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Inhibition of cell proliferation with antibody-targeted liposomes containing methotrexate-γ-dimyristoylphosphatidylethanolamine

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We have prepared liposomes containing methotrexate-γ-dimyristoylphosphatidylethanolamine (MTX-DMPE liposomes), to which protein A was covalently coupled, permitting specific association of these liposomes in vitro with murine cells preincubated with relevant protein A-binding monoclonal antibodies. In the absence of antibody the presence of externally-oriented methotrexate (MTX) in MTX-DMPE liposomes did not result in greater binding to cells than liposomes made without MTX-γ-DMPE. Derivation of methotrexate with phospholipid permits enhanced drug-liposome association. These liposomes are more resistant than conventional liposomes to repeated cycles of freezing and thawing. MTX-DMPE liposomes are comparable to antibody-targeted liposomes made with encapsulated water-soluble methotrexate both with respect to specific binding to target cells and drug effect. The inhibitory effects of MTX-liposomes, as well as free MTX, were reversible by either thiamin pyrophosphate (Tpp) or N^5-formyltetrahydrofolate (F-THF), while the effects of MTX-DMPE liposomes were reversed only by N^5-formyltetrahydrofolate. This suggests that the toxicity of non-targeted MTX-liposomes may be due to leakage of the encapsulated MTX. The absence of an effect of thiamin pyrophosphate on non-targeted MTX-DMPE liposomes indicates that they do not enter into the cell via the normal folate transport system.

Introduction

Water-soluble drugs, such as methotrexate (MTX), have been encapsulated in liposomes, which may be coupled to various ligands, including monoclonal antibodies. We have demonstrated that, when these liposomes are used in vitro, the normally non-specifically toxic MTX can be rendered specific for cells expressing the determinant bound by the monoclonal antibody [1]. Liposome

Abbreviations: MTX, methotrexate; MTX-γ-DMPE, methotrexate-γ-dimyristoylphosphatidylethanolamine; DPPC, dipalmitoylphosphatidylcholine; SPDP, *N*-succinimidyl-3-(2-pyridyldithio)propionate; mAb, monoclonal antibody; ^{3}H-dUrd, tritiated deoxyuridine; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Tpp, thiamin pyrophosphate; F-THF, N^5-formyltetrahydrofolate.

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technology is limited by several inconvenient features. These include low levels of encapsulation of the drug and leakage of the liposome contents, which increases non-specific toxicity and reduces the amount of material available for specific targeting.

One approach to the solution of this problem is the development of water-soluble drug analogs which, if they leak from liposomes, are incapable of entering into cells, and thus have reduced non-specific toxicity [2]. An other alternative is to prepare derivatives of drugs that are covalently associated with phospholipids, so that the drug becomes a stable component of the liposome structure. Liposome association of this category of hydrophobic drug should approach 100% [3]. In previous publications it has been shown that non-targeted liposomes containing methotrexate-γ-dimyristoylphosphatidylethanolamine (MTX-γ-DMPE) are capable of inhibiting the proliferation of cells in vitro when the liposomes are present at relatively high concentrations [4–6]. To assess the potential of this phospholipid prodrug with respect to its capacity for specific targeting MTX-γ-DMPE has been included in liposomes to which protein A was covalently coupled. This permits subsequent association of these liposomes with cells pre-incubated with relevant protein A binding monoclonal antibodies. Evaluation of the drug effect was measured by inhibition of DNA synthesis and of cell proliferation. The results indicate that, when bound to target cells, the activity of antibody-targeted liposomes prepared with MTX-γ-DMPE is comparable to similarly targeted liposomes containing MTX, and that liposomes prepared with MTX-γ-DMPE have augmented stability.

