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Alterations of the Carrier-Mediated Transport of an Anionic Solute, Methotrexate, by Charged Liposomes in Ehrlich Ascites Tumor Cells

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Summary. Interaction of positively charged liposomes with Ehrlich ascites tumor cells increases the bidirectional transmembrane fluxes of the anionic folic acid analog, methotrexate. Negative liposomes reduce methotrexate influx. Stimulation of methotrexate influx by positively charged liposomes is time and concentration dependent, requiring at least a 5-min incubation with 2.5 mM phosphatidylcholine containing 20% stearylamine for maximum effect. Stimulation is not appreciably reversed by washing the cells. Similar increases are observed for influx and efflux so that there is no change in the steady-state methotrexate electrochemical-potential difference across the cell membrane. The increase in influx appears to be a stimulation of the carrier-mediated transport process for methotrexate since both control and stimulated influx are abolished by the competitive inhibitor, 5-formyltetrahydrofolate or the sulfhydryl group inhibitor, *p*-chloromercuriphenylsulfonic acid and the Q_{10} of the system remains unchanged. Influx of 5-methyltetrahydrofolate, which shares the same transport carrier as methotrexate, is also stimulated. However, the transport of folic acid, which is structurally similar to methotrexate but does not utilize the carrier, is unaffected. The kinetic change induced by positively charged liposomes is an increase in the V_{ma}^{in}, while the K_t^{in} remains unchanged. Trans-stimulation of methotrexate influx by 5-formyltetrahydrofolate occurs to the same extent in the presence or absence of positively charged liposomes. The liposomes have no apparent effect on the intracellular water, the extracellular space, or the chloride distribution ratio. The data suggest that interaction of positively charged liposomes with Ehrlich ascites tumor cells accelerates the rate of transposition of the membrane carrier system for methotrexate, altering the kinetics of transport without a change in transport thermodynamics.

Liposomes have been studied as well-defined model membranes [34], as models for cell fusion and adhesion [35, 38], as carriers for introducing drugs and enzymes into cells [13, 19, 39, 48], and as immunological adjuvants [2, 23, 30]. These studies have generated interest in mechanisms of liposome-cell interactions and their consequent effects [33]. Depending on the composition of the liposome, liposome-cell interactions have induced changes in cell membrane composition [7, 10, 28, 44, 45] and a variety of cellular activities [1, 6, 8, 11, 12, 27, 31, 36, 42, 43]. The

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effect of these interactions on carrier-mediated membrane transport processes has not, however, been examined.

Studies from this laboratory have described a carrier-mediated transport process for tetrahydrofolate cofactors and the synthetic analog of folic acid, methotrexate, in mammalian cells [4, 14–18]. In this paper we show that charged liposomes alter the carrier-mediated transport of the bivalent anions, methotrexate and 5-methyltetrahydrofolate, in the Ehrlich ascites tumor. Because methotrexate is not metabolized in this cell system, it was used as a model for characterizing the effects of charged liposomes on this transport process. Positively charged liposomes increase bidirectional methotrexate fluxes without altering the apparent steady-state electrochemical-potential transmembrane difference. Negatively charged liposomes reduce influx of methotrexate. The data clearly indicate a specific effect on the transport carrier and not the induction of a nonspecific increase in membrane permeability. The data suggest that the interaction between positively charged liposomes and the cell membrane results in an acceleration of the rate-limiting step in the transmembrane fluxes of methotrexate, presumably an augmentation in the rate of carrier translocation in the cell membrane rather than an alteration in substrate-carrier affinity.

Materials and Methods

Chemicals

Phosphatidylcholine, cholesterol, stearylamine, and dicetylphosphate were obtained from Sigma Chemical Co. (St. Louis, Mo.). Phosphatidylcholine was purified by column chromatography on alumina [46] and purity confirmed by thin layer chromatography [9]. [3′,5′,9(N)-^{3}H]Methotrexate, [3′,5′,9(N)-^{3}H]folic acid, and 5-[^{14}C]methyltetrahydrofolic acid were obtained from Amersham/Searle Corporation (Arlington Heights, Ill.) and purified by diethylaminoethyl cellulose chromatography [18]. 5-Formyltetrahydrofolic acid was obtained from Lederle Laboratories (Pearl River, N.Y.) and [COOH-^{14}C]-inulin from New England Nuclear Corporation (Boston, Mass.).

Cells, Media, and Incubation Techniques

Ehrlich ascites tumor cells were grown in male CFl mice (Sprague Dawley, Madison, Wisc.) and passed weekly by intraperitoneal inoculation of 0.2 ml of undiluted ascitic fluid. Cells were suspended in buffer composed of 136 mM NaCl, 4.4 mM KCl, 16 mM $NaHCO_3$, 1.1 mM KH_2PO_4, 1.0 mM $MgCl_2$, and 1.9 mM $CaCl_2$. 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 specifically designed flasks inserted in a 37 °C water bath. Unidirectional fluxes, trans-stimulation, and net uptake of [^{3}H]methotrex-

ate were measured as previously described [15, 18]. Uptake of radiolabeled liposomes was determined by similar techniques. 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° [18]. The cell fraction was separated by centrifugation ($2000 \times g$ for 30–60 sec) and washed twice with the 0° NaCl solution. 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 electrobalance (Cahn Instruments, Paramount, Calif.), placed in a scintillation vial, and dissolved in 0.2 ml of 1 N KOH for 1 hr 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.

