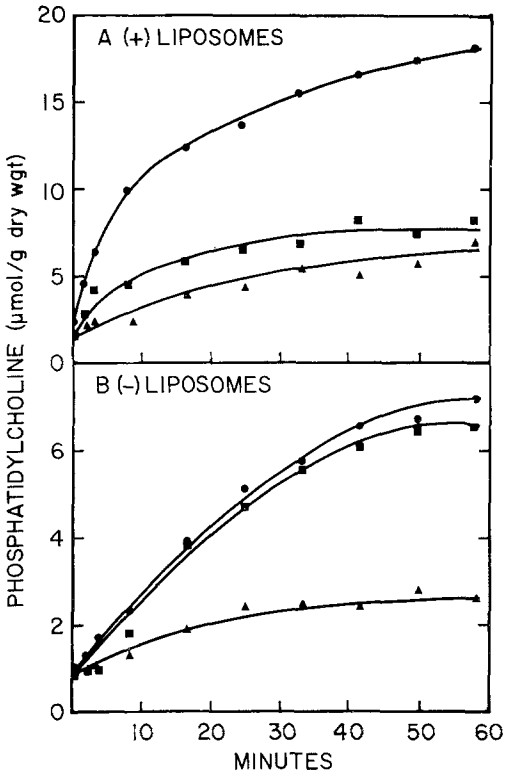

Fig. 6. Effect of temperature or metabolic inhibitors on lipsome-cell association. The uptake of positive or negative liposomes was determined at 37 °C (●), 4 °C (▲) or after cells had incubated for 30 min with 10 mM azide and 50 mM 2-deoxyglucose (■). The phospholipid concentration was 2.5 mM and the specific activity was 0.1 μCi [^{14}C] phosphatidylcholine/μmol

Later uptake of positively charged liposomes (a) was markedly inhibited by the metabolic inhibitors and lowering the temperature to 4 °C. Uptake of negatively charged liposomes (b) was similarly temperature sensitive but was not affected by metabolic poisons. Early association (10-sec determination) of both types of liposomes, however, was not significantly affected by these conditions (Table 4). To establish whether the component of liposomal uptake that was sensitive to temperature, or in the case of positively charged liposomes, to metabolic inhibitors, was involved in the modification of methotrexate transport, positively or negatively charged liposomes were incubated for 5 min with cells at 4 or 37 °C or with cells that had been pretreated for 30 min with glucose or azide and 2-deoxyglucose. After the cells were washed free of the extracellular lipid, MTX influx at 37 °C in the liposome-treated cells was compared with control cells treated in an identical manner but without liposomes. There was no significant difference in the stimulation by positively charged liposomes or inhibition by negatively charged liposomes under any of the experimental conditions suggesting the modifications produced by the liposomes were temperature- and energy-independent (Table 5) and again unrelated to the total level of liposome association with the cells.

Table 4. Effect of temperature or metabolic inhibitors on the 10-sec uptake of positively or negatively charged liposomes

	Control	Azide + 2-deoxy-glucose	4 °C
(+) Liposomes	3.41 ± 1.1	2.82 ± 1.4 ($p > 0.2$)	2.67 ± 0.9 ($p > 0.2$)
(−) Liposomes	1.26 ± 0.18	1.22 ± 0.42	1.15 ± 0.34

Cells were exposed to radiolabeled liposomes at 37 or 4 °C or after cells had preincubated with 10 mM azide plus 50 mM 2-deoxyglucose and the incorporation of radiolabel determined after 10 sec as described in Materials and Methods. The data are expressed as μmol phosphatidylcholine per g of cell dry wt and represent the mean ± SE of four separate experiments.

Discussion

The association of liposomes with cells has received much attention in relation to cellular delivery of pharmacological agents [24, 39], mechanisms of membrane fusion [34, 37] and immunological properties [26]. More recently, interest in liposome-cell interactions has focused on the modification of cellular activities

Table 5. Effect of metabolic inhibitors, glucose, and 4 °C on the alterations of methotrexate transport by charged liposomes

	Control	Azide + 2-deoxyglucose	Glucose	4 °C
(+) Liposomes	171.6 ± 5.9 (8)	171.7 ± 6.5 (7)	177.5 ± 5.1 (4)	161.5 ± 17.1 (3)
(−) Liposomes	65.4 ± 2.1 (5)	64.1 ± 1.9 (5)	64.3 ± 2.6 (4)	64.1 ± 12.8 (3)

Cells at 37 °C (control) or 4 °C or cells that had been preincubated for 30 min with 10 mM glucose or 10 mM azide plus 50 mM 2-deoxyglucose were exposed to positively or negatively charged liposomes (2.5 mM phosphatidylcholine) for 5 min, washed twice, and resuspended into 1 μM [^{3}H] methotrexate. Influx was measured over 100 sec and the data are expressed as a percentage of influx in cells treated identically but not exposed to liposomes. Each value represents the mean ± SE of (*n*) experiments.

[10, 20, 27, 30, 39, 40] and membrane composition [5, 9, 43]. A previous publication [13] reported that exposure of cells to positively charged liposomes altered the transport kinetics of the anticancer agent, methotrexate, in Ehrlich ascites tumor cells. Further studies now demonstrate that positively and negatively charged liposomes produce opposite effect on methotrexate transport. Similarities, however, in the characteristics of these modifications by both liposomes suggest that they may interact at a common site or affect the same physical parameters in the membrane or at the transfer protein.