Materials and Methods

Liposome preparation. Small unilamellar liposomes of 500 Å nominal diameter containing either MTX or MTX-γ-DMPE were made using an 'Extruder' (Lipex Biomembranes, Inc., Vancouver, Canada), mounted with 0.05 μm polycarbonate filters (Nucleopore, Pleasanton, CA), according to the manufacturer's instructions and published references [7]. Liposomes were composed of 20 μmol dipalmitoylphosphatidylcholine (DPPC) (Sigma Chemical Co., St. Louis, MO), 20 μmol cholesterol (Calbiochem-Behring, Inc., La Jolla, CA) and 0.8 μmol dipalmitoylphosphatidylethanolamine (Sigma) modified with *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) (Pharmacia, Uppsala, Sweden), as described earlier [8].

For formation of liposomes containing MTX: to 3 ml of a solution containing 90 μmol purified [9] carboxyfluorescein (Eastman Kodak, Rochester, NY) in 100 mM $NaHCO_3$, pH 8.0 was added 75 μmol MTX (Division of Cancer Treatment, National Cancer Institute, NIH, Bethesda, MD) prior to mixture with the lipid components after evaporation of organic solvent. These liposomes are called MTX-liposomes.

For formation of liposomes containing MTX-γ-DMPE, prepared and characterized as described in Ref. 10: to the lipid preparation was added 0.4 μmol MTX-γ-DMPE, prior to evaporation and the addition of the carboxyfluorescein/$NaHCO_3$ solution. These liposomes are called MTX-DMPE liposomes. Following liposome formation, protein A (Pharmacia) was coupled to liposome preparations as described in Ref. 8. The phospholipid concentration was determined by the ammonium ferrothiocyanate technique of Stewart [11] and the amount of protein A bound to the liposomes was determined by the inclusion of a small amount of ^{125}I-labelled protein A as a tracer. The covalent attachment of protein A to liposomes was unaffected by the presence of the MTX-γ-DMPE. The majority of experiments reported here were performed with aliquots of a single preparation of MTX or MTX-DMPE liposomes, to which were coupled 8 μg protein A per μmol total lipid.

MTX concentration of the liposome preparations. Encapsulated MTX was measured by fluorescence by virtue of its ratio to coencapsulated carboxyfluorescein. MTX-γ-DMPE was evaluated by its ratio to DPPC, measured as phospholipid. These measurements and the concentration of free MTX used were confirmed by a MTX radioimmunoassay (Oris Industrie, St. Quentin, Yvelines, France), using ^{125}I-modified MTX, and a high-affinity rabbit antibody specific for MTX.

Cell lines. The murine cell lines used were the AKR thymoma RDM4, and the hybrid cell line B1.069.3, which is the product of the fusion of

C57Bl/6 B cell blasts with the AKR thymoma BW5147 [12]. T cells were obtained from male CBA/J spleens as described elsewhere [13]. These cells were stimulated with concanavalin A at 2 μg/ml and incubated with liposomes 48 h later. All of these cells express the major histocompatibility complex-encoded H-2K^k molecule, which has been shown to be an effective target for antibody-mediated delivery of liposome-encapsulated MTX for T cells and T-B hybrids [12,14].

Monoclonal antibodies. The monoclonal antibodies (mAbs) used are H100.5.28, which is specific for the H-2K^k molecule, and 20.8.4, specific for the H-2K^b molecule. Both are murine IgG2a antibodies with equivalent affinity for protein A [15].

Cell culture conditions. To flat bottom wells of 96 well tissue culture plates containing 10^4 cells (for the MTT assay (see below)) or 10^5 cells (for ^{3}H-dUrd) in RPMI 1640 medium (Gibco, Cergy Pontoise, France), supplemented with 5% fetal calf serum and antibiotics were added relevant or control mAbs (to a final concentration of 5 μg/ml) and dilutions of stock preparations of free MTX, MTX-liposomes, or MTX-DMPE-liposomes. In some experiments 10 mM NH_4Cl, 150 μM thiamin pyrophosphate (Tpp) (Boehringer-Mannheim, Maylan, France), or 1 μM N^5-formyltetrahydrofolate (F-THF) (National Cancer Inst.), were added to culture wells 30 min prior to the addition of the liposomes. The final volume in the wells was 120 μl.

