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Antibody-targeted liposomes: Increase in specific toxicity of methotrexate-γ-aspartate

(monoclonal antibody/ligand/antifolate/vesicle/antitumor agent)

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ABSTRACT **Liposomes conjugated with anti-H2K^k antibody associate with L929 murine fibroblasts in 6- to 20-fold greater amount than do nonspecific liposomes. The ability of methotrexate-γ-aspartate to inhibit L929 growth is increased 10-fold when encapsulated in targeted liposomes but is decreased to 50% when encapsulated in liposomes with no specificity for the target cells. Ammonium chloride inhibits the effects of the encapsulated but not the free drug. Soluble antibody does not inhibit the efficacy of targeted vesicles, but empty targeted vesicles do inhibit the efficacy. The compound in both targeted and nontargeted vesicles has a minimal effect on BALB/c 3T6 fibroblasts. These results demonstrate the potential of antibody-targeted liposomes and the importance of selecting liposome-dependent cytotoxic agents.**

The production of antibody-directed cytotoxic agents is of prime importance in cell biology and medicine. Use of such agents, which can specifically kill cells bearing the appropriate antigen, may give insight into the mechanisms of receptor-mediated internalization and ultimately may lead to production of tumor-specific cytotoxic agents. Antibody-directed cytotoxic agents have been produced primarily by preparing drug (1) or toxin (2) conjugates with antibodies. More recently, we have developed efficient methods for conjugation of protein to liposomes (3–5). We have proposed the use of such liposomes as targeted cytotoxic agents because targeted liposomes§ bind to the appropriate cells with high efficiency (4, 6).

The efficient use of targeted liposomes in cancer therapy will depend primarily on the difference between their toxicities for target and nontarget cells. Two factors must be optimized to achieve this aim. First, the liposomes must deliver their contents efficiently to the cytoplasmic compartment. Second, the nonspecific toxicity, which may arise through the leakage of the cytotoxic agent from the liposome (7–9), must be minimized. The toxicity of leaked drug could be minimized by using a drug that cannot enter cells but which would be toxic if delivered to the cytoplasm. Such a compound would be analogous to viral nucleic acids, which become infectious when encapsulated in liposomes.

Methotrexate is a pteridine antifolate which inhibits cell growth by inhibiting dihydrofolate reductase (10). Methotrexate has been used successfully in targeted liposomes by Leserman *et al.* (11) but it readily penetrates cells via the folate transport system. Piper *et al.* (12) have examined the effects of structural alterations of methotrexate on cytotoxicity, influx kinetics, and ability to inhibit dihydrofolate reductase. Methotrexate-γ-aspartate proved to be virtually indistinguishable from methotrexate in its ability to inhibit dihydrofolate reductase. However, methotrexate-γ-aspartate is only 1/200th as toxic for L1210 cells compared to methotrexate because its influx K_m is at least 100 times greater. Methotrexate-γ-aspartate therefore fulfills our criteria for a liposome-dependent drug because its ability to enter cells is poor but it should be equipotent to methotrexate if delivered intracytoplasmically.

In this communication we describe the efficacy of methotrexate-γ-aspartate in liposomes targeted with monoclonal anti-H2K^k (13). H2K^k is a protein of the mouse major histocompatibility complex which is expressed at high frequency on L929 fibroblasts. We compare the γ-aspartate to methotrexate and show why the γ-aspartate is more suitable for use with targeted vesicles. We also demonstrate that targeted vesicles interact with cells in an energy-dependent manner and compete effectively for binding with a large excess of soluble antibody.

METHODS

Lipids were obtained or purified as described (3) except for 4-(*p*-maleimidophenyl)butyrylphosphatidylethanolamine which was synthesized (5). Methotrexate was obtained from Bristol Myers, and methotrexate-γ-aspartate was synthesized as described (12). Monoclonal antibodies used in these experiments were anti-H2K^k IgG2A secreted by the clone 11-4.1 (13), antisheep erythrocyte IgG2A (hybridoma S-S.1), and anti-Thy 1.1 IgG2A secreted by the hybridoma 19VE12 (14). 11-4.1 and S-S.1 were supplied by M. Cohn (Salk Institute) and 19VE12 was provided by R. C. Nowinsky (Fred Hutchinson Cancer Center, Seattle, WA). All hybridomas were grown in culture in Dulbecco's modified Eagle medium (DME medium) with antibiotics and 10% fetal calf serum. The cells (10^7) were injected into pristane-primed mice and the ascitic fluid was harvested.

