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In Vitro Uptake and Therapeutic Application of Liposome-encapsulated Methotrexate in Mouse Hepatoma 129*

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Abstract—*The biological activities of liposome-encapsulated and non-encapsulated methotrexate (MTX) were compared* in vitro *and* in vivo *using mouse Hepatoma* 129 *ascites tumor cells. Under* in vitro *conditions, cells accumulated up to* 29% *of* [^{3}H]-*MTX when the drug was incorporated in the lipid bilayers of positively charged, unilamellar liposomes. There was no significant uptake of non-encapsulated MTX under the same conditions. A single intraperitoneal injection of liposome-encapsulated MTX* (3 mg *MTX*/kg) *increased the mean survival time of tumor-bearing mice to* 42.5 ± 11.2 days, *compared to* 23.5 ± 2.2 days *for untreated controls. Non-encapsulated MTX had no significant effect on survival time. Thus the* in vivo *treatment studies appear to agree with the* in vitro *uptake measurements. Addition of galactolipids to the lipid bilayers of liposomes did not increase* in vitro *uptake of encapsulated MTX and gave no additional improvement in therapeutic effectiveness. Encapsulation of MTX in liposomes might thus be used to increase uptake of the drug in cells which may be deficient in MTX transport.*

INTRODUCTION

PRIMARY liver cancer is relatively rare in the United States and Europe, but higher incidences of this cancer occur in parts of Africa and Asia [1]. Most untreated patients die within six months of first diagnosis [2, 3]. Chemotherapy of liver cancer has been relatively ineffective.

In this study, liposomes have been tested as carriers to induce uptake of anti-tumor drug and to cause killing of mouse Hepatoma 129 ascites tumor cells. The Hepatoma 129 cells are used as a model tumor for liver cancer. Methotrexate (MTX) was chosen as the test drug because it is poorly transported by some mouse tumor lines [4], although it is used in treatment of several human malignancies [4–6].

Liposomes have been used as biological carriers for a variety of substances such as enzymes [7], chelators [8], viral nucleic acids [9] and drugs, including anti-tumor agents [10]. Liposome-encapsulated drugs are incorporated more effectively and are retained longer than the corresponding non-encapsulated drugs in a number of organs, particularly the liver [11–13]. Liposomes have also been used to overcome the impermeability of cell membranes to chelating agents [8, 11] and to actinomycin D [14].

Another potential use of liposome-encapsulated drugs is targeting of liposomes with specific surface characteristics to particular tissues. Earlier work done in this laboratory has indicated that drugs encapsulated in different types of liposomes can be directed to different specific organs [12, 13, 15]. In particular, elevated uptake of EDTA encapsulated in liposomes containing galactocerebroside has been observed in liver [15]. For this reason, the possible specific uptake by Hepatoma 129 cells of liposomes made with galactolipids has been studied under *in vitro* and *in vivo* conditions.

The results reported in this study give evidence for *in vitro* and *in vivo* incorporation by Hepatoma 129 cells of liposome-encapsulated MTX but not of the non-encapsulated form of

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the drug. The mechanism of uptake of liposome-encapsulated MTX has also been explored.

MATERIALS AND METHODS

Chemicals

L-α-Dipalmitoyl phosphatidylcholine (synthetic; purity 99%+), trypsin (hog pancreas, Type IX), trypsin inhibitor (soybean, Type I), and cholesterol (purity 99%+) were obtained from Sigma Chemical Co., St. Louis, MO. Monogalactosyl diglyceride (plant) and galactocerebroside (bovine) (both chromatographically pure) were purchased from Supelco, Inc., Bellfonte, PA, and used without further purification. Stearylamine was purchased from K and K Laboratories, Inc., Plainview, NY, cytochalasin B from Calbiochem, San Diego, CA, and methotrexate from Lederle Laboratories, Pearl River, NY. The sodium salt of 3′,5′,9(n)-[^{3}H]-MTX (10 Ci/mmol) was obtained from Amersham/Searle Corp., Arlington Heights, IL. Sephadex G-50-Fine was purchased from Pharmacia Chemicals, Uppsala, Sweden. RPMI 1640 culture medium and fetal calf serum were obtained from Grand Island Biological Co., Grand Island, NY.

Preparation of liposomes

Positively charged, multilamellar liposomes (ML) containing [^{3}H]-labeled MTX were prepared using methods described by Rahman *et al.* [11, 12] and by Kimelberg *et al.* [16]. The amounts of lipids used in the different liposome preparations are shown in Table 1. MTX was encapsulated either in the lipid or in the aqueous compartment of the liposomes. The aqueous-phase liposomes (APL) were prepared by including MTX at a concentration of 55 mM in Kimelberg's histidine buffer [16]. All ML liposomes were prepared at 50–55°C. For the lipid-phase liposomes (LPL), MTX was dissolved in methanol, evaporated to dryness to remove traces of water, redissolved in methanol, mixed with chloroform solutions of lipids and dried as previously described [11, 12]. The molar ratio of dipalmitoyl phosphatidylcholine to MTX in the LPL was 1:1.6. The aqueous phase of the LPL was histidine buffer [16]. Unincorporated MTX was removed from ML preparations by three successive centrifugations (1000 *g*, 15 min) in 0.9% NaCl, The ML was passed through a 0.6-μm Nuclepore filter before use.