Cytotoxicity assays. MTX-mediated inhibition of DNA synthesis was evaluated by its effect on the incorporation of ^{3}H-dUrd (Amersham, Les Ulis, France). After 3 h of exposure to free drug or liposomes, 0.5 μCi ^{3}H-dUrd was added to the culture wells. After an additional 16 h incubation at 37°C, radioactivity incorporated in DNA was determined as described in Ref. 1. Direct measurement of the effect of MTX on cell proliferation was by the MTT assay [16], which measures the production of a blue formazan precipitate following the intracellular reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma). 45 h after addition of MTX-liposomes, MTX-DMPE liposomes or free MTX, cells were resuspended in 100 μl medium containing 500 μg/ml MTT. After 3 h incubation at 37°C plates were centrifuged, the supernatant was removed, and the cells were resuspended in 2-propanol. The resulting blue color, which is directly proportional to the number of living cells in the well [16], was measured spectrophotometrically with an automatic tissue culture plate reader using a test wavelength of 570 nm and a reference wavelength of 630 nm. Data presented are derived from the mean of duplicate cultures expressed as a percentage of control wells incubated without MTX; and are representative of results obtained in at least three independent experiments.

Flow cytometry measurements. Cell-associated fluorescence was evaluated with an EPICS 5 flow cytometer (Coulter Electronics, Inc., Hialeah, FL). Liposome binding data were obtained for 5000 cells sampled from 10^5 cells incubated in 100 μl medium containing 5% fetal calf serum, in the presence or absence of mAbs (50 μg/ml) at 4°C for 1 h, followed by washing and exposure to MTX or MTX-DMPE liposomes for 1 h under the same conditions. In some experiments incubations were performed in the presence of MTX (1 μM), to assess the possible participation of the MTX transport system in the binding of MTX-DMPE liposomes, or with free protein A (50 μg/ml), to assess the specificity of binding. Results are presented as the fluorescence of cell-associated liposomes which are fluorescent as a consequence of the entrapped carboxyfluorescein, with the fluorescence of the cells incubated alone taken as unity.

Freezing and thawing. To test the capacity of liposomes to retain either MTX or MTX-DMPE, we submitted aliquots of the liposome preparations to five cycles of freezing and thawing. The amount of MTX or MTX-γ-DMPE accessible for binding by rabbit anti-MTX antibody in a radioimmunoassay was determined before and after this treatment. The effect on cells of MTX or MTX-γ-DMPE released as a consequence of freezing and thawing was also evaluated in an MTT assay, in the presence and absence of targeting antibodies.

Results

Inhibitory effects of non-targeted liposomes

The inhibitory effect of non-targeted liposomes containing MTX-γ-DMPE has been reported

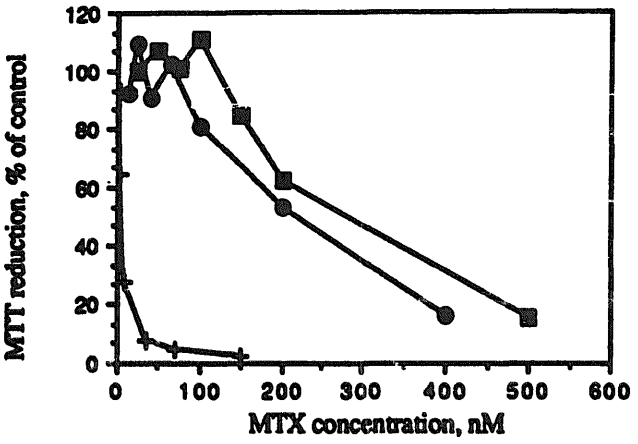

Fig. 1. Inhibition of B1.069.3 cell proliferation by liposomes containing encapsulated MTX (MTX-liposomes) and liposomes sensitized with MTX-γ-DMPE (MTX-DMPE liposomes). Evaluation by the MTT assay. The A_{570} for control cells incubated without drug was 0.360±0.053. +, MTX; ●, MTX-liposomes; ■, MTX-DMPE liposomes.