Antibody was purified from ascitic fluid by affinity chromatography on protein A-Sepharose (15). Liposomes were prepared by the method of Szoka and Papahadjopoulos (16) in 5 mM methotrexate-γ-aspartate or in 0.22 mM methotrexate with 50 mM 4-morpholineethanesulfonic acid/50 mM 4-morpholinepropanesulfonic acid/40 mM NaCl, pH 6.7.

The vesicles were prepared from a 10:10:1 molar mixture of phosphatidylcholine, cholesterol, and 4-(*p*-maleimidophenyl)butyrylphosphatidylethanolamine. Excess drug was removed on a 1 × 10 cm Sephadex G-50 column (equilibrated with the same buffer) and the vesicles were conjugated to monoclonal antibod-

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Abbreviation: DME medium, Dulbecco modified Eagle medium.

§"Targeted liposomes" will refer to vesicles that contain antifolate and are conjugated to anti-H2K^k antibody. "Targeted empty liposomes" will refer to vesicles that do not contain a drug and are conjugated to anti-H2K^k. Similarly, "nonspecific liposomes" and "nonspecific empty liposomes" will be the terms applied to vesicles conjugated to an antibody with no specificity for the H2K^k antigen (either anti-sheep erythrocyte antibody or anti-Thy 1.1 antibody). "Uncoated liposomes" and "uncoated empty liposomes" will refer to liposomes that have not been conjugated with an antibody.

ies by the method of Martin and Papahadjopoulos (5) with modifications. Antibody was thiolated with *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) because the generation of Fab' from mouse monoclonal antibody is inefficient. The antibody was thiolated for conjugation (17) at a SPDP/protein ratio of 20:1 which gave 5 pyridyldithiols per IgG molecule. After overnight reaction the vesicles were separated from unbound antibody by flotation in metrizamide (3) and analyzed for lipid (18), protein (3), and drug content. Drug concentration was determined after extraction of a vesicle sample by the method of Bligh and Dyer (19). The absorbance of the methanolic aqueous phase was measured at 370 nm, and the molar extinction coefficient of the drug was assumed to be 7,943. Sterility was maintained throughout the entire conjugation and separation procedure. For interaction studies, targeted vesicles were prepared by the method of Heath *et al.* (3). The properties of the products are given in the legends to the figures.

Cells were obtained and grown as follows. L929 murine fibroblasts were provided by L. B. Epstein (University of California, San Francisco) and grown in DME medium with 5% fetal calf serum, penicillin, and streptomycin. R1.1, a T lymphoma from the C58 mouse (20, 21), was provided by M. Cohn (Salk Institute) and was grown in DME medium with 10% newborn calf serum and antibiotics. BALB/c 3T6 fibroblasts were obtained from W. Eckhardt (Salk Institute) and grown in DME medium with 10% fetal calf serum.

For interaction studies, 0.2 ml of liposomes (1–100 nmol of lipid) was incubated for 1 hr at 37°C with 5×10^6 L929 or BALB/c 3T6 cells as a confluent monolayer in 6-cm culture dishes or with 2×10^6 R1.1 cells in suspension. The cells were washed four times and taken up in Liquiscint scintillant (National Diagnostics) for measurement of the amount of [^{3}H]dipalmitoylphosphatidylcholine-labeled vesicles associated with the cells.

For growth inhibition studies, 3×10^4 cells (L929 or BALB/c 3T6) were plated per well in 1 ml of fresh medium in 24-well plates (Costar). After incubation for 12 hr, the cells were treated as indicated in Fig. 2 and incubated a further 48 hr in serum-containing medium. For L929 fibroblasts, the medium was aspirated and the cells were resuspended in 1 ml of phosphate-buffered saline with 1 mM EDTA before counting. BALB/c 3T6 cells were trypsinized, resuspended, and counted with a Coulter Counter (model Fn).