Unilamellar liposomes (UL) were prepared by sonication of unwashed ML for 30 min in a bath sonicator (Model G11 2SPIG, Laboratory Supplies Co., Hicksville, NY). Non-encapsulated MTX was removed from UL by the method of Fry *et al.* [17]. Both the APL and the LPL liposomes could be sonicated to produce UL.

Stability of liposomes containing MTX was investigated by suspension of liposomes in either 0.9% NaCl or RPMI 1640 medium containing 20% fetal calf serum, followed by immediate dialysis against a standard volume of the same solution. Aliquots of the dialysis solution were removed at various times and the percentage leakage of the labeled drug was determined using a Beckman LS-233 liquid scintillation counter.

Maintenance of Hepatoma 129 ascites tumor

An ascites form of Hepatoma 129 was obtained from DCT Tumor Bank, Worcester,

Table 1. Liposomal lipid composition and incorporation of [^{3}H]-MTX in unilamellar liposomes

Liposomal lipid composition*	Location of encapsulated MTX*	Approximate molar ratio of lipids	Incorporation of [^{3}H]-MTX‡ (%)	Concentration of [^{3}H]-MTX in *in vitro* assay mixture (mg/ml)§
DpCP/chol/StAm†	LPL	34:23:10	5.53 ± 0.48	0.12 ± 0.01
DpPC/chol/StAm	APL	34:23:10	7.48 ± 1.39	0.15 ± 0.03
DpPC/chol/StAm/MGDG	LPL	34:23:10:17	5.01 ± 1.92	0.08 ± 0.02
DpPC/chol/StAm/gal-cer	LPL	34:23:10:18	6.25 ± 0.36	0.12 ± 0.02
DpPC/chol/StAm/gal-cer	APL	34:23:10:18	9.45 ± 1.52	0.18 ± 0.03

*Abbreviations: DpPC, dipalmitoyl phosphatidylcholine; chol, cholesterol; StAm, stearylamine; MGDG, monogalactosyl diglyceride; gal-cer, galactocerebroside; MTX, methotrexate; LPL, lipid-phase liposomes; APL, aqueous-phase liposomes.

†All liposomes positively charged.

‡Measurements of incorporation of MTX into each type of liposome were made from four separate liposome preparations used in four independent *in vitro* experiments. Each value is the mean ± S.E. for percentage incorporation.

§Concentrations calculated from incorporations reported in adjacent column.

MA. The tumor was maintained in C3H mice by weekly transfers of 0.3–0.4 ml of undiluted ascites fluid into healthy mice by intraperitoneal injection.

In vitro *studies*

Hepatoma 129 ascites cells were removed from mice between 6 and 9 days after their initial transfer. Cells taken from different mice were incubated in separate flasks (Fig. 1 legend). Cells were washed twice in 5–7 ml RPMI 1640 medium and filtered through two layers of cotton gauze. Viability of cells was determined with trypan blue stain; 92–98% viability was consistently observed with the washed cells.

Freshly washed cells and UL containing MTX were incubated on a rocking platform at 37°C in a 5% CO_2 atmosphere. The concentrations of liposome-encapsulated MTX in the assay mixtures are indicated in Table 1. The concentration of non-encapsulated MTX used in the assay ranged from 0.13 to 0.32 mg/ml; [^{3}H]-MTX activity ranged from 6×10^4 to 7.5×10^4 counts/min/ml. The final reaction mixture contained 2×10^7 viable cells/ml and liposomes (5–7 mg lipid/ml) suspended in RPMI 1640 medium. All *in vitro* studies were done with UL because the hepatoma cells tended to aggregate in the presence of ML.

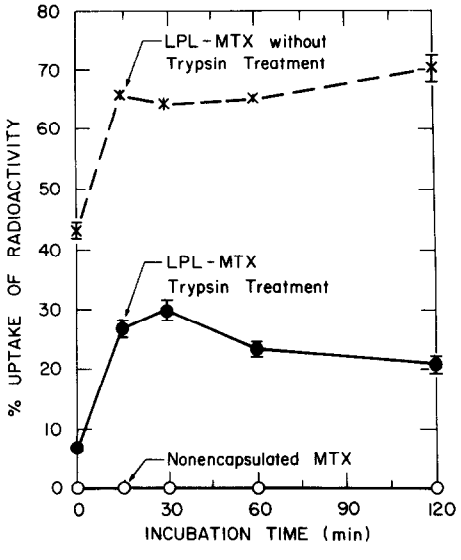

Fig. 1. In vitro association of free and liposome-encapsulated [^{3}H]-MTX with mouse Hepatoma 129 cells. LPL-MTX without trypsin treatment (×---×); LPL-MTX with trypsin treatment (●——●); non-encapsulated MTX (○——○). Duplicate incubation flasks were prepared using Hepatoma 129 cells from two different mice. Each point represents the mean of four values: duplicate samples of 4×10^6 cells from each flask. Vertical bars represent two standard errors of the mean. Figs 1–4 illustrate representative data from three or four independent experiments.

