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Inhibition of cell proliferation and dihydrofolate reductase by liposomes containing methotrexate-dimyristoylphosphatidylethanolamine derivatives and by the glycerophosphorylethanolamine analogs

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Liposomes, which were prepared with the three methotrexate (MTX)-dimyristoylphosphatidylethanolamine (DMPE) derivatives described in the preceding paper, were tested for their ability to block proliferation of mouse 3T3 and L1210 cells. Tritiated deoxyuridine incorporation into DNA could be completely inhibited by liposomes sensitized with MTX-DMPE I (MTX-γ-DMPE). Under similar conditions, liposomes containing MTX-DMPE II (MTX-α-DMPE) and MTX-DMPE III (MTX-α,γ-diDMPE) produced partial and no inhibition, respectively. These effects on cell growth were paralleled by the capacity of liposomes, prepared with each of the DMPE derivatives, to inhibit dihydrofolate reductase isolated from L1210 cells. Analogous experiments with the three corresponding glycerophosphorylethanolamine (glyceroPE) analogs also indicated that MTX-glyceroPE I was the most effective inhibitor of both cell proliferation and enzymatic activity. However, MTX-DMPE I sensitized liposomes apparently enter target cells as a consequence of phagocytosis, and not via the ubiquitous methotrexate transport system that is employed by MTX-glyceroPE I. For example, novel use of thiamine pyrophosphate showed that this compound had no influence on inhibition of cell proliferation due to liposomes, whereas thiamine pyrophosphate could completely antagonize the inhibitory effects of methotrexate and MTX-glyceroPE I. The results are discussed with reference to possible therapeutic advantages of these liposomes.

Introduction

In the preceding paper [1], we have described the synthesis of three methotrexate derivatives of dimyristoylphosphatidylethanolamine (DMPE) and presented evidence to show that these compounds corresponded, respectively, to MTX-γ-DMPE (I), MTX-α-DMPE (II), and MTX-α,γ-di-DMPE (III). For reasons previously detailed [1], these derivatives were prepared to examine the biochemical and pharmacological properties of liposomes in which an otherwise water-soluble drug is attached to the lipid bilayers via an appropriate non-polar anchor.

As a potent cytotoxic agent, whose mode of action has been firmly established, methotrexate has been used extensively by several laboratories to investigate the properties of liposomes that contain a drug trapped in the aqueous compartments. These studies [2–8] generally have shown that encapsulation prevents access of methotrexate to the transport system responsible for its entry into cells, and thereby almost completely prevents growth inhibition by methotrexate. Cell proliferation is, however, inhibited if the liposome-encapsulated drug is directed to appropriate antigens

Abbreviations: MTX, methotrexate; DMPE, dimyristoylphosphatidylethanolamine; glyceroPE, glycerophosphorylethanolamine; DOPC, dioleoylphosphatidylcholine.

on the target cell membrane by means of antibodies that have been bound or covalently coupled to the liposome surface. Under such circumstances, methotrexate enters the cell by a pathway that initially involves phagocytosis of the liposomes via lysosomes.

With these studies as reference point, the present experiments were designed to answer the following questions (answers in parentheses). Can untargeted liposomes sensitized with these MTX-DMPE derivatives inhibit cell growth in vitro? (Yes, depending on the derivative.) Does inhibition of cell proliferation correlate with the ability of the sensitized liposomes, as well as the ability of the corresponding glycerophosphorylethanolamine (glyceroPE) analogs, to inhibit dihydrofolate reductase (EC 1.5.1.3) isolated from the target cells? (Yes.) Do liposomes prepared with the MTX-DMPE derivatives enter cells by the same system that is responsible for the transport of methotrexate? (No.) Do the MTX-glyceroPE analogs utilize this system? (Yes.) The results obtained are discussed with regard to the therapeutic potential of these liposomes.

Materials and Methods

Chemicals. These were purchased from commercial sources as follows: dioleoylphosphatidylcholine (Avanti Polar Lipids, Birmingham, AL); 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and thiamine pyrophosphate (Calbiochem-Behring, La Jolla, CA); cholesterol, folic acid, NADPH, *N*-hydroxysuccinimide, methotrexate, and ω-aminohexyl-Sepharose 4B (Sigma Chemical Co., St. Louis, MO); dicetyl phosphate (Sigma or K and K Labs., Plainview, NY); tritiated deoxyuridine ([^{3}H]-dUrd, 15–30 Ci/mmol) (New England Nuclear, Boston, MA). The MTX-DMPE derivatives (I, II and III) and the MTX-glyceroPE analogs (I, II and III) were synthesized and isolated as described in the companion paper [1].

Liposome preparation. Liposomes were generated from dried lipid films containing dioleoylphosphatidylcholine, cholesterol, and dicetylphosphate in a molar ratio of 2 : 1.5 : 0.2, respectively. Unless otherwise indicated, the films were also supplemented with 2.5 mol% of MTX-DMPE I or II, or 5 mol% of MTX-DMPE III, on the basis of phosphate content. The lipid films were dispersed by vortexing in sufficient balanced salt solution to give a 10 mM liposomal (DOPC) suspension, and further diluted with balanced salt solution when necessary. As reported previously [1], MTX-DMPE I and MTX-DMPE II both had a phosphate : methotrexate ratio of 1, whereas MTX-DMPE III had a ratio of 2. Accordingly, the final methotrexate density in liposomes prepared with each of the derivatives was identical (2.5 mol% methotrexate).