[4–6]. In the present study they were compared to liposomes containing the encapsulated drug by the MTT assay. Fig. 1 shows that, for the cell line B1.069.3, which is quite MTX sensitive, inhibition of proliferation by both MTX- and MTX-DMPE liposomes required the addition of 20-times more MTX for an effect equivalent to that of free drug (amount of MTX giving 50% growth inhibition: ID_{50}). Other cells tested, such as T cell blasts, are less sensitive both to free and liposome-associated MTX (see below).

The toxicity of non-targeted liposomes was investigated in the presence of various reagents. B1.069.3 cells were incubated with concentrations of free MTX or MTX- or MTX-DMPE liposomes sufficient to cause about 90% inhibition of cell growth. To some wells was added 150 μM Tpp, to others 1 μM F-THF. Tpp is a competitor for the folate transport system by which MTX enters into cells, though it has no other effect on the action of MTX [17]; F-THF is also a competitor for transport, and additionally is an analogue of the product whose synthesis is blocked by MTX, permitting it to reverse the toxicity of MTX [14]. The results of a representative experiment, presented in Table I, show that the inhibitory effects of MTX-liposomes, as well as free MTX, are reversible by either Tpp or F-THF, while the effects of MTX-DMPE liposomes are reversed only by F-THF. This suggests that the toxicity of non-targeted MTX-liposomes may be due to leakage of the encapsulated MTX. The absence of an effect of Tpp of non-targeted MTX-DMPE liposomes indicates that they do not enter into the cell via the normal folate transport system.

Liposome binding studies

The fluorescence of RDM4 cells incubated with equal amounts of MTX- or MTX-DMPE liposomes, determined by flow cytofluorometry is shown in Table II. Low levels of binding were observed in the absence of antibody, or when cells had been incubated with the control antibody, 20.8.4, for which there is no binding site on the cells. Binding was increased to the same extent for both liposome preparations when cells were preincubated with the relevant anti-H-2 antibody (H100.5.28), indicating that the binding of the MTX-DMPE liposomes was as efficient as that of MTX-liposomes. Similarly, the cell association of both liposome preparations was inhibited to the same extent by an excess of free protein A, as expected by its competition with the liposome-bound protein A for cell binding (not shown). Neither liposome preparation was inhibited in its binding by 1 μM free MTX in the medium. These experiments indicate that the presence of externally oriented MTX in MTX-DMPE liposome preparations does not augment their binding to cells.

TABLE I

THE EFFECT ON B1 069.3 CELL PROLIFERATION OF Tpp AND F-THF

The effect of Tpp and F-THF on B1 069.3 cell proliferation in the presence of MTX (15 nM), MTX-liposomes (400 nM) or MTX-DMPE liposomes (500 nM) was evaluated by the MTT assay. The numbers refer to the percentage of proliferation relative to control cells incubated in medium alone. Tpp and F-THF had no effect on cell proliferation in the absence of MTX (not shown).

	Percentage of proliferation		
	control	Tpp	F-THF
MTX	7	71	85
MTX-liposomes	16	49	55
MTX-γ-DMPE liposomes	15	13	70

TABLE II

FLOW CYTOFLUOROMETRIC STUDIES ON RDM4 CELLS

Results are presented as mean fluorescence (arbitrary units) relative to the fluorescence of unlabelled cells taken as unity. The concentration of MTX and of MTX-γ-DMPE was 10 μM for both liposome preparations. The control mAb was 20.8.4, the relevant mAb H 100.5.28.