Determination of Intracellular Water and Chloride Distribution Ratio

Intracellular water was determined from the difference between wet wt and dry wt of a cell pellet less the [^{14}C]inulin space as described in detail [4, 18]. The chloride distribution rate was measured as an indication of changes in membrane potential [4, 18].

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-ethane-sulfonic 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 the solution cleared. The liposome preparation was then centrifuged for 1 hr at $100{,}000 \times g$ and the supernatant fraction used for these studies. Negatively charged liposomes were prepared in the same manner except 25 μmol of dicetylphosphate was substituted for the stearylamine. Neutral liposomes contained only phosphatidylcholine. Phospholipid content was assayed by measuring lipid phosphorous [3].

Results

Positively Charged Liposomes Enhance Methotrexate Influx

Influx of methotrexate in Ehrlich ascites tumor cells is easily and accurately determined because dihydrofolate reductase sites within the cell bind the agent so tightly and rapidly that net uptake of [^{3}H]metho-

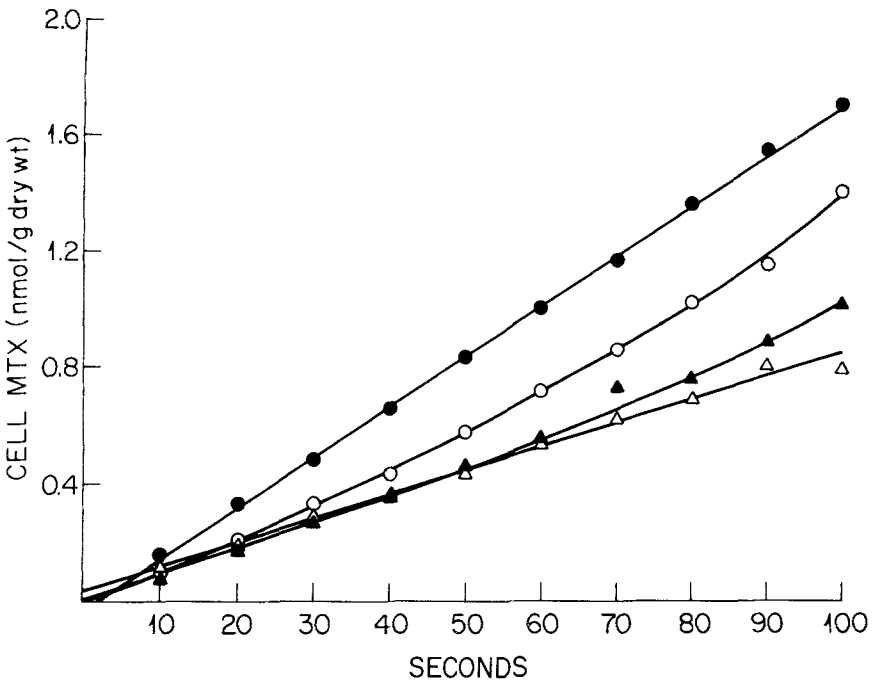

Fig. 1. Effect of positively charged liposomes on methotrexate (MTX) influx. Cells were exposed to liposomes (phosphatidylcholine/stearylamine, 5:1) (▲) 50 sec after, (○) simultaneously, or (●) 5 min before methotrexate. Control influx is indicated by △. The concentration of methotrexate and phospholipid was 1 μM and 2.5 mM, respectively

trexate is unidirectional for at least the interval required to saturate this enzyme [17]. Hence, alterations in net uptake of methotrexate prior to saturation of dihydrofolate reductase reflect changes in the influx process unaffected by changes in the intracellular disposition of this agent. Exposure of Ehrlich ascites tumor cells to positively charged liposomes (phosphatidylcholine/stearylamine) increased methotrexate influx (Fig. 1). Maximum stimulation did not occur instantaneously, but increased with time when the cells were exposed to liposomes and methotrexate simultaneously. Stimulation occurred irrespective of the sequence of addition of liposomes and methotrexate to cells. As seen in Fig. 1, when liposomes were added 50 sec after methotrexate, an increase in influx was detected within 10 sec. When liposomes were added to cells 5 min before methotrexate, the lag phase was eliminated. To further assess the duration required for maximum stimulation of methotrexate influx by positively charged liposomes, cells were incubated with or with-