Cell culture. Mouse 3T3 cells for the proliferation experiments were grown in tissue culture flasks (Corning No. 25116) containing 25 ml of Dulbecco's modified Eagle's medium (DMEM; Grand Island Biological Co., Grand Island, NY) supplemented with 10% fetal calf serum (FCS; Reheis Chemical Co., Phoenix, AZ). The flasks were initially seeded with 10^6 cells. After 3 days incubation (37°C, 5% CO_2), the cells (approx. 10^7 per flask) were harvested with 5 ml of a trypsin-EDTA solution diluted with Hanks' balanced salt solution lacking Ca^{2+} and Mg^{2+} (Gibco). The cells were spun down (4°C; 10 min; $750 \times g$), resuspended in 2 ml of cold DMEM-FCS, and counted. Viability was generally in excess of 95% as determined by Trypan blue exclusion.

Essentially the same procedure was employed to obtain L1210 cells for the proliferation experiments except that: (a) the flasks contained 50 ml of RPMI 1640 medium supplemented with penicillin-streptomycin (PS) antibiotic mixture (Gibco) and 10% FCS; (b) the flasks were initially innoculated with $5 \cdot 10^6$ cells; (c) after two days incubation, the cells (approx. $5 \cdot 10^7$ per flask) were collected by centrifugation, washed with balanced salt solution, and resuspended in 10 ml of RPMI-FCS prior to counting.

The L1210 cells for isolation of dihydrofolate reductase were grown in spinner flasks containing 1000 ml RPMI 1640-PS-FCS, which had been seeded with 10^8 cells. Two days later, the cells (approx. $1.5 \cdot 10^9$) were harvested by centrifugation (4°C; 20 min; $1500 \times g$), washed in 0.15 M NaCl, and resuspended in the latter at a concentration of 10^9/ml. They were stored in this manner at −70°C until needed.

Deoxyuridine incorporation. Proliferation of 3T3 cells was determined in wells of a 24-well tissue

culture plate (Costar No. 3524) that initially contained $5 \cdot 10^4$ washed cells in 1 ml of DMEM-FCS. After overnight incubation at 37°C in 5% CO_2, methotrexate or liposomes prepared with the MTX-DMPE derivatives, and other materials (as required by the individual experiments), were added to triplicate wells in 100 µl of balanced salt solution. The final concentrations are specified in the figure and table legends. Four hours later, 1 µCi of [^{3}H]dUrd in 10 µl of balanced salt solution was added to each well. Incubation was continued for another 24 h at which time the culture medium was filtered through, and the trypsinized cells collected onto, glass fiber paper using an automatic harvester (Brandel Labs., Gaithersburg, MD). The paper discs were washed with water, dried with ethanol and then counted in Aquasol-2 (New England Nuclear) using a Beckman LS-7500 scintillation spectrometer. Results are expressed as percent of control incorporation, which was calculated from the arithmetic means of the radioactivity taken up by the cells. Standard errors invariably fell within 10% of the mean values and therefore have not been presented in the figures.

For proliferation experiments with L1210 cells, the preceding protocol was modified as follows. Each well of a 96-well tissue culture plate (Costar No. 3596) received $2 \cdot 10^4$ washed cells in 180 µl of RPMI 1640-FCS. Other additions were then made in 20 µl of balanced salt solution (the overnight incubation step was omitted). Four hours later, 10 µl (1 µCi) of the tritiated deoxyuridine was added to triplicate wells and, after 24 h further incubation, the cells were harvested as above but without trypsinization.

Purification and assay of dihydrofolate reductase. L1210 cells which had been washed and frozen, were diluted with 0.15 M NaCl to give 30 ml of a suspension containing 10^8 cells/ml. This suspension was sonicated on ice for eight 15-s time periods with cooling in between, and centrifuged twice (4°C; 20 min; 25 000 × *g*). The clear supernatant solution (crude extract) subsequently was dialyzed overnight against two changes (2 liters each) of cold 50 mM potassium phosphate buffer (pH 6). The copious precipitate, which had formed, was removed by centrifugation as described above.

Enzyme in the supernatant solution (dialyzed extract) was isolated by affinity chromatography using a combination of the procedures described by in Refs. 9 and 10. The absorbent was prepared by incubation of 1 gram of packed ω-aminohexyl-Sepharose 4B (previously washed sequentially with 0.5 M NaCl, water, and methanol) with 80 µmol each of methotrexate, *N*-hydroxysuccinimide, and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide in 30 ml of methanol/dioxane (1 : 1, v/v). After 20 h at room temperature, unconjugated methotrexate was removed by successive washes with the methanol/dioxane mixture, methanol and water. The MTX-Sepharose was transferred to a 5 ml glass syringe (column dimensions: 1.2 × 5 cm) and equilibrated with 50 mM potassium phosphate buffer (pH 6). The dialyzed extract (approx. 28 ml) in this buffer was loaded onto the column followed by the passage of 80 ml of the same phosphate buffer, 80 ml of 50 mM Tris (pH 7), and 80 ml of 50 mM Tris (pH 8.5) containing 400 mM KCl. Dihydrofolate reductase was then eluted from the column with 20 ml of 0.2 mM dihydrofolic acid in 50 mM Tris (pH 8.5)/400 mM KCl; the dihydrofolic acid was prepared as described in ref. 11. This fraction was concentrated to 2 ml (in a sac that had been presoaked in 0.05% bovine serum albumin) by overnight vacuum dialysis against two changes (2 liter each) of 50 mM Tris (pH 7.4). Using this procedure, the enzyme was purified an average of 75-fold (range of four preparation 55–100 ×) with a 40% recovery of activity (range 30–55%).