	Relative fluorescence			
	liposomes	liposomes + irrelevant mAb	liposomes + relevant mAb	liposomes + relevant mAb + methotrexate
MTX-liposomes	1	4	32	32
MTX-γ-DMPE liposomes	1	2	31	32

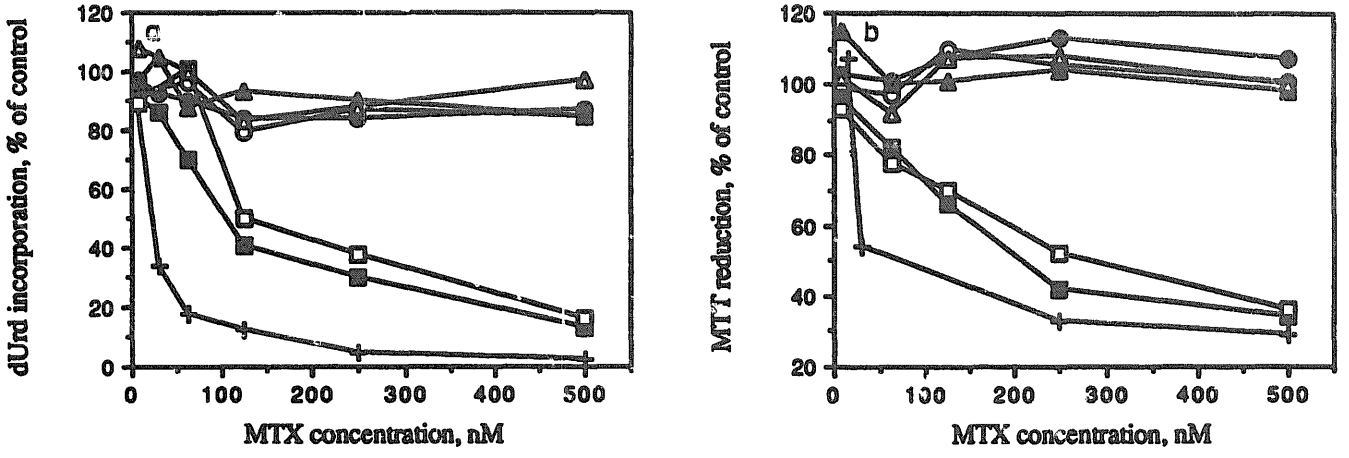

Fig. 2. Inhibition of T cell blast proliferation by antibody-targeted preparations of MTX-liposomes and MTX-DMPE liposomes. (a) The inhibition of cell proliferation was evaluated by the incorporation of ^{3}H-dUrd. Control incorporation was 37400 ± 4800 cpm. (b) Evaluation of cell proliferation by the MTT assay. The control A_{570} was 0.385 ± 0.051. +, MTX; ●, MTX-liposomes; ○, MTX-DMPE liposomes; ■, MTX-liposomes with relevant mAb (H100.5.28); □, MTX-DMPE liposomes with the relevant mAb; ▲, MTX-liposomes with the control mAb (20.8.4); △, MTX-DMPE liposomes with the control mAb.