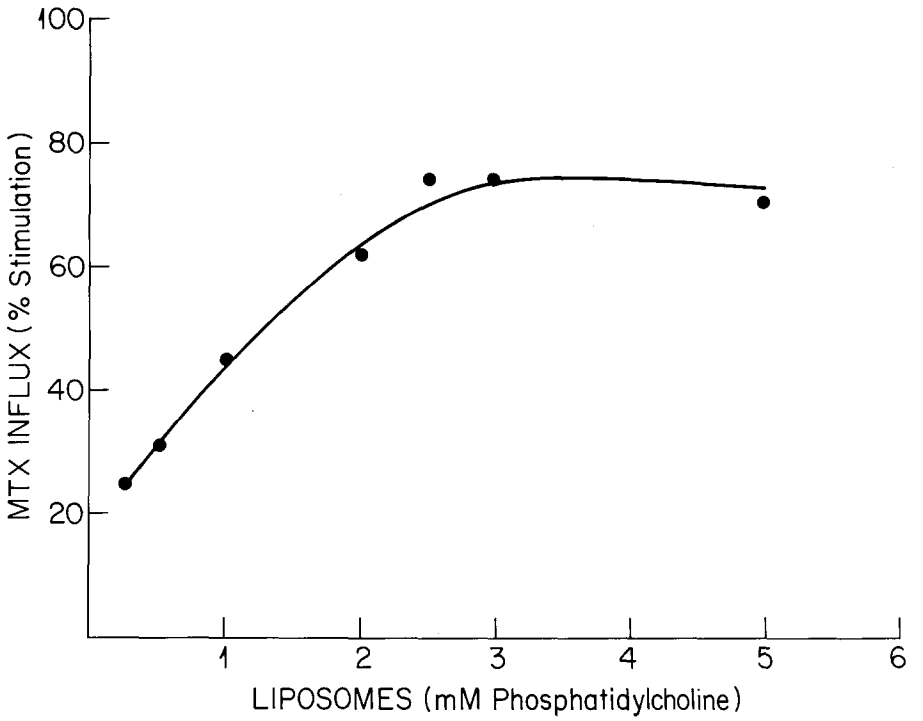

Fig. 2. Effect of liposome concentration on the degree of stimulation of methotrexate (MTX) influx. Cells were exposed to liposomes (phosphatidylcholine/stearylamine, 5:1) 5 min before the addition of 1 μM methotrexate and influx over 100 sec compared with that of control cells

out liposomes and portions were removed at 50 sec intervals for 100 sec influx determinations. Maximum stimulation was achieved when the cells were exposed to liposomes for 5 min prior to addition of [^{3}H]methotrexate, after which the extent of influx stimulation was constant for up to 35 min. Influx stimulation was dependent upon the concentration of liposomes (Fig. 2). Stimulation increased with increasing liposome concentration to 2.5 mM phosphatidylcholine containing 20% stearylamine. In 28 experiments, a 5-min exposure to liposomes at this concentration stimulated methotrexate influx by $84.8 \pm 5.6\%$.[1] The stimulatory effect of positively charged liposomes was only slightly reversible. When cells were exposed to liposomes for 5 min, washed, and resuspended into liposome-free buffer, stimulation was decreased by only $15.7 \pm 4.1\%$ ($n=3$, $P<0.1$).

[1] Data is expressed as mean ± se.

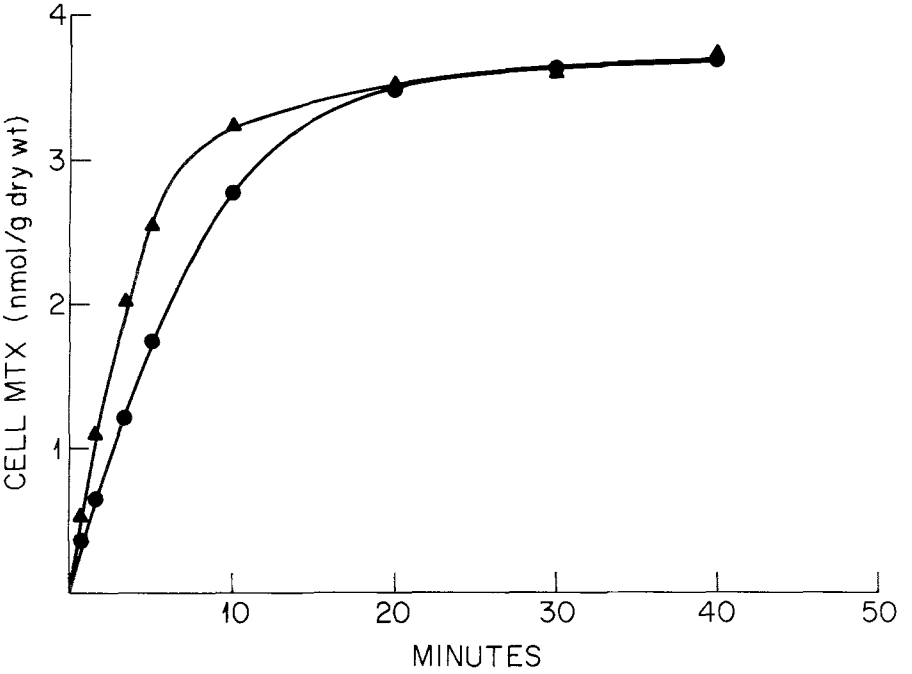

Fig. 3. Effect of positively charged liposomes on methotrexate (MTX) influx and the steady-state level achieved. Cells were incubated with 1 μM methotrexate with (▲) and without (●) liposomes (phosphatidylcholine/stearylamine, 5:1) at a concentration of 2.5 mM phospholipid

Positively Charged Liposomes do not Alter the Steady-State Methotrexate Level

Although methotrexate influx was increased in the presence of positively charged liposomes, the steady-state intracellular methotrexate level was not altered (Fig. 3). The difference in net uptake of methotrexate declined as uptake decreased from initial rates so that the steady-state levels achieved were identical. In 18 experiments, the steady-state methotrexate level in liposome-treated cells differed from the control cells by only $1.2 \pm 2\%$. The absence of any effect by positively charged liposomes on net methotrexate transport was not related to the long incubation required to bring the cells to a steady state since no change in net methotrexate transport was observed over 75 min when cells were exposed to liposomes after intracellular methotrexate was first brought to the steady state.