Duplicate 0.2 ml samples containing 4×10^6 cells were withdrawn at intervals up to 120 min and gently layered on top of 3 ml chilled 10% sucrose in 0.9% NaCl. Cells were separated from free liposomes by centrifugation at 410 **g** for 5 min. The liposomes remained suspended on the top of the denser sucrose solution. The cell pellet was usually re-suspended in 2 ml RPMI 1640 medium containing 5 μg trypsin/ml. After 10 min of trypsinization at 37°C, which removed non-specifically adsorbed liposomes [18], 8 μg trypsin inhibitor in 0.2 ml RPMI 1640 medium was added and the trypsinized cells were again centrifuged (410 **g**, 5 min). The cell pellet was washed in 2 ml chilled sucrose–saline and transferred to scintillation vials in a final volume of 0.7 ml water for determination of radioactivity by liquid scintillation counting. The percentage uptake of liposome-encapsulated MTX was calculated by comparing radioactivity in the samples with that in 0.2 ml of the corresponding initial reaction mixture.

Study of mechanisms of in vitro *uptake of liposomes*

In addition to assays performed at 4°C, the effect of the microfilament disruptor, cytochalasin B, on *in vitro* uptake of liposomes was investigated by preincubating cells with cytochalasin B at a concentration of 0.25 mg per 10^7 cells for 30 min at 37°C. Liposomes were added to the cells after the preincubation, and the usual assay was carried out in the presence of cytochalasin B. In both studies, cell viability, as measured by trypan blue exclusion, was not affected by cytochalasin B.

In vivo *treatment of mice bearing Hepatoma* 129 *ascites tumor*

Hepatoma 129 cells used in each comparative treatment study were all taken from a single C3H mouse. Seven-day-old tumor cells were filtered through two layers of cotton gauze, counted and diluted in 0.9% NaCl. Three to four-month-old C3H mice were each injected intraperitoneally with 10^6 viable cells in 0.2 ml. These mice were then given MTX in non-encapsulated or liposome-encapsulated forms at various doses and at different intervals after the inoculation of tumor cells. Both intraperitoneal and intravenous treatments were tested. The mean survival times of mice were calculated according to Geran *et al.* [19]. Statistical analyses of significances of differences in mean survival times were made by Duncan's modified paired test, using the Statistical Analysis System program [20, 21].

RESULTS

Characterization of liposomes

The incorporation of [^{3}H]-MTX into UL of various compositions is indicated in Table 1. A slightly higher incorporation of MTX was generally seen in APL (7.5–9.4%) than in LPL (5.0–6.4%). The size of the UL liposomes was measured from electron micrographs of negatively stained liposome preparations. The diameters ranged from 0.05 to 0.14 μm, with an average of 0.08 ± 0.02 μm.

Measurements of leakage of encapsulated [^{3}H]-MTX from liposomes showed that both APL and LPL lacking glycolipids lost less than 5% of the encapsulated MTX within 6 hr when dialyzed against 0.9% NaCl. When these liposomes were dialyzed against RPMI 1640 medium with 20% fetal calf serum, leakage of encapsulated MTX reached 11% in 6 hr. Addition of glycolipids to the liposomal membranes increased leakage of MTX from LPL, but leakage was still less than 10% in 4 hr when dialyzed against 0.9% NaCl.

In vitro *uptake of free and liposome-encapsulated MTX by Hepatoma* 129 *cells*

The conditions used for the *in vitro* assay did not significantly affect viability of the hepatoma cells. Before incubation, the cells were $94.4 \pm 0.5\%$ viable; after 3 hr of incubation with non-encapsulated MTX or with MTX in LPL, cell viabilities were $95.6 \pm 0.2\%$ and $93.7 \pm 0.3\%$ respectively.

The molar amounts of MTX used in the different incubation mixtures varied somewhat because of variations in incorporation of drug into the liposomes. The concentrations of liposome-encapsulated MTX used ranged from 0.04 to 0.09 mg MTX per 10^7 viable cells. The concentration of non-encapsulated MTX ranged from 0.065 to 0.16 mg MTX per 10^7 viable cells (amounts calculated from Table 1). To allow comparisons between these assays, data for the *in vitro* uptake studies (Figs 1–4) are presented as percentage uptake of [^{3}H]-MTX radioactivity.