The ability of various substances (methotrexate, liposomes with MTX-DMPE derivatives, MTX-glycero PE analogs) to inhibit dihydrofolate reductase was routinely determined by the following procedure; basically, the same protocol was used to measure enzyme activity during purification except that the inhibitors were omitted. Cuvets (10 mm light path) initially contained 650 µl of 0.5 M sodium acetate buffer (pH 6)/0.6 M KCl, and 50 µl of 100 mM 2-mercaptoethanol and 100 µl of 1 mM NADPH (each dissolved in the buffer-KCl mixture). Enzyme (75 µl of an appropriate dilution; see below) and inhibitor (25 µl; final concentration indicated in legends) were then added. After preincubation for 5 min at 23°C, the reaction was started by the addition of 100 µl of 0.7 mM dihydrofolic acid in 50 mM Tris (pH 7.4). The decrease in absorbance (340 nm) per minute

was then determined from 8 successive 15-s interval readings (Beckman DU-8 spectrophotometer). Under the above conditions, the amount of enzyme used in the assays produced a decrease of approx. 0.06 A/min in the absence of any inhibitor, and results have been expressed as percent of this control rate.

Results

Inhibition of DNA synthesis by liposomes containing MTX-DMPE derivatives and by MTX-glyceroPE analogs

When prepared without any derivative, unsensitized liposomes did not inhibit the ability of 3T3 cells to incorporate tritiated deoxyuridine into DNA up to a final concentration of 400 μM DOPC (Fig. 1). Liposomes sensitized with the MTX-DMPE derivatives blocked DNA synthesis to different extents, depending on which compound had been incorporated. Thus, liposomes containing MTX-DMPE III had no effect. Liposomes prepared with MTX-DMPE II produced partial inhibition (approx. 25%) at a relatively high concentration (20 μM on the basis of methotrexate content). In contrast, significant inhibition (approx. 85%) occurred in the presence of 2 μM liposomal MTX-DMPE I. This concentration was 10-fold higher than the concentration of methotrexate giving the same degree of inhibition (Fig. 1).

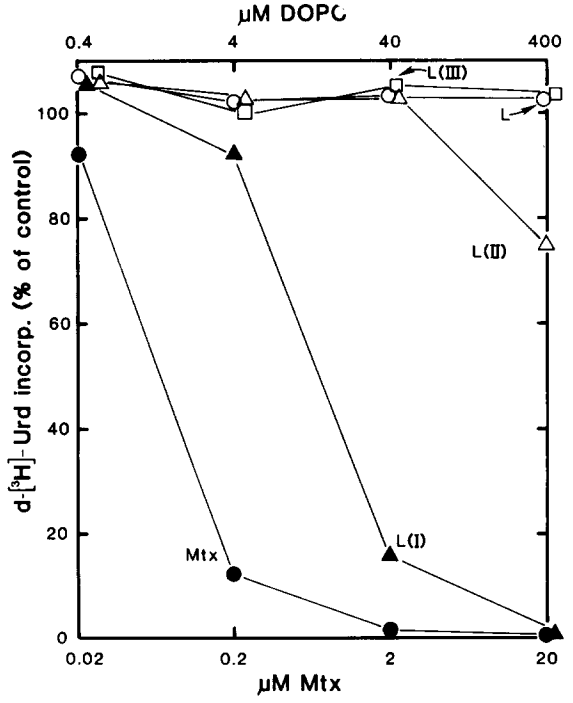

Fig. 1. Inhibition of 3T3 cell proliferation. Methotrexate, unsensitized liposomes prepared without any MTX-DMPE derivative (L), and liposomes prepared with each of the derivatives (L(I), L(II), and L(III)), were serially diluted with balanced salt solution prior to addition to the culture wells. The final concentration of methotrexate (Mtx) and the derivatives is shown on the bottom abscissa, and the final concentration of liposomal phospholipid (DOPC) is specified on the top abscissa. Control incorporation (no addition) was 432567 ± 4548 cpm.

The in vitro cytotoxic effect of the liposomes also was tested on L1210 cells in anticipation of future in vivo studies involving these murine leukemic cells. As shown in Fig. 2 (top), the same inhibitory sequence was obtained. Thus, at a concentration of 20 μM, the derivatives in liposomes blocked deoxyuridine incorporation in the following order: MTX-DMPE I (complete inhibition) > MTX-DMPE II (partial inhibition) > MTX-DMPE III (little or no inhibition).

The effect of the MTX-glyceroPE analogs, which were readily soluble in balanced salt solution (unlike the parent compounds), on DNA synthesis by L1210 cells is illustrated in Fig. 2 (bottom). MTX-glyceroPE I was most inhibitory and, in this regard, resembles MTX-DMPE I. At a concentration greater than 2 μM, MTX-glyceroPE II and MTX-glyceroPE III were essentially equally effective. The somewhat greater potency of MTX-glyceroPE III than liposomes sensitized with MTX-DMPE III may reflect the fact that these substances probably enter cells by different pathways (see below).