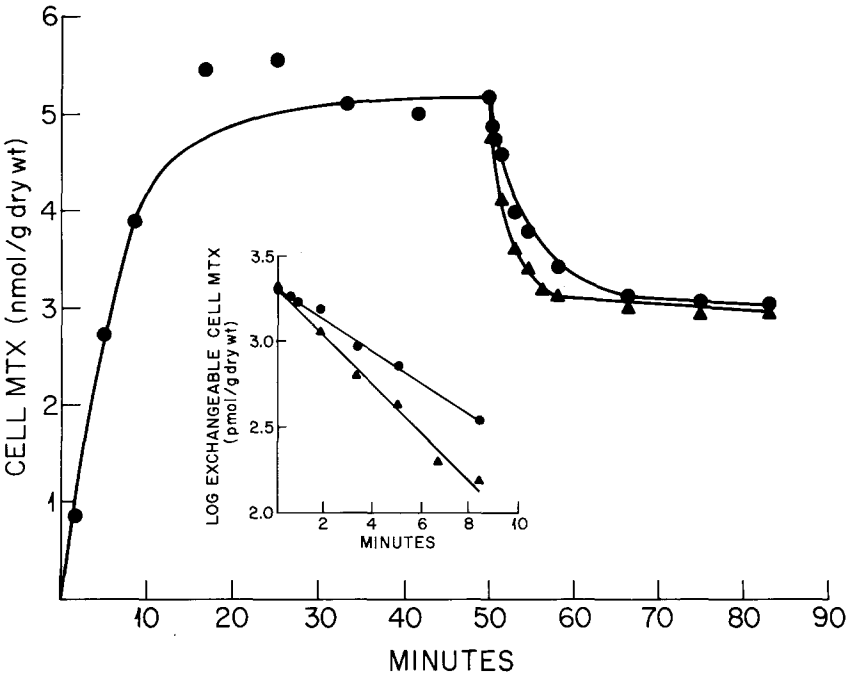

Fig. 4. Effect of positively charged liposomes on methotrexate (MTX) efflux. Cells were incubated with 1 μM methotrexate for 50 min then washed twice in 0° buffer. Portions were then resuspended into methotrexate-free buffer at 37 °C with (▲) or without (●) liposomes (phosphatidylcholine/stearylamine, 5:1), 2.5 mM phospholipid. The inset indicates the log of exchangeable intracellular MTX (total less the component tightly bound to dihydrofolate reductase established from the linear washout analysis) as a function of time

Positively Charged Liposomes Accelerate Methotrexate Efflux

Cells were brought to the steady state with 1 μM methotrexate, separated by centrifugation, resuspended into methotrexate-free buffer with or without positively charged liposomes, and efflux was monitored (Fig. 4). Intracellular methotrexate consists of an exchangeable fraction which rapidly leaves the cells and a fraction tightly bound to dihydrofolate reductase. As seen in the inset, the efflux of the major portion of exchangeable methotrexate can be characterized by a single exponential. Treatment of cells with positively charged liposomes increased the methotrexate efflux rate constant by $78 \pm 10\%$ ($n=4$). Further, the ordinate intercepts were identical in control and liposome-exposed cells (inset) indicating that the onset of efflux stimulation was rapid. This was further confirmed by studies which indicate that there is no significant difference

in efflux whether cells were exposed to liposomes at the time of resuspension into methotrexate-free buffer or 5 min prior to efflux determination. Hence, the onset of efflux stimulation by positively charged liposomes is more rapid than the onset of influx stimulation. Analysis by the unpaired *t*-test showed that there is no significant difference between maximum liposomal stimulation of methotrexate influx and efflux ($P > 0.2$), which accounts for the lack of effect on the steady-state methotrexate level.

Liposomal-Stimulation of Methotrexate Influx is Mediated by the High-affinity Methotrexate-Tetrahydrofolate Cofactor Transport Carrier

Methotrexate transport in Ehrlich ascites tumor cells is mediated by a high-affinity carrier system which is utilized by naturally occurring tetrahydrofolate cofactors such as 5-formyltetrahydrofolate and 5-methyltetrahydrofolate. Four lines of evidence indicate that the liposomal-stimulated methotrexate flux is also mediated by this high-affinity carrier rather than accelerated transport by some secondary low-affinity process or enhanced passive diffusion due to a nonspecific increase in membrane permeability. (i) Both control and liposomal-stimulated influx was abolished by the competitive inhibitor, 5-formyltetrahydrofolate (Fig. 5). (ii) *p*-Chloromercuriphenylsulfonic acid, a sulfhydryl group inhibitor and potent inhibitor of the carrier-mediated influx of methotrexate [41], abolished the liposomal-stimulated influx. (iii) The Q_{10} for methotrexate flux via the high-affinity carrier system is high in comparison to other mediated transport mechanisms or passive diffusion. Thus, if the flux stimulated by positively charged liposomes is related to transport via a secondary route or passive diffusion, the Q_{10} for the combined process should be less than that observed for the high-affinity carrier alone. However, temperature dependence for methotrexate influx in control cells and cells in which influx was stimulated by positively charged liposomes was identical. The average Q_{27-37} for methotrexate influx in control cells was 5.75 ± 0.72 while the Q_{27-37} in the presence of liposomes was 6.12 ± 0.53 ($P > 0.2$, $n = 3$). (iv) Positively charged liposomes did not stimulate the initial uptake rate of folic acid in these cells, a compound structurally similar to methotrexate with a comparable charge but which is not transported appreciably by the high-affinity carrier [29, 41]. Consistent with an effect of positively charged liposomes on the methotrexate-