Figure 1 shows the *in vitro* association of non-encapsulated and liposome-encapsulated MTX with hepatoma cells. Only background levels of the non-encapsulated MTX were associated with cells during the 2-hr incubation period. Addition of positively charged 'empty' liposomes which contained no MTX had no effect on the amount of non-encapsulated MTX associated with cells. However, when MTX encapsulated in LPL was incubated with cells, up to 66% of the liposome-encapsulated MTX became associated with cells within 15 min.

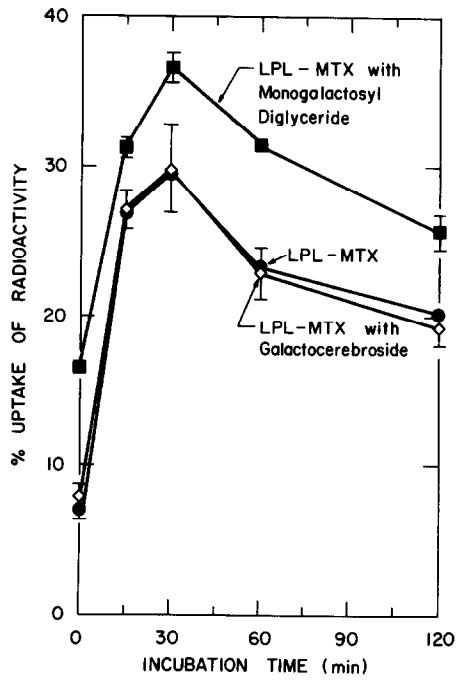

Fig. 2. Effect of inclusion of galactolipids in LPL-MTX liposomes on in vitro *uptake by mouse Hepatoma* 129 *cells. LPL-MTX with monogalactosyl diglyceride (■——■); LPL-MTX without galactolipid (●——●); LPL-MTX with galactocerebroside (◇——◇). See legend of Fig.* 1 *for description of points and error bars. Trypsin treatment used.*

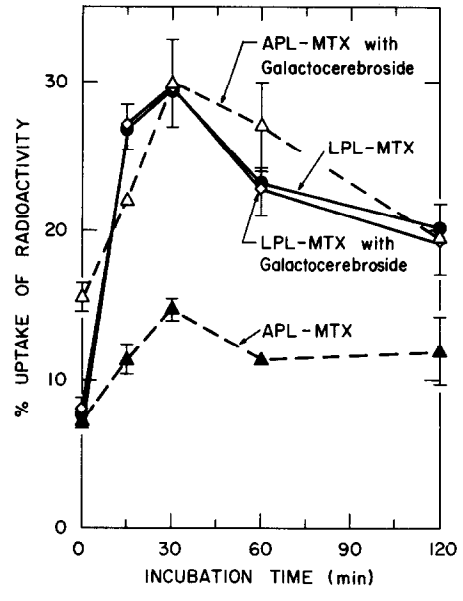

Fig. 3. Effect of location of MTX in liposomes on in vitro *uptake by mouse Hepatoma* 129 *cells. APL-MTX with galactocerebroside (△---△), APL-MTX without glycolipid (▲---▲), LPL-MTX with galactocerebroside (◇——◇), LPL-MTX without glycolipid (●——●). See legend of Fig.* 1 *for description of points and error bars. Trypsin treatment used.*

Treatment of the cells with trypsin after incubation to remove surface-adsorbed liposomes reduced the amount of MTX in LPL associated with cells from 66 to 29% at 30 min incubation (Fig. 1). The initial 7–10% of liposome-encap-