Inhibition of dihydrofolate reductase by liposomes containing MTX-DMPE derivatives and by MTX-glyceroPE analogs

It has been well established that the effect of methotrexate on cell proliferation is a consequence of its ability to inhibit dihydrofolate reductase, the enzyme responsible for the reduction of 7,8-dihydrofolic acid to 5,6,7,8-tetrahydrofolic acid. The latter is an essential cofactor in the methylation reaction by which deoxyuridylic acid (derived from deoxyuridine) is converted to deoxythymidylic acid prior to synthesis of DNA.

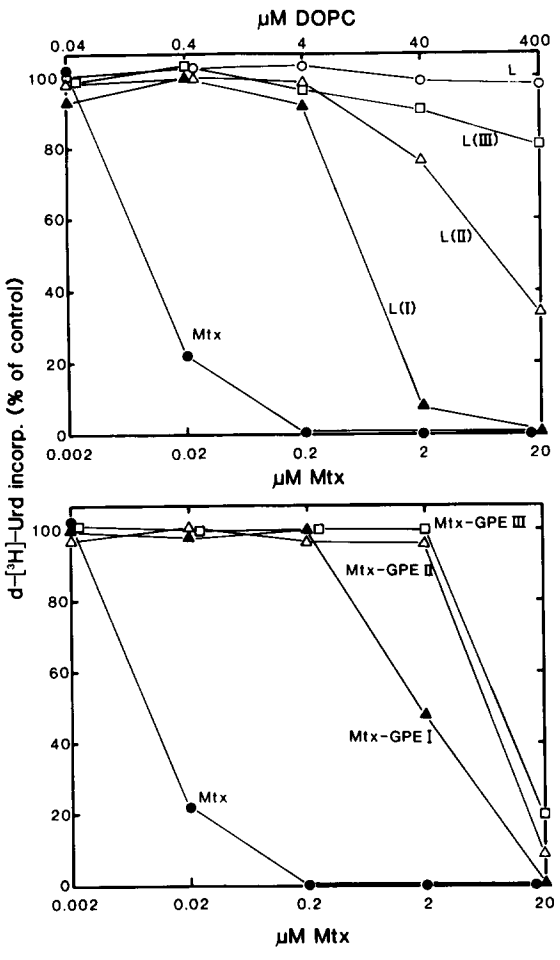

Fig. 2. Inhibition of L1210 cell proliferation. Top panel: serial dilutions of methotrexate, unsensitized liposomes (L), and liposomes sensitized with each of the MTX-DMPE derivatives (L(I), L(II), and L(III)), were added to the wells to give the final concentrations of methotrexate equivalents and DOPC indicated. Bottom panel: serial dilutions of methotrexate (Mtx) and each of the MTX-glyceroPE analogs were added to the wells to give the final concentrations indicated. Control incorporation (no addition) was 85960 ± 1025 cpm.

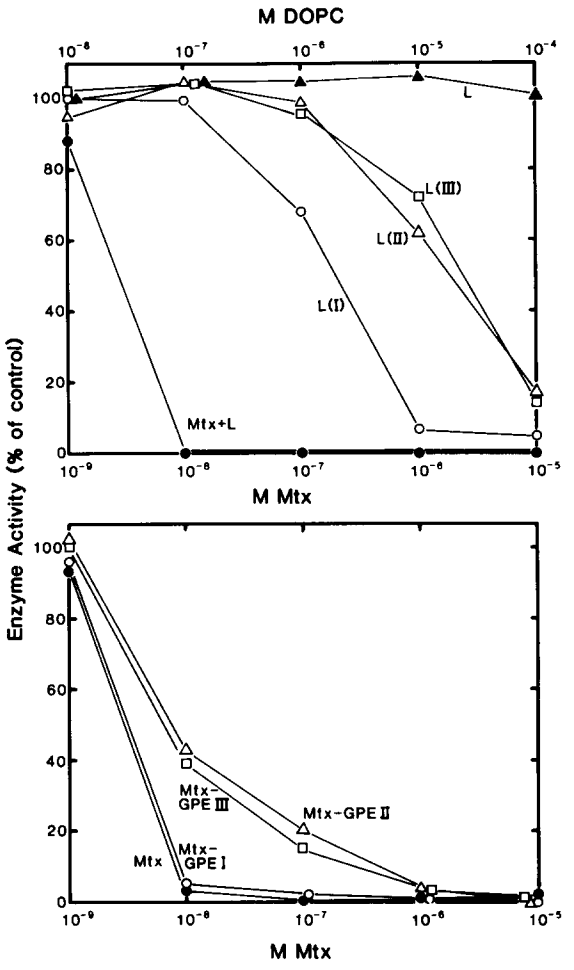

Fig. 3. Inhibition of dihydrofolate reductase activity. Top panel: serial dilutions of methotrexate (Mtx), unsensitized liposomes prepared without any MTX-DMPE derivative (L), and liposomes prepared with each of the derivative (L(I), L(II), and L(III)), were present in the cuvets at the final concentrations of methotrexate equivalents and DOPC indicated. Bottom panel: serial dilutions of methotrexate and MTX-glyceroPE analogs I, II, and III were present at the final concentrations shown.