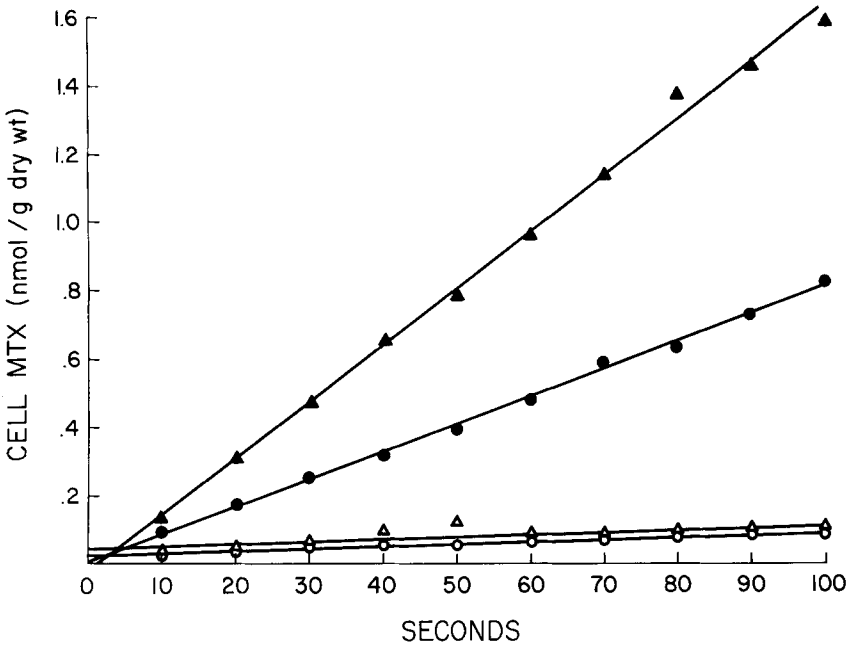

Fig. 5. Effect of 5-formyltetrahydrofolate on liposomal stimulation of methotrexate (MTX) influx. Cells were exposed to 1 μM methotrexate in the presence and absence of liposomes (phosphatidylcholine/stearylamine, 5:1, 2.5 mM phospholipid) and/or 320 μM 5-formyltetrahydrofolate. (●) control; (○) 5-formyltetrahydrofolate; (▲) liposomes; (△) liposomes + 5-formyltetrahydrofolate

tetrahydrofolate carrier system rather than an interaction with methotrexate alone was the observation that positively charged liposomes stimulated influx of 5-methyltetrahydrofolate, the naturally occurring tetrahydrofolate cofactor that this carrier transports under physiological conditions.

Positively Charged Liposomes Enhance the Maximum Transport Velocity for Methotrexate Without a Change in the Influx K_m

The effect of liposomes on influx kinetics was determined by measuring methotrexate influx over 100-sec intervals in control cells and cells treated with liposomes. To maintain a maximum and constant influx stimulation, treated cells were exposed to liposomes 5–15 min prior to exposure to methotrexate. To insure that measurements were initial rates, the flux interval was sufficiently brief so that the cell methotrexate level did not exceed the dihydrofolate reductase binding capacity. Figure 6

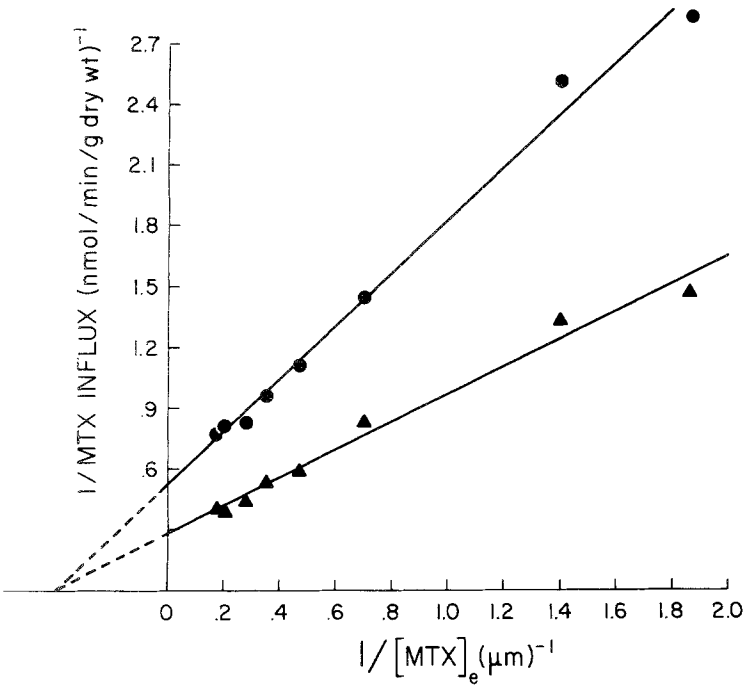

Fig. 6. A double reciprocal plot of methotrexate (MTX) influx kinetics in control cells (●) and cells preincubated for at least 5 min with positive liposomes (phosphatidylcholine/stearylamine, 5:1, 2.5 mM phospholipid) (▲)