RESULTS

Cell Association Studies. Nonspecific empty liposomes showed a low level of association that was not affected by azide and 2-deoxy-*D*-glucose (Table 1). Liposomes coated with monoclonal anti-H2K^k showed a 6-fold higher interaction; this interaction was decreased by 55% in the presence of azide and 2-deoxy-*D*-glucose. The energy dependence of the targeted interaction suggests that either targeted vesicles are internalized or optimal interaction requires an energy-dependent redistribution of the H2K^k antigen. The difference between targeted and nonspecific or uncoated liposome interaction was increased by the inclusion of serum. In the presence of 50% newborn calf serum, interaction of targeted vesicles with the cells was 20-fold greater than interaction of liposomes coated with anti-sheep erythrocyte antibody. This increase in the difference between targeted and nonspecific liposome interaction was largely due to a decrease in the interaction of nonspecific liposomes. The specificity of targeted liposomes is confirmed by the low level of interaction of both vesicle preparations with BALB/c 3T6 fibroblasts. This cell line does not react with either anti-H2K^k or anti-sheep erythrocyte antibodies. The two liposome preparations showed a low level of interaction comparable to the interaction of the nonspecific liposomes with L929 fibroblasts.

Table 1. Interaction of antibody-targeted liposomes with L929 and BALB/c 3T6 fibroblasts

Cell	Serum*	Inhibitors†	% bound		
			Uncoated	Nonspecific	Targeted
L929	−	−	2.7	2.1	12.4
L929	+	−	1.2	0.6	10.6
L929	−	+	—	1.8	5.6
BALB/c 3T6	+	−	0.4	0.5	0.4

Liposomes were prepared from phosphatidylcholine/cholesterol/gangliosides (45:45:10) and conjugated to anti-sheep erythrocyte antibody (nonspecific) or anti-H2K^k antibody (targeted) by the method of Heath *et al.* (3). The final antibody/lipid ratio was 80 g/mol, and the vesicles contained 2,000 cpm of [^{3}H]dipalmitoylphosphatidylcholine per nmol of lipid.

* Cells were incubated without or with 50% (vol/vol) newborn calf serum.

† Cells were incubated without or with 5 mM sodium azide/50 mM 2-deoxy-D-glucose.

Neither anti-H2K^k nor anti-sheep erythrocyte antibody affected the uptake of nonspecific liposomes (Fig. 1). In addition, soluble anti-sheep erythrocyte antibody had no effect on the association of targeted liposomes with the cells. Soluble anti-H2K^k antibody inhibited the interaction of targeted liposomes with both cell types. The inhibition was greatest for the interaction of targeted liposomes with R1.1, for which addition of 30 μg of antibody inhibited liposome–cell association by 75%. Inhibition of interaction (approximately 30% inhibition) with L929 was seen only when 30 μg of soluble anti-H2K^k was added. The 20 nmol