Experiments were therefore carried out to compare the abilities of methotrexate, MTX-DMPE sensitized liposomes, and MTX-glyceroPE analogs to inhibit dihydrofolate reductase isolated from L1210 cells (Fig. 3). The following order was observed in the case of methotrexate and the derivatives in liposomes: methotrexate (complete inhibition at 10^{-8} M) > MTX-DMPE I (complete inhibition at 10^{-6} M) > MTX-DMPE II = MTX-DMPE III (complete inhibition at 10^{-5} M). With the analogs, the sequence was: methotrexate = MTX-glyceroPE I (complete inhibition at 10^{-8} M) > MTX-glyceroPE II = MTX-glyceroPE III (complete inhibition at 10^{-6} M). In general, the results reveal a close correlation between the effect of these materials on enzymatic activity and their capacity to inhibit deoxyuridine incorporation by L1210 cells. For example, liposomes prepared with MTX-DMPE I were less effective than methotrexate, but more effective than liposomes containing

either MTX-DMPE II or MTX-DMPE III in inhibiting both dihydrofolate reductase and cell proliferation.

The present results are also significant insofar as they bear on the postulated structures of the MTX-DMPE derivatives and analogs. Piper et al. [12] previously have demonstrated that the α glutamyl carboxyl group of methotrexate must be free to obtain maximum inhibition of L1210 cell DHFR. Thus, various γ-substituted amide and peptide analogs of methotrexate proved as potent as methotrexate, whereas the comparable α-substituted compounds were less effective by several orders of magnitude. In the preceding paper, we have presented circumstantial evidence that MTX-DMPE I (MTX-glyceroPE I) possesses a single DMPE (glyceroPE) residue linked via an amide bond to the γ carboxyl of methotrexate. This conclusion is supported by the current finding that MTX-glyceroPE I (i.e., MTX-γ-glyceroPE) and methotrexate were not only equipotent enzyme inhibitors but more potent than both MTX-glyceroPE II and MTX-glyceroPE III (MTX-α-glyceroPE and MTX-α,γ-diglyceroPE, respectively).

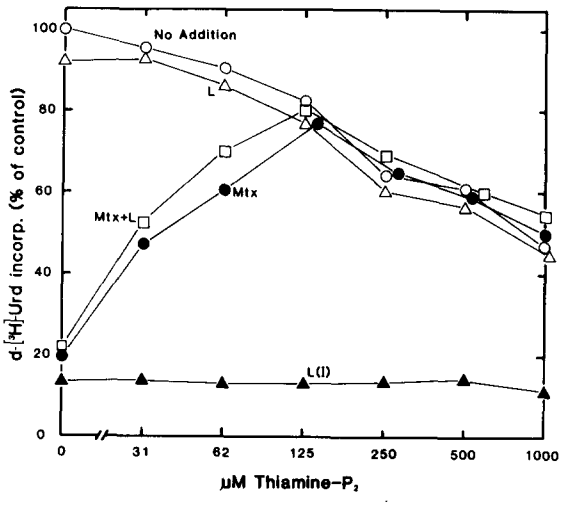

Fig. 4. Effect of thiamine pyrophosphate on inhibition by methotrexate (Mtx) and liposomes containing MTX-DMPE I. 3T3 cells were preincubated for 30 min with varying concentrations of thiamine pyrophosphate (as indicated on the abscissa), and the following (alone or in combination as specified in the figure) were then added: methotrexate (0.2 μM); unsensitized liposomes prepared without derivative (L, 40 μM DOPC); sensitized liposomes prepared with derivative (L(I), 40 μM DOPC, 2 μM MTX-DMPE I). Control incorporation (no addition; no thiamine pyrophosphate) was 287159 ± 6314 cpm.

Comparative effects of thiamine pyrophosphate on inhibition by methotrexate, MTX-DMPE derivatives in liposomes, and MTX-glyceroPE analogs

Methotrexate entrance into mammalian cells is known to be mediated by a specific transport system. Henderson and Zevely [13–15] have shown that thiamine pyrophosphate is a rather potent inhibitor of methotrexate influx via this system, and that it can block affinity labeling by *N*-hydroxysuccinimidylmethotrexate of a protein component involved in transport. Although the basis for this phenomenon has not been established, we have extended these findings in experiments utilizing thiamine pyrophosphate to determine whether methotrexate, liposomes prepared with the MTX-DMPE derivatives, and the MTX-glyceroPE analogs enter 3T3 and L1210 cells by the same pathway.

As illustrated in Fig. 4, thiamine pyrophosphate per se inhibited deoxyuridine incorporation by 3T3 cells in a dose dependent manner. Thus, 125 μM thiamine pyrophosphate reduced uptake of radioactivity by 20% both in the absence and presence of unsensitized liposomes. In this experiment, 0.2 μM methotrexate produced 80% inhibition when thiamine pyrophosphate was omitted. However, only 20% inhibition was observed in wells containing 0.2 μM methotrexate and 125 μM thiamine pyrophosphate indicating complete antagonism of the effect of methotrexate by thiamine pyrophosphate. In contrast thiamine pyrophosphate concentrations up to 1 mM did not block the inhibition produced by 2 μM MTX-DMPE I in liposomes. This differential effect of thiamine pyrophosphate was also apparent when cell number, instead of DNA synthesis, was measured (Table I). Thus, both 2 μM MTX-DMPE I in liposomes and 0.2 μM methotrexate blocked cell multiplication to the same extent (approx. 59%) in the absence of thiamine pyrophosphate. In the presence of 250 μM thiamine pyrophosphate, the sensitized liposomes still inhibited cell division (by approx. 64%) where methotrexate had no effect.