is a double reciprocal plot of a representative experiment. Influx of methotrexate follows Michaelis-Menten kinetics in liposome-treated as well as control cells. The kinetic change was an increase in the maximum influx velocity, V^{in}_{max}, while the K^{in}_t was unchanged (the methotrexate concentration at which the influx velocity was one-half of maximum). In 6 such experiments, the V^{in}_{max} for control cells and liposome-treated cells was 2.25 ± 0.25 and 3.67 ± 0.42 nmol/g dry wt/min ($P < 0.001$), respectively. The K^{in}_t were 3.71 ± 0.56 and 3.72 ± 0.36 ($P > 0.2$) μM, respectively.

Trans-Stimulation of Methotrexate Influx by 5-Formyltetrahydrofolate is not Affected by Positively Charged Liposomes

Cells were loaded with 50 μM 5-formyltetrahydrofolate for 45 min, washed in folate-free buffer at 0°, then resuspended into buffer containing 1 μM methotrexate with or without positively charged liposomes. Although there was appreciable variability in the data, trans-

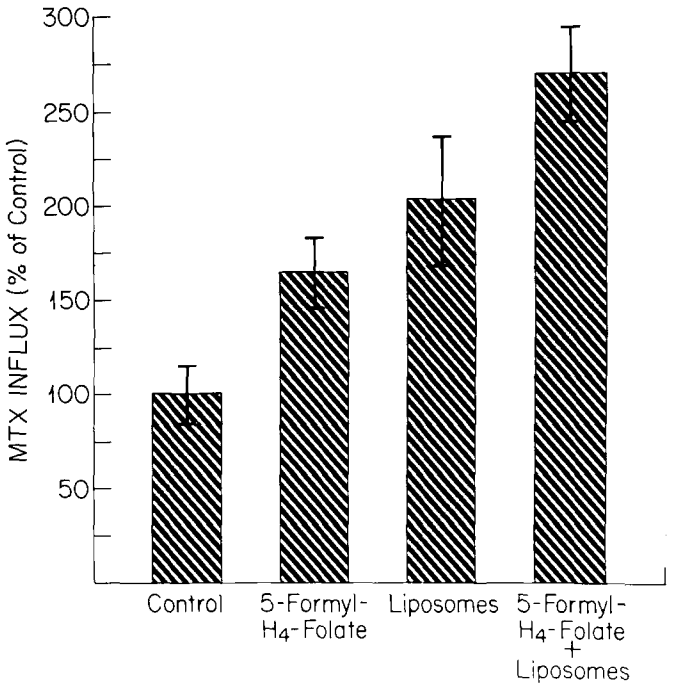

Fig. 7. The effect of positively charged liposomes on the trans-stimulated influx of methotrexate (MTX). Cells were loaded with 50 μM 5-formyltetrahydrofolate for 45 min, washed twice in 0° buffer, then resuspended into buffer containing 1 μM methotrexate with or without liposomes (phosphatidylcholine/stearylamine, 5:1) 2.5 mM phospholipid. Data is expressed as the mean of 4 separate experiments ± se

stimulation of methotrexate influx by 5-formyltetrahydrofolate was the same with (66 ± 16%) or without (64.6 ± 18.2%) treatment with positively charged liposomes (Fig. 7). Similarly, stimulation by positively charged liposomes during trans-stimulation of methotrexate influx was the same (104 ± 20.1%) as in control cells (102 ± 34.2%). Hence, stimulation by positively charged liposomes and transeffects are additive and one process does not appear to influence the other.

Positively Charged Liposomes do not Alter the Intracellular Water, Extracellular Space, or Chloride Distribution Ratio of Ehrlich Ascites Tumor Cells

Positively charged liposomes had no effect on several physical properties of Ehrlich ascites tumor cells under these experimental conditions

Table 1. Effect of positively charged liposomes on the physical properties of Ehrlich ascites tumor cells

	Control	Liposomes
DW/WW[a]	0.1584 ± 0.0065	0.1569 ± 0.0090
$[H_2O]_e$/WW (μl/mg)[b]	0.2610 ± 0.0348	0.2739 ± 0.0524
$[H_2O]_i$/DW (μl/mg)[c]	3.665 ± 0.0277	3.622 ± 0.0681
$[Cl]_i/[Cl]_e$[d]	0.5360 ± 0.0394	0.5466 ± 0.0119

Cells were incubated 5 to 15 min with and without liposomes (phosphatidylcholine/stearylamine, 5:1), 2.5 mM phospholipid. Data is expressed as mean ± SE of the average of 5 measurements in 6 separate experiments.

[a] The ratio of the dry wt to wet wt of a cell pellet.