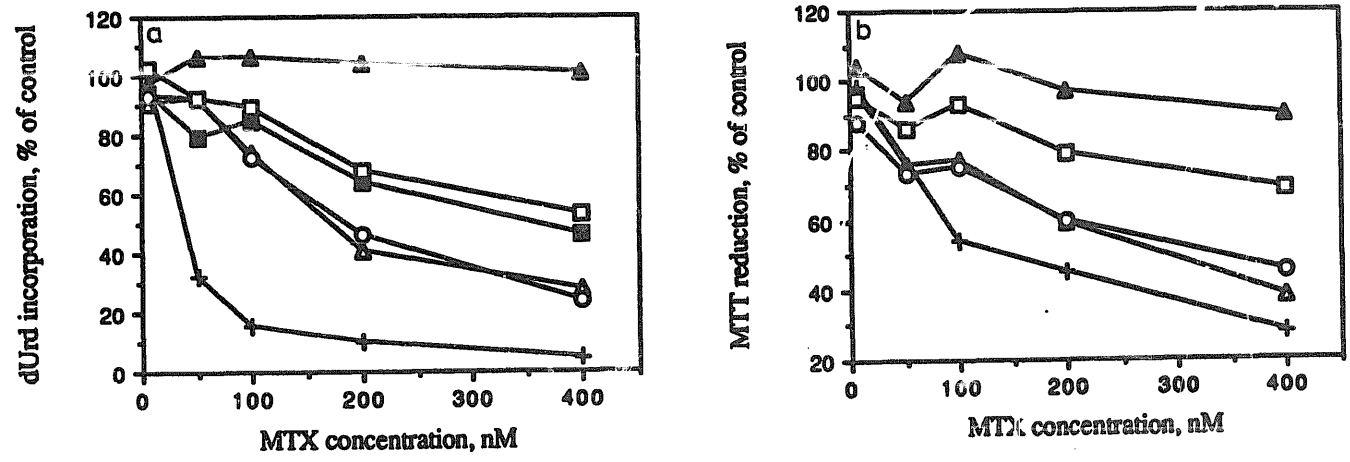

Fig. 3. Effect of NH_4Cl, Tpp and F-THF on the cell toxicity of antibody-targeted MTX-DMPE liposomes for T cell blasts. (a) Evaluation by incorporation of ^{3}H-dUrd. Control incorporation was 35200 ± 5100 cpm. (b) Evaluation by the MTT assay. Only the effect of Tpp and F-THF was studied because of the toxicity of NH_4Cl in a 48 h incubation. The control A_{570} was 0.502 ± 0.064. +, MTX; ▲, MTX-DMPE liposomes; △, MTX-DMPE liposomes with relevant mAb without any inhibitor; ■, with NH_4Cl; ○, with Tpp; □, with F-THF.

Inhibitory effects of antibody-targeted liposomes

The effect on cells of antibody-targeted preparations of MTX-DMPE liposomes and MTX-liposomes, containing the same quantity of MTX, and coupled to the same quantity of protein A per μmol phospholipid, was compared on T cell blasts. The results, presented in Fig. 2, indicate a marked enhancement of the inhibition of cell proliferation by both liposome preparations when they were incubated in the presence of the relevant mAb (H100.5.28), as compared to liposomes incubated without antibody, or in the presence of the control mAb 20.8.4. MTX-DMPE liposomes were as efficient as MTX-liposomes for an equivalent amount of MTX, as shown both by DNA synthesis in the overnight ^{3}H-dUrd assay (Fig. 2a), and by the 48 h MTT assay (Fig. 2b). In no case was there inhibition by the targeting antibody alone, or by antibody-bearing liposomes made without free MTX or MTX-γ-DMPE (data not shown).

The antibody-targeted MTX-DMPE liposomes were further examined in the presence of Tpp, and F-THF in both the ^{3}H-dUrd and MTT assays. NH_4Cl, which we have previously reported as antagonizing the effect of liposome-encapsulated, but not free MTX [1] was studied only in the overnight ^{3}H-dUrd assay, because of its toxicity in the longer MTT assay (not shown). Results are presented in Fig. 3. The effects of targeted MTX-DMPE liposomes were partially reversed by F-THF and NH_4Cl. There was no effect of the Tpp on their action. Tpp similarly did not reduce the toxicity of targeted liposomes containing MTX (data not shown).

Characterization of MTX in liposome preparations, and cytotoxicity of liposomes exposed to repeated cycles of freezing and thawing

The concentration of MTX which was actually liposome-encapsulated or associated was confirmed by a MTX radioimmunoassay, which also permitted an evaluation of the integrity of the liposomes. For liposomes containing encapsulated MTX (MTX-liposomes), 95% of the MTX was latent, and was measurable only after lysis of the liposome preparation with detergent (1% Triton X-100). This is consistent with low leakage of MTX from the MTX-liposomes under normal conditions of storage (at 4°C after sterilization by passage through 0.45 μm filters (Gelman, Ann Arbor, MI). After five cycles of freezing and thawing about half of the MTX was liberated, as demonstrated by the immunoassay (data not shown), and by the non-specific effects of these liposomes on cells, as shown by the decrease in the number of MTX-containing liposomes required to inhibit MTT reduction in the presence of both relevant and control antibodies (Fig. 4a).