Fig. 5 (top) shows that, in the case of L1210 cells, concentrations of thiamine pyrophosphate

TABLE I

EFFECT OF THIAMINE PYROPHOSPHATE ON GROWTH OF 3T3 CELLS IN PRESENCE OF METHOTREXATE AND LIPOSOMES CONTAINING MTX-DMPE I

Plates were set up as in the proliferation experiment (see legend to Fig. 4) except that radioactive deoxyuridine was omitted; instead, mean number of viable cells per well was determined after 32 h incubation. L, unsensitized liposomes prepared without derivative; L(I), sensitized liposomes prepared with 2.5 mol% MTX-DMPE I; MTX, methotrexate; TP_2, thiamine pyrophosphate.

Addition (and final concentration)	Cells/well ($\times 10^{-4}$)	
	$-TP_2$	$+TP_2$ (250 μM)
None	13.8	16.0
L (40 μM DOPC)	13.5	13.2
L(I) (40 μM DOPC; 2 μM derivative)	5.3	4.6
MTX (0.2 μM)	6.0	16.1

per se up to 125 μM had no effect on the uptake of deoxyuridine. In this range, thiamine pyrophosphate did not antagonize the inhibition that was produced by liposomes containing MTX-DMPE I (2 μM) or MTX-DMPE II (20 μM). Fig. 5 (bottom) demonstrates, however, that increasing concentrations of thiamine pyrophosphate influenced significantly the inhibition obtained with either methotrexate or the MTX-glyceroPE analogs. Thus, 125 μM thiamine pyrophosphate completely prevented the effect of methotrexate (0.02 μM) and MTX-glyceroPE I (2 μM) on deoxyuridine incorporation, and partially antagonized inhibition by MTX-glyceroPE II (20 μM) and MTX-glyceroPE III (20 μM). Altogether, these results indicate that liposomes prepared with the MTX-DMPE derivatives do not utilize the transport system that is responsible for the entrance of methotrexate into cells, whereas this system is employed by the MTX-glyceroPE analogs.

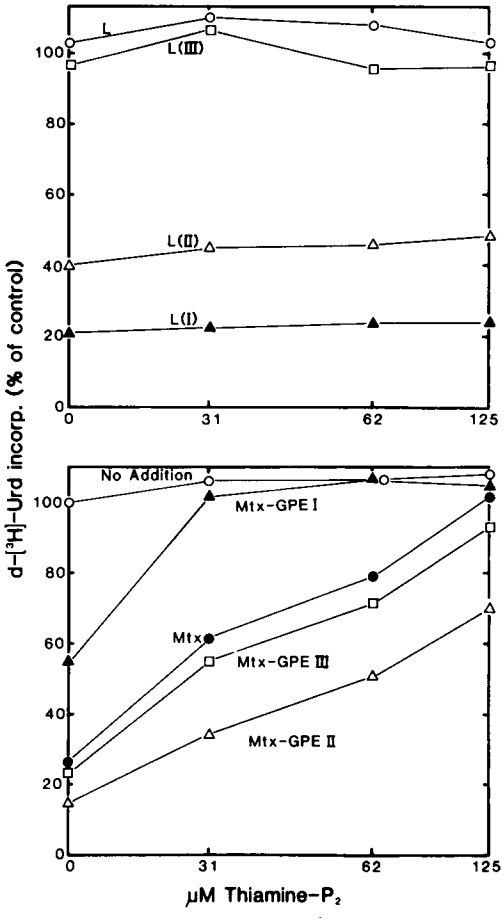

Fig. 5. Effect of thiamine pyrophosphate on inhibition by methotrexate, MTX-DMPE sensitized liposomes, and MTX-glyceroPE analogs. L1210 cells wre preincubated for 30 min with varying concentrations of thiamine pyrophosphate (as indicated on the abscissa), and the following were then added: unsensitized liposomes (400 μM DOPC); liposomes sensitized with either MTX-DMPE II or MTX-DMPE III (400 μM DOPC, 20 μM derivative); liposomes sensitized with MTX-DMPE I (40 μM DOPC, 2 μM derivative); methotrexate (Mtx) (0.02 μM); MTX-glyceroPE I (2 μM); MTX-glyceroPE II (20 μM); MTX-glyceroPE III (20 μM).Control incorporation (no addition; no thiamine pyrophosphate) was 158849 $\pm$ 941 cpm.

Effect of MTX-DMPE I density on inhibition

In the initial experiment (Fig. 1), the density of the MTX-DMPE derivatives in the liposomes was held constant (2.5 mol% methotrexate equivalents) and the concentration of the derivatives (as methotrexate) and liposomes (as DOPC) was varied in parallel. The converse experiment is described in Fig. 6 in which the concentration of MTX-DMPE I was kept constant (2 μM) while varying the density of this derivative in the liposomes. As indicated by the bottom curve (solid circles), maximum inhibition of deoxyuridine incorporation occurred with liposomes that contained between 0.85 and 2.6 mol% MTX-DMPE I. Inhibition was significantly less with liposomes that were prepared with either lower or higher densities of derivative.