[b] The ratio of the extracellular water to wet wt of a cell pellet.

[c] The ratio of the intracellular water to dry wt of a cell pellet.

[d] The ratio of the concentration of chloride in the intracellular $[Cl^-]_i$ to extracellular $[Cl^-]_e$ water.

(Table 1). Liposomes did not alter the ratio of the intracellular water to dry wt (a measure of membrane permeability to inulin and cell surface properties) or the dry wt to wet wt of the cell pellet. Positively charged liposomes did not alter the chloride distribution ratio, an indication that the transmembrane electrical-potential difference is unchanged. Trypan blue exclusion [22] did not decrease in relation to control cells after a three-hour incubation, further indicating the integrity of the cell membrane.

The Effect of Liposomes on Methotrexate Influx is Determined by the Liposomal Charge

To evaluate the role of liposome charge in the stimulation of methotrexate influx, studies were undertaken to assess the effects of neutral and negatively charged liposomes. Cells were exposed to neutral (phosphatidylcholine), negatively charged (phosphatidylcholine/dicetylphosphate, 5:1), or positively charged (phosphatidylcholine/stearylamine, 5:1) liposomes for 5 or 30 min following which methotrexate influx was measured. Significant effects by negative and neutral liposomes were observed only after a prolonged incubation (~30 min); a representative experiment is illustrated in Fig. 8. In 4 experiments, negatively charged and neutral liposomes inhibited methotrexate influx by 47.9 ± 1.1% and 22.2 ± 0.56%, respectively. When cells were first exposed to positively charged liposomes

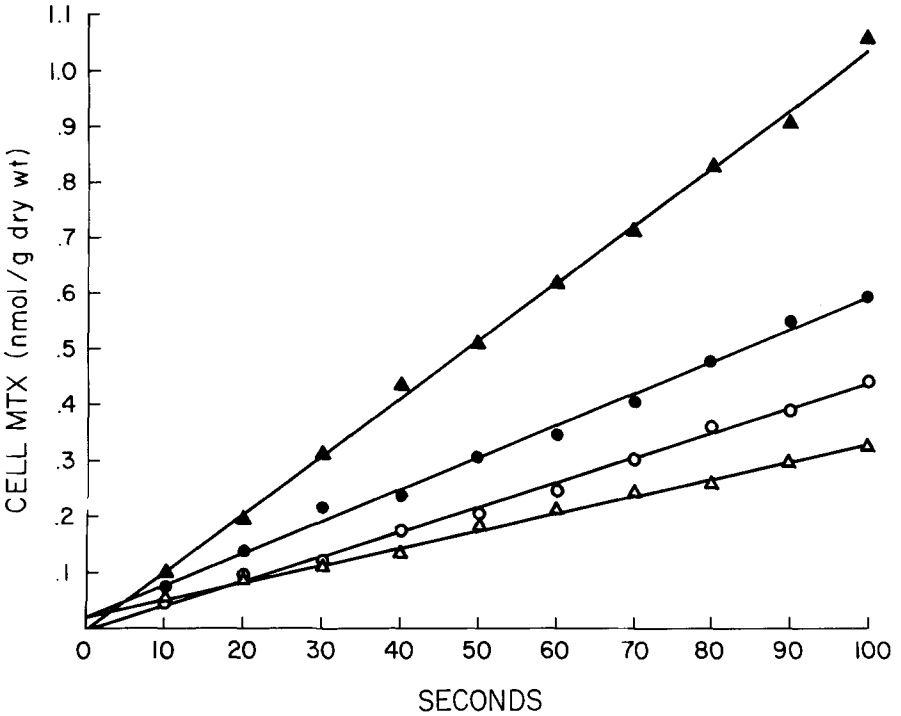

Fig. 8. The effect of positive, negative, and neutral liposomes on methotrexate influx. Cells were exposed to neutral (phosphatidylcholine), positive (phosphatidylcholine/stearylamine, 5:1), or negative (phosphatidylcholine/dicetylphosphate, 5:1) liposomes 30 min before addition of 1 μM methotrexate. The phospholipid concentration was 2.5 mM. (●) control; (▲) positive; (△) negative; (○) neutral liposomes

for 5 min, followed by exposure to negatively charged liposomes, stimulation was abolished. Exposure of cells to an emulsion of stearylamine increased influx by only 22% as compared to 90% stimulation when the same amount of stearylamine was incorporated into a phosphatidylcholine bilayer. Exposure of cells to the multivalent cation, spermine, in amounts equal to the liposomal stearylamine had no effect on influx.

Discussion

The potential utilization of liposomes for the delivery of a variety of pharmacologic and physiologic agents across lipid membrane bilayers has stimulated interest in the mechanism of liposome-cell interactions and their consequent effects on membrane properties [25, 32, 33, 40]. Previous studies have shown that liposomes may modify cell membrane

cholesterol and phospholipid content resulting in alterations of membrane functions. Such properties as osmotic fragility, glycerol and K^+ permeability, and membrane microviscosity have been altered by liposome interactions with erythrocytes, platelets, normal lymphocytes, and leukemia cells [6, 7, 11, 28, 36, 44, 45]. In this report, we demonstrate that charged liposomes alter the carrier-mediated transport of the folic acid analog, methotrexate, and the naturally occurring derivative, 5-methyltetrahydrofolate, and expolore some of the properties of this phenomenon.