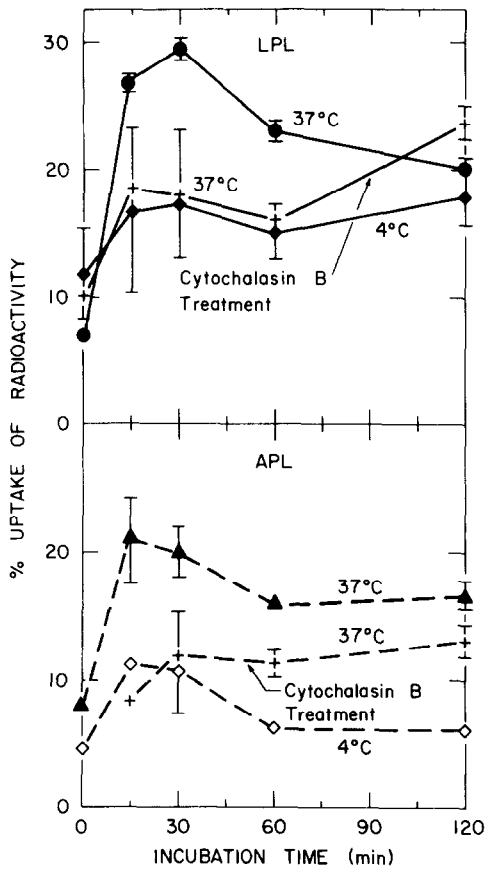

Fig. 4. Effect of temperature and cytochalasin B on in vitro *uptake of liposomes by mouse Hepatoma* 129 *cells. LPL-MTX,* 37°C *incubation* (●——●); *LPL-MTX, cytochalasin B preincubation,* 37°C *incubation* (+——+); *LPL-MTX,* 4°C *incubation* (◆——◆); *APL-MTX* 37°C *incubation* (▲---▲); *APL-MTX, cytochalasin B preincubation,* 37°C *incubation* (+---+); *APL-MTX,* 4°C *incubation* (◇---◇). *See legend of Fig.* 1 *for description of points and error bars. Trypsin treatment used.*

sulated MTX remaining after trypsin treatment (Fig. 1) was consistently observed and could not be removed, even by using trypsin concentrations up to five times higher than those of Pagono and Takeichi [18]. There was no decrease in cell viabilities at the end of the assay when the higher trypsin concentrations were tested. All subsequent results reported for *in vitro* MTX uptake studies are of measurements made after trypsinization of the cells.

Effect of galactolipids on in vitro *uptake by Hepatoma* 129 *cells of liposome-encapsulated MTX*

Inclusion of a plant galactolipid, monogalactosyl diglyceride, in the membranes of LPL increased the maximum MTX uptake to 36% in 30 min, compared with 29% uptake in 30 min for the corresponding liposomes without glycolipid (Fig. 2). However, when a common galactolipid of animal membranes, galactocerebroside, was tested in the LPL membranes, the uptake of MTX was virtually identical with that observed for the liposomes without glycolipid (Fig. 2).

Effect of site of encapsulation of MTX in liposomes on in vitro *uptake of MTX by Hepatoma* 129 *cells*

The site of encapsulation of the MTX in the aqueous or lipid phases of the liposomes affected the *in vitro* uptake of the drug by the hepatoma cells. The maximum uptake of MTX from APL was only 15% in 30 min (Fig. 3). Addition of galactocerebroside to the APL increased the *in vitro* MTX uptake to 30% in 30 min, essentially the same as that observed for MTX in LPL with or without galactocerebroside (Fig. 3).

Mechanism of liposome uptake by Hepatoma 129 *cells* in vitro

Figure 4(A) shows the effect of temperature and cytochalasin B on uptake of MTX in LPL. At 37°C, the maximum uptake of MTX by hepatoma cells was 29% at 30 min incubation. Decreasing the temperature to 4°C reduced MTX uptake to 17% in the same time period. Thirty-min preincubation of the cells with cytochalasin B followed by incubation of cells and liposomes at 37°C also reduced the uptake of MTX in LPL (19% in 15 min), but only at the earlier time points.

Figure 4(B) shows uptake of MTX from APL under the corresponding conditions. In this experiment, the maximum uptake of MTX at 37°C was 21% at 15 min incubation. At 4°C, the maximum uptake was reduced to 11% in 15 min. Preincubation of cells with cytochalasin B also reduced MTX uptake (12% in 30 min) but, as with LPL, the decreased uptake was more evident at earlier time points.

Treatment of mouse Hepatoma 129 *ascites tumor* in vivo

To correlate the *in vitro* observations with an *in vivo* system, we tested *in vivo* treatment of mouse Hepatoma 129 ascites tumor with non-encapsulated or liposome-encapsulated MTX. Untreated C3H mice with a tumor burden of 10^6 viable hepatoma cells had a mean survival time of 21.5 ± 1.3 days after injection of tumor cells (Table 2). Treatment of tumor-bearing mice with 2 mg non-encapsulated MTX/kg by either intravenous or intraperitoneal injection on day 1 after injection of hepatoma cells had no therapeutic effect (Table 2). Intravenous treatment of tumor-bearing mice with 2 mg MTX/kg encapsulated in ML-LPL also failed to improve the mean survival time over that of the untreated mice (Table 2). However, in-

Table 2. Effect of non-encapsulated and liposome-encapsulated MTX on survival time of mice bearing Hepatoma 129 ascites tumor