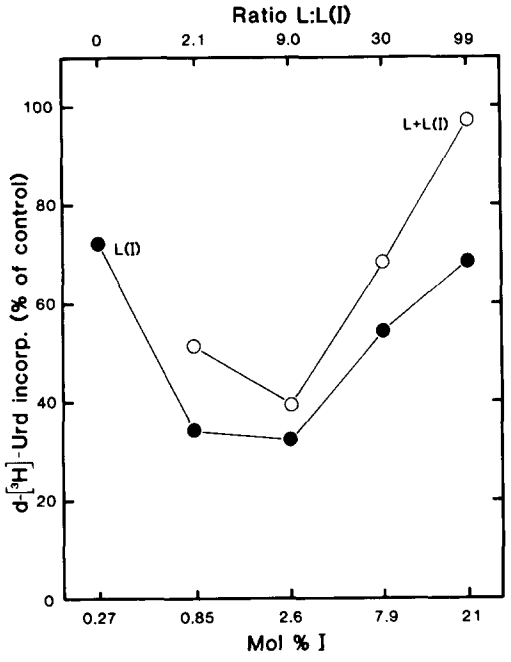

Fig. 6. Effect of MTX-DMPE I density on inhibition of 3T3 cell proliferation by liposomes. Liposomes (L(I)) were prepared as described in Materials and Methods except for the addition of various amounts of MTX-DMPE I to give the mol% values indicated on the abscissa. Inhibition was determined in the absence and in the presence of unsensitized liposomes (L); in the latter case, the total liposome concentration (sensitized plus unsensitized) was 400 μM DOPC. See text for additional details. Control incorporation (no addition) was 334304 $\pm$ 2889 cpm.

It is important to note that, under the conditions employed, liposome (DOPC) concentration was inversely related to the density of MTX-DMPE I in the following manner: 400 μM (0.27 mol%), 127 μM (0.85 mol%), 40 μM (2.6 mol%), 12.7 μM (7.9 mol%), 4 μM (21 mol%). Several studies previously have shown that the cellular uptake of liposomes is dependent on liposome concentration as manifested by a hyperbolic saturation curve (see, for example, Ref. 16). On this basis, we anticipated a reduction in inhibition at high densities of MTX-DMPE I, i.e., at low concentrations of liposomes. Conversely, liposome-cell interaction may be maximal at high liposome concentrations, but the amount of MTX-DMPE I taken up by the cells could be insufficient to produce marked inhibition due to its low density in the liposomes.

As indicated in Fig. 6 by the top curve (open circles), the same phenomenon was observed when appropriate amounts of unsensitized liposomes, prepared without the derivative, were added to the culture wells so that the total liposome concentration remained constant (400 μM DOPC). Furthermore, at each density of MTX-DMPE I, the presence of unsensitized liposomes reduced the extent by which the unsensitized liposomes inhibited incorporation of deoxyuridine. Indeed, essentially no inhibition occurred at a 99 : 1 ratio of unsensitized to sensitized liposomes. These results suggest that unsensitized liposomes may sterically hinder interaction of sensitized liposomes with 3T3 cells, and/or that both types of vesicles compete for a common pathway that is responsible for liposome uptake.

Comparative effects of NH_4Cl on inhibition by methotrexate and MTX-DMPE I in liposomes

Inhibition of cell proliferation by MTX-DMPE I may occur as a consequence of phagocytosis of the liposomes or fusion of the liposomes with the cell membrane. These alternatives are not mutually exclusive and both are consistent with the results described in the preceding section (Fig. 6). Regardless of mechanism, however, MTX-DMPE I or a biologically active metabolite would eventually have to gain access to dihydrofolate reductase which is located in the cytoplasm. The effect of various lysosomotropic agents suggests that, in the case of antibody-targeted liposomes containing methotrexate in the aqueous compartments, the drug enters the cytoplasm after the liposomes have been phagocytosed and processed within phagolysosomes [4–7]. Thus, NH_4Cl, which is known to elevate intralysosomal pH and thereby reduce the activity of lysosomal enzymes, can antagonize inhibition of cell proliferation by methotrexate trapped in liposomes, but not inhibition due to free methotrexate.

The inability of NH_4Cl to influence inhibition by methotrexate, both in the absence and presence of unsensitized liposomes, is confirmed by the experiment shown in Fig. 7. This was apparent inspite of the fact that, under identical conditions, NH_4Cl per se caused a significant inhibition of deoxyuridine incorporation. In contrast, NH_4Cl at concentrations between 1–16 mM could, at least partially, reduce the inhibition produced by liposomes that were prepared with MTX-DMPE I.

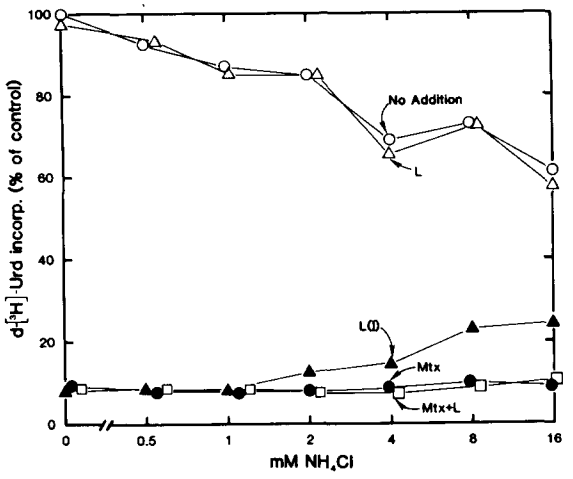

Fig. 7. Effect of NH_4Cl on inhibition by methotrexate and liposomes containing MTX-DMPE I. 3T3 cells were preincubated for 30 min with varying concentrations of NH_4Cl (as indicated on the abscissa), and the following (alone or in combination as specified in the figure) were then added: methotrexate (Mtx) (0.2 μM); unsensitized liposomes prepared without derivative (L, 40 μM DOPC); sensitized liposomes prepared with derivative (L(I), 40 μM DOPC, 2 μM I). Control incorporation (no addition; no NH_4Cl) was 336550 ± 2252 cpm.