Although several possibilities exist for the mechanism of these liposome-induced alterations in the transport of methotrexate and tetrahydrofolate cofactors, some can be eliminated by the available data. Thus, whereas liposomes in some cases have been shown to be cytotoxic [32], which ultimately may affect membrane integrity, the modifications in methotrexate transport by charged liposomes do not appear to be caused by membrane damage or a nonspecific leak since in the presence of positively or negatively charged liposomes (*i*) methotrexate influx can be totally inhibited by the competitive inhibitor 5-CHO-H_4-folate or the sulfhydryl group inhibitor *p*-chloromercuriphenylsulfonate, (*ii*) cell viability, as determined by Trypan blue exclusion, does not decrease (*see* Materials and Methods), (*iii*) the Q_{10} of influx is not changed, and (*iv*) transport of folic acid, a compound similar in structure and charge to methotrexate but not significantly transported by the same carrier [21, 41] is unaffected. Further, most deleterious effects by liposomes occur only after prolonged exposure to cells [32] while the alterations in methotrexate transport are at maximum within minutes.

Since methotrexate influx is sensitive to the presence of metabolic inhibitors [14–16] the possibility existed that the changes in methotrexate transport were related to liposome-induced perturbations of one or more metabolic pathways such as the inhibition of glycolysis which has been observed in the brain by liposomes composed of phosphatidylserine [2]. This, however, does not seem likely since the steady-state levels of intracellular methotrexate, a parameter markedly affected by changes in energy metabolism [14–16], remains unchanged in the presence of liposomes, and glucose, which reverses metabolic effects on methotrexate transport has no effect on the lipsome-induced alterations of methotrexate transport.

Since methotrexate is a bivalent anion at physiological pH [36], another possibility that must be considered is a change in the surface potential of the cell membrane. According to the Maxwell-Boltsmann distribution, the concentration of an ion in the unstirred layer adjacent to a membrane depends on the surface potential of the membrane and indeed it has been shown that the surface charge of artificial and biological membranes can influence their passive and energy-linked permeability to ions [33, 19] and metabolites [29, 40]. Kinetically, this change in local concentration of the substrate would alter the apparent K_m^{in} of transport but not the V_{max}^{in} [22]. Since charged liposomes alter only the V_{max}^{in} of methotrexate influx but not the K_m^{in}, this explanation does not appear to be applicable. Furthermore, treatment of cells with neuraminidase which lowers the surface potential of the Ehrlich cells [51] does not alter methotrexate transport nor the changes produced by charged liposomes.

Several reports have demonstrated the extraction of proteins [5, 18], cholesterol [6] or other lipids [31] from biological membranes by liposomes with subsequent alterations in membrane permeability or fluidity. This possibility for the observed changes in methotrexate transport produced by liposomes, however, does not seem likely since the transport alterations in cells that have been exposed to charged liposomes and then washed free of the extracellular media (consequently removing any extracted components) can be reversed by subsequent exposure of the cell to liposomes of opposite charge. However, the possibility still exists that extracted components could be associated with liposomes absorbed to the cell surface and therefore still available to the cell when the oppositely charged liposomes are added.

The data suggest that only a small component of the total liposomal uptake with the cell is responsible for the changes in methotrexate transport. This is supported by the observations that (*i*) maximal effect on methotrexate transport occurs within minutes while liposome uptake continues for at least 1 hr, and (*ii*) neither this rapid component of liposome uptake nor the alterations in methotrexate transport are affected by metabolic inhibitors or low temperature while the latter stages of liposome uptake are sensitive to these conditions.

In light of the complex nature by which liposomes interact with cells [31], it is difficult to derive a specific mechanism for the liposome-induced alterations in methotrexate transport. However, one possibility is that the liposomes through their association with the cell may be altering the intrinsic properties of the cell membrane. Since oppositely charged liposomes produce opposite effects on methotrexate transport, this indicates that it may be change in charge density within the membrane that governs these alterations. Lipid transition temperatures have been shown to be sensitive to changes in charge density [47] and the interaction of cationic lipid-soluble molecules with membranes can increase membrane fluidity [11, 35]. Since the V_{max}^{in} of transport but not the K_m^{in} is affected

by charged liposomes it must be assumed that this alteration ensues from a change in the number of carrier sites or an increase or decrease in the rate-limiting step of the translocation process. Should the transport of methotrexate be dependent on the mobility or conformation of a specific protein, changes in the fluidity or packing of the membrane may markedly affect its function. Indeed, Burns et al. [7] found that the membranes of L1210 cells grown in mice fed on either a highly polyunsaturated or saturated fat diet correspondingly exhibited an increase or decrease in fluidity. These changes could be correlated with a change in methotrexate transport characteristics, thus providing evidence that this transport process is sensitive to its lipid environment.

In conclusion, this paper demonstrates that liposome-cell interactions can specifically alter the methotrexate-tetrahydrofolate transport carrier and that the properties of these alterations are governed by the net charge of the liposome. Further, these changes appear to result from an initial rapid association of lipsomes with the cell that is not related to changes in cell surface potential.

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