Route of administration	Treatment* type of liposome	Dose per injection (mg/kg)	Dose schedule	Mean survival time† (days)	Range of survival time (days)
Untreated	—	—	—	21.5 ± 1.3	20–23
i.p.	Non-encap	2	single injection, day 1	22.4 ± 0.7	21–23
i.v.	Non-encap	2	day 1	21.0 ± 0.8	20–22
i.v.	ML-LPL	2	day 1	20.8 ± 0.8	20–22
i.p.	ML-LPL	2	day 1	32.6 ± 6.1‡	24–43
Untreated	—	—	—	23.5 ± 2.2	19–27
i.p.	Non-encap	3	single injection, day 1	23.0 ± 1.1	22–25
i.p.	ML-LPL	3	day 1	36.8 ± 7.0‡	31–52
i.p.	UL-LPL	3	day 1	42.5 ± 11.2‡	25–58
Untreated	—	—	—	18.9 ± 2.5	14–22
i.p.	Non-encap	3	two injections, days 1 and 3	24.3 ± 4.5	14–29
i.p.	UL-LPL	3	days 1 and 3	40.2 ± 13.2‡	18–58§ (+2 long-term survivors)
i.p.	UL-LPL + gal-cer	3	days 1 and 3	44.6 ± 12.7‡	10–61‖ (+2 long-term survivors)
i.p.	UL-APL	3	days 1 and 3	36.8 ± 7.2‡	29–53
i.p.	UL-APL + gal-cer	3	days 1 and 3	41.4 ± 14.3‡	24–69¶ (+1 long-term survivor)
i.p.	UL-LPL + MGDG	3	days 1 and 3	35.1 ± 10.0‡	20–51

*Abbreviations: Non-encap, non-encapsulated; ML, multilamellar liposome; UL, unilamellar liposome; i.p., intraperitoneal; i.v., intravenous; other abbreviations as in Table 1.

†Ten mice were used in each treatment group. Calculations of mean survival time were made from all 10 mice unless otherwise noted. Mean survival times are reported with standard deviations.

‡$P = 0.0001$ (highly significant) for statistical significance of differences in mean survival times between groups of mice treated i.p. with liposome-encapsulated MTX and control mice or mice treated with non-encapsulated MTX. *P*-values calculated separately for each treatment study shown (see text).

§Experiment terminated after 120 days, with 2 surviving mice. Eight mice used for calculation of mean survival time.

‖Three mice died before controls from drug toxicity; experiment terminated after 120 days, with 2 surviving mice. Five mice used for calculation of mean survival time.

¶Experiment terminated after 120 days, with 1 surviving mouse. Nine mice used for calculation of mean survival time.

traperitoneal injection of the same dose of MTX encapsulated in ML-LPL increased the mean survival time of the tumor-bearing mice to 32.6 ± 6.1 days (Table 2). All subsequent treatments were given by the intraperitoneal route.

The effectiveness of liposomes of different sizes is compared in Table 2. Intraperitoneal injection of 3 mg non-encapsulated MTX/kg on day 1 after hepatoma injection again produced no increase in survival time. When MTX in ML-LPL was administered at the same dose, the mean survival time of the tumor-bearing mice was increased to 36.8 ± 7.0 days. The same dose of MTX given in UL-LPL produced a mean survival time of 42.5 ± 11.2 days. The sonicated UL were used for all subsequent tumor treatment studies because the size of these liposomes can be better controlled.

Results of treatment of tumor-bearing mice with liposomes containing galactolipids are also shown in Table 2. The MTX treatments were increased to two doses of 3 mg MTX/kg given on days 1 and 3 after injection of hepatoma

cells. Treatment of tumor-bearing mice at this dosage with LPL containing galactocerebroside gave a mean survival time of 44.6 ± 12.7 days. (Following the method of Geran *et al.* [19], the three mice that died from an apparent toxic effect of the MTX were not included in this calculation.) MTX in the corresponding LPL without galactocerebroside produced a similar mean survival time with no evidence of drug toxicity (Table 2). MTX in APL with or without galactocerebroside also gave mean survival times that were not significantly different from those observed for the mice treated with LPL (Table 2).

Monogalactosyl diglyceride was also tested in another treatment study with LPL. Use of LPL containing this galactolipid for treatment of hepatoma, using the MTX dose and dose schedule described above, gave a mean survival time of 35.1 ± 10.0 days, less than that observed for the corresponding liposomes without galactolipid (Table 2).

The increase in number of drug treatments thus did not significantly change the mean survival time of mice treated with liposome-encapsulated MTX. The increased drug dosage did slightly increase the mean survival time of mice treated with non-encapsulated MTX (24.3 ± 4.5 days) compared with that of the untreated controls (18.9 ± 2.5 days), which in this particular test had an unusually short mean survival time. When these two groups of mice are compared alone, this difference in survival time is statistically significant ($P = 0.0001$), but when these groups are compared with all of the control mice and mice treated with non-encapsulated MTX in all of the treatment studies, there are no significant differences between the above survival times. As noted in the footnotes of Table 2, a few of the mice treated with liposome-encapsulated MTX at the above dosage did survive for more than 120 days. None of the untreated control mice in any of the treatment studies reported in Table 2 survived longer than 27 days. The longest survival time for mice treated with non-encapsulated MTX was 29 days.