Positively charged liposomes stimulate bidirectional transmembrane fluxes of methotrexate without a change in the net intracellular level at the steady state. Since the chloride distribution ratio does not change, the data suggest that there is no change in the electrochemical-potential difference for methotrexate across the cell membrane. Hence, the phenomenon is related to symmetrical alterations in the kinetics of methotrexate flows across the cell membrane without an effect on the thermodynamics of transport. The data establish that alterations in the bidirectional fluxes of methotrexate caused by positively charged liposomes are not due to the induction of a nonspecific leak across the cell membrane, but are related to a stimulation of the carrier-mediated transport process since the liposome-stimulated influx of methotrexate can be totally abolished by 5-formyltetrahydrofolate, a competitive inhibitor of the carrier transport process [16, 18] and by the sulfhydryl group inhibitor, *p*-chloromercuriphenylsulfonic acid, which abolishes methotrexate transport by the carrier system [41]. Furthermore, the temperature dependence of methotrexate transport remains unchanged during liposomal stimulation, indicating that the stimulated and unstimulated fluxes are mediated by the same highly temperature-sensitive mechanism. Finally, liposomes have no effect on the initial uptake of folic acid, a compound with similar structure and charge as methotrexate, but which is not transported significantly by the methotrexate carrier [29, 41]. Similarly, the stimulated influx cannot be related to changes in cell volume or a loss of cell viability since positive liposomes do not alter the inulin space, the ratio of intracellular water to dry wt or Trypan blue exclusion.

The kinetic change in methotrexate influx induced by positively charged liposomes, provides some information on the mechanism of this alteration. Since the K_t^{in} remains unchanged, this eliminates the possibility that stimulation by positively charged liposomes is caused by an increased concentration of methotrexate at the unstirred pericellular layer due to neutralization of the negatively charged groups on the Ehrlich cell membrane [49]. Should this increase in methotrexate concentration

have occurred, there would have been an apparent decrease in the K_t^{in} since the Michaelis equation for the carrier system would be altered by the Boltzmann factor [47]. The increase in V_{max} suggests that positively charged liposomes may either cause an activation or opening of new carrier sites available for the transport of methotrexate or an increase in the velocity of the rate-limiting step for the carrier process. The rate-limiting step for this system appears to be the rate of reorientation of the unloaded carrier from the intracellular to extracellular interphase since trans-stimulation of methotrexate influx can be demonstrated by loading cells with 5-formyltetrahydrofolate [17]. It is of interest then that influx stimulation by positively charged liposomes is the same whether or not there is concurrent trans-stimulation. This suggests (i) that liposomes accelerate the rate of translocation of both the loaded or unloaded carrier, but the latter still remains the rate limiting step, and (ii) the interaction between 5-formyltetrahydrofolate and the unloaded carrier is unperturbed by the presence of liposomes.

While a precise mechanism to explain why charged liposomes alter methotrexate fluxes is unknown, there are several possibilities. Since the polycation spermine and emulsions of stearylamine do not significantly increase methotrexate influx, it seems unlikely that stimulation is caused by the presence of positively charged species at the extracellular membrane surface. The insertion of liposomal components into cell membranes has been shown to induce membrane microviscosity [28, 44, 45], and a change in V_{max}^{in} might be induced by altering lipid composition and allowing a greater mobility of the methotrexate carrier. Alternatively, it is possible that the insertion of charge itself into the membrane by the liposome may be the primary factor in altering the transport process. Another possibility is the extraction of proteins or other components from the cell membrane and into the liposomes which might alter transport without actual liposome-cell association. Extraction of membrane proteins by liposomes has been observed in erythrocytes [5, 26]. Finally, ionic liposomes may alter the charge density of the membrane producing changes in perm-selectivity. Studies with liposomes have shown that the presence of acidic groups (dicetylphosphate, phosphatidic acid, phosphatidylserine) within the bilayer increase permeability to cations and decrease permeability to anions while the presence of cationic groups (stearylamine) have the opposite effect [24]. Reversal of perm-selectivity from cationic to anionic in the presence of the positive particles, thorium, ferric iron, or the second amine, Amberlite LA-2, has been observed in lipid membranes from sheep red blood cells [20]. Since methotrexate

is a bivalent anion at physiological pH [37], an increase in positive charge density within the cell membrane might allow a greater rate of penetration of methotrexate. The inhibition of methotrexate transport by neutral liposomes is not inconsistent with the importance of membrane charge since there is evidence for a coplaner orientation of the phosphatidylcholine head group in which the negative phosphate moiety may manifest its charge more prominently than the positive quaternary amine group [21]. However, to identify a definite mechanism by which liposomes alter methotrexate transport would require not only precise knowledge of the mode of interaction between liposomes and the cell membrane, but a better understanding of the biochemical composition of the methotrexate carrier and its interaction with cell membrane constituents.

These studies indicate that the interaction between liposomes and cells are not inconsequential events but can have profound effects on the properties of cell membranes. These observations may provide a better understanding of the nature of the interaction between membrane transport carriers and their lipid environment in the cell membrane and may illuminate the role of electrostatic forces on the carrier-mediated transport of charged solutes across the cell membrane.

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