In contrast, for MTX-DMPE liposomes, the quantity of MTX-γ-DMPE measurable before addition of detergent was 60% of that measured after its addition. This is what is expected for liposomes

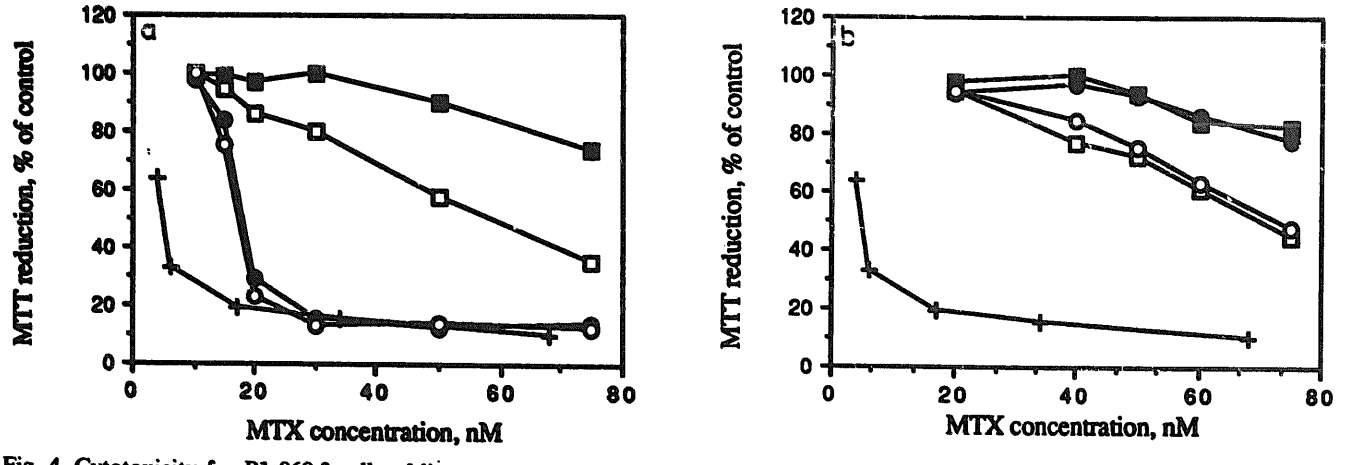

Fig. 4. Cytotoxicity for B1 069.3 cells of liposomes exposed to repeated cycles of freezing and thawing. Evaluation by MTT assay. The control A_{570} was 0.383±0.047. (a) +, MTX. ■, Untreated MTX-liposomes with control mAb; □, with relevant mAb. ●, Frozen/thawed MTX-liposomes with control mAb; ○, with relevant mAb. (b) +, MTX. ■, Untreated MTX-DMPE liposomes with control mAb; □, with relevant mAb. ●, Frozen/thawed MTX-DMPE liposomes with control mAb; ○, with relevant mAb.

containing a phospholipid-derivatized drug, about half of which is oriented toward the inside, and about half toward the outside, in predominately unilamellar vesicles. Freezing and thawing did not appreciably increase the binding of MTX-γ-DMPE by the anti-MTX antibody. After this treatment these liposomes did not show either increased toxicity in the presence of the control antibody, or reduced toxicity in the presence of targeting antibody (Fig. 4b).