DISCUSSION

This study has demonstrated that liposome encapsulation of MTX produced a significant increase in apparent uptake of the drug by Hepatoma 129 cells *in vitro*. A significant increase in mean survival time of mice bearing ascites Hepatoma 129 tumor was also observed after intraperitoneal treatment with liposome-encapsulated MTX. Little or none of the non-encapsulated MTX was taken up by the cells *in vitro*, and the non-encapsulated drug was generally ineffective in the *in vivo* treatment studies.

Tumors of the liver are known to be rather insensitive to chemotherapy [2, 3]. Our results suggest that the use of liposomes as drug carriers for certain antitumor drugs may be one possible way of overcoming this drug insensitivity of hepatomas. Small unilamellar liposomes given by intravenous injection have been shown to be effectively taken up by the parenchymal cells of the liver (Rahman *et al.* [22]). Selective delivery of antitumor drugs into certain types of hepatoma cells may therefore be achievable by the use of suitable liposomes.

The enhancement of antitumor activity observed in the present study might be due to a slow release of the liposome-encapsulated drug, as has been suggested by Kaye *et al.* in their studies using a MTX-resistant osteosarcoma [23]. Metabolic breakdown of non-encapsulated MTX in plasma and tissues has been shown to be greatly reduced by liposome encapsulation [24], thus allowing prolonged retention of a single injection of the encapsulated drug in the tissues. Our own tests of liposome stability and those of Kimelberg [24], using very similar liposomes, have indicated relatively low permeability of positively charged liposomes to MTX. Both of these observations would therefore support a slow release mechanism for liposome-encapsulated MTX. However, the results of the multiple dose regimen do not indicate slow drug release as a sole mechanism for the enhanced cell killing. When two intraperitoneal injections of the same dosages of non-encapsulated and encapsulated MTX were given on day 1 and day 3 (see Table 2), there was little or no increase in the mean survival time of mice treated with non-encapsulated MTX compared with that of untreated mice. There was also little change seen in mean survival times of the mice treated with either single or double injections of liposome-encapsulated MTX. However, when two injections of liposome-encapsulated MTX were given by the intraperitoneal route, there were long-term survivors in three groups of mice (treated with LPL, LPL with galactocerebroside, and APL with galactocerebroside), as well as early deaths due to drug toxicity in one group of mice (treated with LPL with galactocerebroside); these data were not used in calculations of mean survival times [19]. Although complete methotrexate toxicity studies have not been conducted with Hepatoma 129 cells, Kimelberg and Atchison [10] have obser-

ved toxicity of liposome-encapsulated MTX in mice under some conditions.

These results and the *in vitro* uptake tests, as well as considerable evidence of rapid internalization of liposomes into cells [8, 11, 18, 25, 26], indicate that direct interaction of hepatoma cells and the liposome-transported antitumor drug may be one of the mechanisms for the observed cell killing.

The cytotoxicity of MTX depends on both the carrier-mediated transport of the drug into the target cell and on the subsequent action of this growth-phase-specific antimetabolite on the intracellular dihydrofolate reductase [4, 27–29]. However, MTX transport is generally considered to be the greatest determinant of MTX toxicity [29]. There have been no other studies of the uptake or cytotoxicity of MTX in Hepatoma 129 cells, as far as the authors are aware. Fry *et al.* [30] have reported stimulation of an MTX carrier in the membranes of Ehrlich ascites cells by positively charged, 'empty' liposomes. These workers have suggested an interaction between positively charged liposomes and the cell membrane which could alter membrane structure and MTX carrier function [30]. Addition of positively charged, 'empty' liposomes did not increase the *in vitro* uptake of non-encapsulated MTX by the Hepatoma 129 cells, however.

In order to study an *in vitro* system that would be roughly comparable to an effective *in vivo* treatment, our *in vitro* uptake studies employed MTX concentrations at least one hundred-fold higher than are generally used in tests of MTX transport and inhibition of dihydrofolate reductase [31, 32]. The use of MTX concentrations above the saturating levels for dihydrofolate reductase or the MTX membrane carrier measured in L1210 murine leukemia cells [31, 32] might have produced cytostatic effects. However, studies of inhibition of dihydrofolate reductase by MTX have all noted that a very high intracellular concentration of this drug must be maintained for prolonged times for this growth-phase-specific drug to have cytotoxic activity [27, 29]. In the *in vitro* system of White and Goldman [33], a 30-min exposure of cells to MTX concentrations ten times higher than normally used was needed to produce adequate intracellular MTX levels for testing of enzyme saturation. Our *in vitro* assays of uptake of liposome-encapsulated [^{3}H]-MTX usually reached a maximum within 30 min, while all corresponding measurements of non-encapsulated [^{3}H]-MTX uptake were not considered to be significantly above background levels, within the limits of error of the assay system. At the end of all of the *in vitro* incubations, the Hepatoma 129 cells were still viable by the trypan blue test, indicating relatively intact membrane functions and structure [34]. Todd *et al.* [35] have reported enhanced uptake of liposome-encapsulated MTX by human chronic leukemia cells *in vitro*, but they observed enhancement of biological activity (inhibition of nucleotide uptake) only with MTX doses at least ten times higher than those used in our *in vivo* studies. Metabolism of MTX proceeds at comparable rates in human and mouse cells [10].