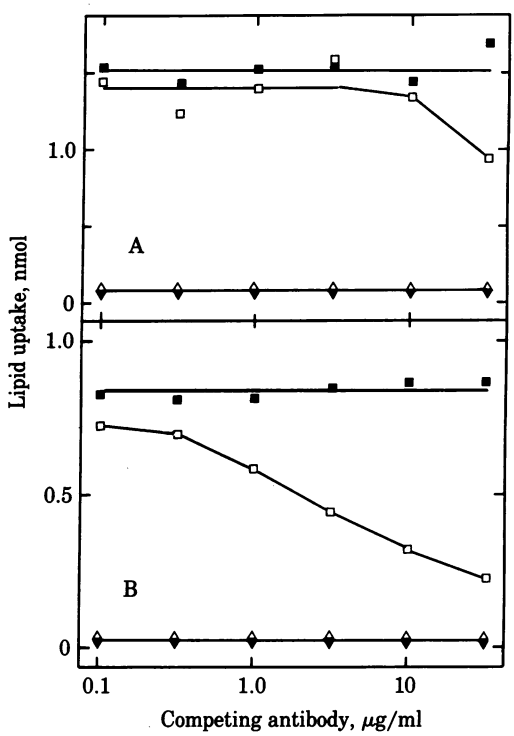

FIG. 1. Effect of soluble antibody on the interaction of targeted vesicles with cells. ■, Anti-H2K^k targeted vesicles inhibited with anti-sheep erythrocyte antibody; □, anti-H2K^k targeted vesicles inhibited with anti-H2K^k antibody; ▼, anti-sheep erythrocyte targeted vesicles inhibited with anti-sheep erythrocyte antibody; ▽, anti-sheep erythrocyte targeted vesicles inhibited with anti-H2K^k antibody. The vesicle preparations were as in Table 1. To 20 nmol of lipid (with 1.6 μg of attached antibody) was added soluble antibody and the mixture was incubated with 5×10^6 L929 fibroblasts (*A*) or 2×10^6 R1.1 T-lymphoma cells (*B*) in phosphate-buffered saline containing 50% newborn calf serum (1 hr; 37°C). The cells then were washed and assayed.

of lipid used for these experiments had 1.6 μg of antibody attached. Hence, a 19-fold excess of soluble antibody was not sufficient to eliminate targeted interaction.

Effects on Cell Growth. Our early studies on growth inhibition of L929 were done with targeted liposomes containing methotrexate (22). In these studies, a comparison of the growth curves indicated that the IC_{50} for free methotrexate was 0.018 μM whereas that of targeted liposomes was 0.05 μM and that of uncoated liposomes was 1.5 μM. In this case, therefore, methotrexate in targeted liposomes was 25 times more effective than methotrexate in uncoated liposomes but only 1/4 as effective as free methotrexate. This result agrees qualitatively with the findings of Leserman *et al.* (11). We have examined the efficacy of targeted and uncoated liposome preparations with various drug/lipid ratios. The experiment discussed above (drug/lipid ratio, 0.78 mmol/mol) gave the greatest difference between targeted and nontargeted vesicles.

Fig. 2*A* shows the growth inhibition of L929 fibroblasts by various preparations containing methotrexate-γ-aspartate. The free drug had an IC_{50} of 0.68 μM. Targeted liposomes were 10.3 times more effective than the free drug (IC_{50} = 0.066 μM). In contrast, nonspecific liposomes (bearing anti-Thy 1.1 antibody) were 60% as effective as the free drug (IC_{50} = 1.2 μM). Consequently, targeted liposomes were 18 times more effective than nonspecific liposomes.

Fig. 2*B* shows that growth inhibition of BALB/c 3T6 fibroblasts by free methotrexate-γ-aspartate, targeted liposomes, and nonspecific liposomes. The cells showed comparable sensitivity to L929 for the free drug (IC_{50} = 0.58 μM). Both vesicle preparations were less effective than free drug and had similar IC_{50} values (2.0 μM for nonspecific and 2.5 μM for targeted). This demonstrates that the superior efficacy of targeted liposomes on L929 cells is due to the specificity of the anti-$H2K^k$ antibody.

Fig. 2*C* shows the inhibition of L929 growth by free methotrexate-γ-aspartate, targeted empty liposomes, and mixtures of the two containing drug and lipid in the same ratio as in the encapsulated preparations shown in Fig. 2 *A* and *B*. Targeted empty liposomes showed no inhibitory effects in the range of concentrations used. In addition, targeted empty liposomes did not increase the inhibitory effects of the free drug. This confirms that the growth inhibitory effects of targeted liposomes are due to the drug and demonstrate a requirement for encap-