Discussion

Historically, two problems limited the use of targeted liposomes: the potential leakage of the encapsulated drug and the problem of associating the targeting moiety stably with the liposome. Drugs have usually been selected in screening tests on their ability to act in the absence of exogenous carriers, such as liposomes. This means that the majority of these reagents have the capacity to pass lipid membranes to enter into cells, and, by extension, their leakage from liposomes is unacceptably high for pharmaceutical purposes. Additionally, the earliest experiments which attempted to attach antibody to liposomes for the purpose of targeting were performed with non-derivatized antibody. For some classes of antibody, the association with liposomes was possible, but the technique was too inconsistent to be generally useful.

The strategy of association of drugs and targeting ligands with phospholipids has improved the efficacy of both entities. Further, because almost all amphipathic lipid participates in the formation of the bilayer structure, the insertion of phospholipid-associated drugs is practically quantitative, in contrast to the low encapsulation efficiency of water-soluble drugs. In the present study, 0.4 μmol of MTX-γ-DMPE in the initial lipid preparation became liposome associated to the same extent as 75 μmol of MTX in the aqueous phase. While the efficiency of encapsulation of water-soluble compounds can be augmented by various techniques, it seldom exceeds 5% for small liposomes, such as those used here [3]. The ability of Tpp, which competes for entry into cells with free MTX, to protect against high concentrations of MTX- but not MTX-DMPE liposomes confirms that the former liposomes can leak their contents in culture, with inhibitory effects on non-targeted cells.

It is important to point out that the absence of leakage is not the same as the absence of non-specific toxicity. Given that much of the effect of MTX from MTX-liposomes was Tpp inhibitable, we might have expected the non-leaky preparation of MTX-DMPE liposomes to be less toxic in the absence of targeting antibody, unless their cell association was greater than that of conventional liposomes. However, measurements by flow cytofluorometry indicate that MTX- and MTX-DMPE liposomes become non-specifically cell-associated to the same limited extent, and that their specific binding is equivalent in the presence of the targeting antibody. This means that the 60% of MTX-γ-DMPE molecules which are externally oriented, and so could be bound by high-affinity rabbit antibody to MTX in our immunoassay, are not bound by the membrane transport mechanism responsible for the entry of free MTX. The comparable toxicity of MTX- and MTX-DMPE liposomes under conditions of equivalent binding, in the absence of the possibility of leakage from MTX-DMPE liposomes, thus suggests that most of the non-specific toxicity of MTX-liposomes comes from cell-bound, rather than from the large excess of non-bound, liposomes present in solution, which is consistent with studies reported by Van Renswoude and Hoekstra for liposomes containing carboxyfluorescein [18]. The effect of the leaked MTX would be non-specific in mixed culture or in vivo, since the diffusion of the leaked MTX away from the target cell would be expected to be rapid with respect to its capture by that cell's folate transport system [19]. In contrast to non-targeted liposomes, specifically-targeted liposomes containing MTX were not inhibited by Tpp, which could potentially be due to their rapid internalization or to their binding to sites on the cell not inducing leakage.

The specific effect of MTX- and MTX-DMPE liposomes was inhibitable by NH_4Cl, which neutralizes acidic intracellular compartments [20]. The passage of MTX from liposomes to the cytoplasm requires its protonation, which increases the lipophilicity of the drug [21]. MTX-DMPE liposomes must first be cleaved by cellular enzymes, includ-

ing phospholipases, since liposomes coupled to non-metabolizable phospholipid analogues of MTX were less effective in vitro [6]. This process may be pH dependent.

The coupling of MTX to phospholipid molecules is associated with improvement in storage capacity of liposomes as demonstrated by their resistance to repeated freezing and thawing. It does not reduce the pharmacologic action of liposomes made with this reagent, which in the present study is shown to be nearly as active as that of liposomes containing MTX. The combination of stability, efficient encapsulation and significant pharmacologic activity of MTX-DMPE liposomes, which has also been reported for muramyl tripeptide [22], cytosine arabinoside [23], and fluorodeoxyuridine [24], together with the efficient targeting reported here suggests that this form of coupling may be of general interest for the delivery of reagents in targeted liposomes.

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