Discussion

The experiments described above show that liposomes, which have been sensitized with MTX-DMPE I (MTX-γ-DMPE), can completely block proliferation of 3T3 or L1210 murine cells as measured by the uptake of tritiated deoxyuridine into DNA. In contrast, liposomes containing either MTX-DMPE II (MTX-α-DMPE) or MTX-DMPE III (MTX-α,γ-diDMPE) are significantly weaker cytotoxic agents, which produce either partial or no inhibition, respectively, at a comparable concentration (2 μM) of methotrexate equivalents. The relative potency of the MTX-DMPE derivatives in liposomes is essentially duplicated by the ability of the corresponding glycero PE analogs to inhibit cell proliferation (i.e., MTX-glyceroPE I was the most effective). Additionally, we have shown that liposomes containing MTX-DMPE I are better inhibitors of dihydrofolate reductase than liposomes prepared with either MTX-DMPE II or MTX-DMPE III. Similarly, MTX-glyceroPE I is a more potent enzyme inhibitor than either MTX-glyceroPE II or MTX-glyceroPE III.

Liposomes, which have been sensitized with MTX-DMPE I, are nevertheless less effective inhibitors of cell proliferation than free methotrexate by an order of magnitude. The greater potency of methotrexate most likely reflects the fact that the free drug enters cells by a different, and probably more efficient, pathway than that utilized by the liposomes. Thus, the effect of NH_4Cl, and the lack of an influence of thiamine pyrophosphate, on inhibition by the liposomes strongly suggest that MTX-DMPE I is taken up by the cells as a consequence of liposome phagocytosis, and not by the ubiquitous methotrexate transport system.

These results do not, however, rule out the possibility that liposomes prepared with MTX-DMPE I can bind to a protein component of the transport system that has a high affinity for methotrexate. It must be emphasized that, in the present experiments, the liposomes have not been directed to either 3T3 or L1210 cells by an antibody. Under these conditions, liposomes with MTX-DMPE I incorporated in the lipid bilayers are significantly better inhibitors of cell proliferation than non-targeted liposomes containing the same concentration (approx. 1–2 μM) of methotrexate trapped in the aqueous compartments (see, for example, Ref. 5). Therefore, the possibility must be considered that binding to a membrane-localized protein (a methotrexate 'receptor') might promote more extensive phagocytosis of MTX-DMPE I sensitized liposomes than the phagocytosis of liposomes in which methotrexate is encapsulated. MTX-DMPE I present in liposomes does have access to other proteins, which possess methotrexate binding sites, as evidenced by inhibition of enzymatic (dihydrofolate reductase) activity. Also, as demonstrated in a subsequent paper (in preparation), liposomes made with any of the MTX-DMPE derivatives can interact with anti-methotrexate antibodies and thereby prevent the agglutination of erythrocytes coated with methotrexate groups.

The available data further indicate that MTX-DMPE I is not degraded extracellularly to either methotrexate and/or MTX-glyceroPE I. This conclusion is also based on the failure of thiamine pyrophosphate to antagonize inhibition caused by liposomes, whereas it clearly abrogates the effects

of both methotrexate and MTX-glyceroPE I. However, the 'active form' of methotrexate, which is responsible for blocking dihydrofolate reductase located in the cytoplasm, has not been identified, and the possibility of intracellular degradation of the derivative should be examined. Metabolism of MTX-DMPE I could, for example, occur in the lysosomal compartment where it might be a substrate for phospholipase A, phospholipase C, acid peptidase, acid phosphatase, etc.

In a broader context, the present results provide the basis for our interest in the in vivo effectiveness of these liposomes. In this regard, it should be recalled (see Introduction to Ref. 1) that the MTX-DMPE derivatives were originally synthesized so that this water-soluble drug could be anchored to lipid bilayers and, consequently, circumvent the problem of methotrexate leaking from the aqueous liposomal compartments. Subsequent experiments (to be published) have, in fact, revealed that liposomes sensitized with MTX-DMPE I retain their ability to inhibit L1210 cell proliferation for a significantly longer time period than do liposomes with encapsulated methotrexate when stored at 37°C in the presence of serum. These findings have also been borne out by an in vivo trial showing that liposomes with MTX-DMPE I protected mice, which had been challenged with L1210 cells, at least as well as free methotrexate *. In view of the evidence indicating that MTX-DMPE I sensitized liposomes do not enter cells via the system utilized by methotrexate, it would be expected, therefore, that these liposomes would be a significantly better therapeutic agent in mice that have been infected by tumor cells, which are resistant to the free drug due to a defect in its transport. Additionally, future in vivo experiments will focus on comparing the effectiveness of liposomes containing MTX-DMPE I incorporated in the bilayers with liposomes containing encapsulated methotrexate in the aqueous compartments, using both non-targeted and antibody-targeted vesicles.

* In this experiment, one million L1210 cells were given i.p. to BDF_1 mice (5 animals/group) on day 0. On days 1 and 7, the first experimental group received 0.89 μmol of methotrexate/mouse i.p. (approx. 15 mg drug/kg). Simultaneously, a volume of liposomes containing the same dose of MTX-DMPE I was administered to the second experimental group, whereas the control group received an equal volume of liposomes prepared without any derivative. The results (50% survival times) were: control group (unsensitized liposomes), 18 days; group one (methotrexate), 28 days; group two (liposomes sensitized with MTX-DMPE I), 31 days.

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