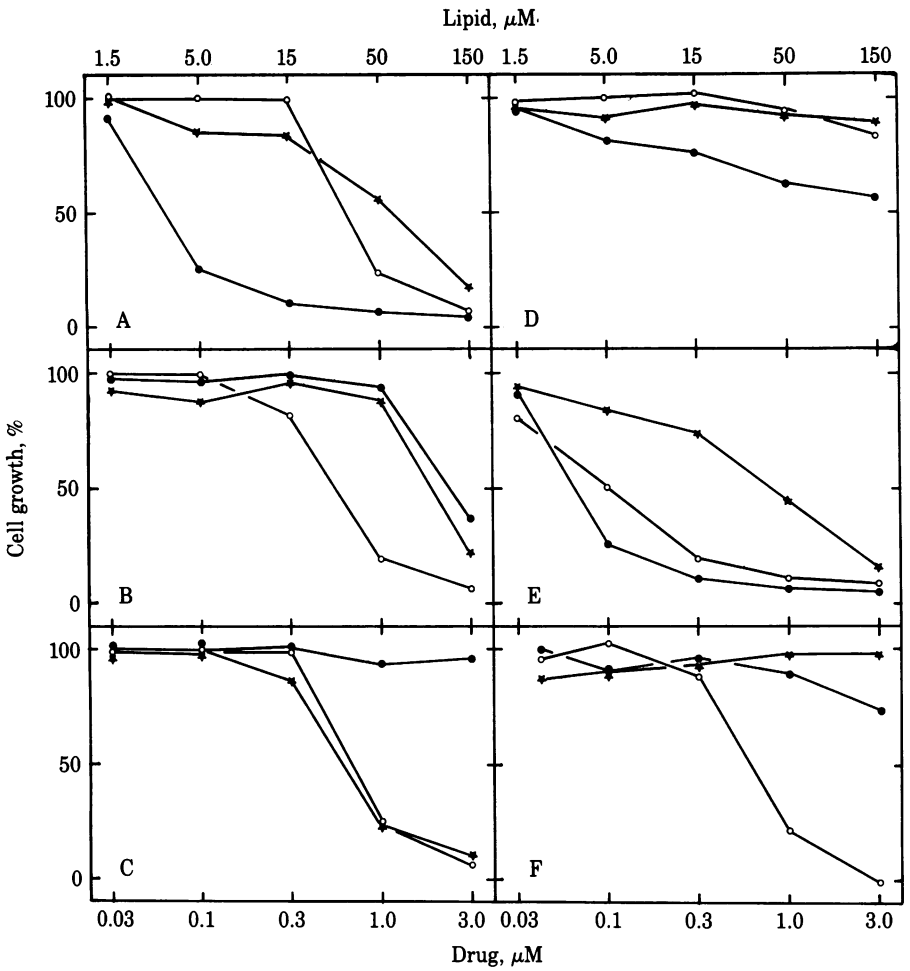

FIG. 2. Growth inhibition by methotrexate-γ-aspartate in targeted vesicles. Fibroblasts were treated with methotrexate-γ-aspartate in various preparations at the concentrations shown. The lipid concentration, where applicable, is also shown. Vesicles were prepared from a 10:10:1 mixture of phosphatidylcholine/cholesterol/4-(*p*-maleimidophenyl)butyrylphosphatidylethanolamine and conjugated with thiolated antibody. Targeted liposomes contained 50 g of anti-$H2K^k$ per mol of phospholipid; nonspecific liposomes contained 62 g of anti-Thy 1.1 per mol of phospholipid; targeted empty liposomes contained 112 g of anti-$H2K^k$ per mol of phospholipid; nonspecific empty liposomes contained 156 g of anti-sheep erythrocyte per mol of phospholipid. (*A*) L929 fibroblasts treated with targeted liposomes (●); free methotrexate-γ-aspartate (○), and nonspecific liposomes (★). (*B*) BALB/c 3T6 fibroblasts treated as in *A*. (*C*) L929 fibroblasts treated with targeted empty liposomes (●), free methotrexate-γ-aspartate (○), and free methotrexate-γ-aspartate with targeted empty liposomes (★). (*D*) As in *A* with 58 μM 5-formyl-5,6,7,8,-tetrahydrofolic acid added 30 min before treatment. (*E*) L929 cells incubated with targeted liposomes (●), targeted liposomes with 0.1 μmol of nonspecific empty liposomes per well added 30 min before treatment (○), and targeted liposomes with 0.1 μmol of targeted empty liposomes per well added 30 min before treatment (★). (*F*) As in *A* with 5 mM NH_4Cl added 30 min before treatment.

sulation of the drug. In the same experiment with uncoated empty liposomes and nonspecific empty liposomes, the same result was obtained (not shown).

Fig. 2*D* shows the effect of 58 μM 5-formyltetrahydrofolate on growth inhibition of L929 by the various drug preparations. Free drug and both encapsulated preparations showed a substantial reduction in their effects on cell growth. This confirms that the inhibition of cell growth by targeted liposomes was due to an inhibition of 5-formyltetrahydrofolate production, presumably as a result of dihydrofolate reductase inhibition.

Fig. 2*E* shows the effects of various preparations on the growth inhibition of L929 cells by targeted liposomes. Soluble anti-H2K^k antibody did not inhibit the effects of these targeted vesicles (not shown). This is consistent with the data from Fig. 1 which shows only a minimal inhibition of L929 cell binding of targeted vesicles by the soluble antibody. Nonspecific empty liposomes inhibited targeted vesicle efficacy by 50%. However, inhibition of the effect to 10% was produced by preincubation with an excess of targeted empty liposomes. This further confirms the role of the anti-H2K^k antibody in vesicle delivery and also suggests that the liposomes bearing the antibody may bind more effectively to the cells than the soluble antibody itself.

Fig. 2*F* shows the effect of ammonium chloride on the inhibition of L929 cell growth by free and encapsulated methotrexate-γ-aspartate. Ammonium chloride did not decrease the effects of the free drug but completely blocked the growth inhibitory effects of both encapsulated preparations. This suggests that the inhibitory effects of encapsulated methotrexate-γ-aspartate involve the endocytosis of the liposomes and may be critically dependent on the low pH of the post-endocytic compartment.

The experiments shown in Fig. 2 were performed over a 1-month period with the same four liposome preparations. Targeted vesicles were tested several times up to 1 month after preparation and showed little change in the efficacy. It therefore appears that, if stored at 4°C, the vesicles do not leak appreciable amounts of drug and do retain their covalently bound antibody. These experiments have now been performed with four different batches of anti-H2K^k targeted vesicles (loaded with methotrexate-γ-aspartate), and all gave similar results.

DISCUSSION

The results presented here demonstrate the ability of liposomes targeted with anti-H2K^k antibody to bind and deliver encapsulated methotrexate-γ-aspartate to L929 fibroblasts. Specificity is confirmed by the poor efficacy of nonspecific liposomes for L929 cells and by the poor efficacy of both liposome preparations against BALB/c 3T6 cells. The effect is not due to growth inhibitory effects of liposomal lipid or antibody because targeted empty vesicles have no effect in the range of concentrations used. Encapsulation of the drug is essential because a mixture of free drug with targeted empty vesicles is no more effective than the free drug. The effects of targeted liposomes are due to folate antagonism because they are substantially inhibited by 5-formyltetrahydrofolate.

Soluble anti-H2K^k antibody has very little effect on the association of targeted empty liposomes with L929 fibroblasts. In addition, prior incubation of cells with 30 μg of soluble anti-H2K^k antibody has no effect on the growth-inhibitory effects of targeted liposomes. In contrast, prior incubation with targeted empty liposomes decreases the effects of targeted liposomes to 10%. These results suggest that the liposomes bind effectively to L929 fibroblasts and are internalized in the presence of a 10- to 30-fold excess of the antibody from which they are derived. This effect could be important if liposome binding were similarly resistant to inhibition by monovalent antigen which has been shed by tumor cells because the efficacy of targeted vesicles may be substantially unaffected by high levels of circulating antigen. It will be important in future experiments to examine whether soluble target antigens can inhibit liposome–cell interactions.

The mechanism of liposome-mediated increase of methotrexate-γ-aspartate efficacy is important. It is clear that liposomes must undergo endocytosis to achieve delivery of the drug because the delivery is inhibited by ammonium chloride. Ammonium chloride is known to increase lysosomal pH, which implies a lysosomal involvement and rules out delivery by fusion of the liposomes with the plasma membrane. The mechanism of drug transfer from the lysosomes to the cytoplasm is unknown but may involve one or more of three different processes. First, the lysosomal membrane may contain a specific folate transport system that is permissive for methotrexate-γ-aspartate. Second, the lysosomes may contain degradative enzymes that convert the γ-aspartate to free methotrexate. Methotrexate could then be transported into the cell by a folate transport protein. Third, the low pH of the lysosomal compartment may accelerate the diffusion of methotrexate-γ-aspartate through the lipid bilayer of the lysosomal membrane. DeDuve *et al.* (23) have suggested this mechanism for delivery of weakly acidic molecules to the cytoplasm via the liposomes. Moreover, Leserman *et al.* (11) have suggested that methotrexate in liposomes reaches the cytoplasm in this way.

All of these potential mechanisms are feasible in view of the inhibition of drug delivery by ammonium chloride. If lysosomal membranes contain a transport system for the γ-aspartate, ammonium chloride might block drug delivery either by inhibiting the lysosomal breakdown of liposomes or possibly by blocking drug transport. If methotrexate-γ-aspartate is converted to methotrexate by an amidase or protease, the enzyme may be inhibited either by the increase of lysosomal pH or by direct ammonium chloride inhibition. If methotrexate-γ-aspartate escapes from the lysosomes because it is a weak acid, increase of lysosomal pH would block its escape.

It is interesting to speculate on the number of drug-laden liposomes required to inhibit cell growth. The IC_{50} of methotrexate is approximately 10 nM for most cells (12). We therefore may assume that an intracellular methotrexate concentration of 10 nM is required for growth inhibition. If the cells are regarded as spheres 10 μm in diameter and the liposomes are 0.1 μm in diameter, then the cell volume is 10^6 times bigger than the liposome volume. If a single 0.1-μm liposome were required to deliver an effective dose to a cell, it would have to contain antifolate at a concentration of 10 mM in order to produce 10 nM antifolate on dilution in the cell. We encapsulated methotrexate-γ-aspartate at 5 mM in liposomes whose mean diameter was 0.4 μm. In consequence, these liposomes contained approximately 15 times the minimal effective dose of antifolate. It therefore, may only be necessary for one liposome or, at most, several liposomes to deliver their contents for effective inhibition of cell growth. In our experiments, substantial inhibition of cell growth occurred when 50,000 vesicles (5 nmol) were added per cell. Hence, the frequency of vesicle uptake may be low.

Various methods have been developed for producing antibody-directed cytotoxic agents (1, 2). Although all of these approaches doubtless will prove to be useful, it is appropriate to consider here some of the advantages of targeted liposomes. Our competition studies show that targeted liposomes associate with and deliver their contents to cells even when a 19-fold excess of soluble antibody is included in the incubation mixture. In contrast, toxin or drug conjugates that contain only a single antibody molecule would not compete with free antibody in this way. Liposomes might also be conjugated to a mixture of an-

tibodies that have specificities for several different membrane antigens. This would enable the vesicles to interact with cells bearing appropriate combinations of antigens rather than a single specificity. A monovalent antibody conjugate again could not give such an effect. Molecules encapsulated in liposomes are protected from agents that may inactivate or destroy them. This is particularly useful for molecules such as DNA or RNA. In addition, the number of drug molecules per liposome can be large and may be varied over a wide range of concentrations. (We calculate that one unilamellar vesicle 0.4 μm in diameter made in the presence of 5 mM drug will encapsulate approximately 1×10^5 drug molecules per liposome.) Such variation is often not possible for direct antibody–drug conjugates.

In conclusion, antibody-targeted liposomes possess several significant advantages compared with the soluble antibodies directly conjugated with drugs or toxins. The work presented in this paper describes the design of antibody-targeted liposomes which are more toxic than the compound they encapsulate, a significant advance in the production of specific cytotoxic agents.

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