The greatly increased association of liposome-encapsulated MTX with Hepatoma 129 cells *in vitro*, even after treatment with trypsin, would not be adequately explained by a simple, non-specific adsorption mechanism. Although up to 64% of the initially associated liposome-encapsulated MTX could be removed from the cells by the type of trypsin treatment recommended by Pagano and Takeichi [18], there was little difference in the amounts of cell-associated liposome-encapsulated MTX after trypsin treatment with four increasing trypsin concentrations (up to five times greater than those used in Pagano's laboratory) [18]. There was also no apparent decrease in the viability of cells incubated at the higher trypsin concentrations. The electron micrographs and uptake data of Pagano and Takeichi [18] indicated that this method was quite effective for removal of liposomes adsorbed on the cell surface. These workers also observed that components of specifically adsorbed liposomes were rapidly internalized by cells and were not removed by trypsin [18]. All of our other tests of *in vitro* uptake of liposome-encapsulated MTX did utilize the trypsin treatments and thus are measurements of only the liposomes which were not trypsin-labile and were firmly associated with the Hepatoma 129 cells.

The mechanism of *in vitro* uptake of the liposomes remaining with the washed cells after trypsin treatment is not yet clear. Pagano and Takeichi showed surface adhesion to be a primary mode of association of 'solid' liposomes prepared with dipalmitoyl phosphatidylcholine well below the transition temperature of the saturated phospholipid, but not at 37°C [18]. Electron microscopy done in our laboratory [25; unpublished data] has given evidence for entry of liposomes into liver cells *in vivo* by the process of endocytosis. The decreased *in vitro* uptake of MTX from both APL and LPL in the presence of the microfilament disruptor, cytochalasin B, as well as at 4°C indicates that

endocytosis could be an important mechanism of entry of MTX in UL into the hepatoma cells. The 5–10% initial association of liposome-encapsulated MTX measured in all the *in vitro* studies, which could not be removed by high trypsin concentrations, may reflect a very rapid, irreversible association of liposomes with the cells. (Separation of cells and liposomes at zero time in the assays could not be done instantaneously; the initial uptake represents a binding that could occur in a chilled tube in approximately 2–3 min.) There have been some contradictory conclusions in the literature about possible fusion of 'solid,' charged UL with cells, but it has been generally accepted that 'solid' liposomes are mainly taken up into cells by endocytosis [36–40].

Inclusion of specific galactolipids in the liposomes had little or no effect on *in vitro* uptake or *in vivo* therapeutic effectiveness of liposome-encapsulated MTX. Previous studies in this laboratory have indicated an increased *in vivo* uptake of liposomes containing galactocerebroside by liver cells, but these observations have all been made for drugs which could only be encapsulated in the aqueous phase of liposomes [15]. Liposomes made with and without galactolipid and containing [^{3}H]-MTX in the aqueous compartments show small but similar differences in their uptake. Other unpublished studies of uptake by Hepatoma 129 cells of drugs other than MTX encapsulated in the aqueous phase of liposomes containing galactolipids are also in good agreement with our previous findings. The structural position of the galactose residues incorporated in the lipid bilayers of the liposomes has not yet been conclusively demonstrated. It is possible that the galactose moiety may not be readily available on the surface of these liposomes for recognition by cellular receptors. It is also possible that the Hepatoma 129 cells have fewer plasma membrane galactose receptors, as has been observed in other tumor cells [41–43].

Contrary to our observations, Segal *et al.* [44] did not observe selective uptake of liposome-encapsulated bleomycin by a solid, human hepatoma. The work of Freise *et al.* [45] indicated no uptake of liposome-encapsulated MTX by solid, chemically induced, rat hepatoma tissue. However, Freise and co-workers used negatively charged liposomes to encapsulate MTX [45], and thus their results are not comparable to those of this study. The differences in the animal and tumor systems used by each group make these negative observations difficult to correlate with our results.

On the other hand, Kosloski *et al.* [46] have observed enhanced *in vitro* uptake and *in vivo* therapeutic activity of liposome-encapsulated MTX in a solid, MTX-resistant rodent tumor. Although the transport of MTX by Hepatoma 129 cells is not yet well elucidated, our results suggest the potential value of liposome-encapsulated MTX as an antitumor agent against tumors previously considered resistant to MTX due to inefficient transport of the drug [4]. This is also a first study demonstrating the therapeutic effectiveness of a liposome-encapsulated antitumor agent against a hepatoma cell model.

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REFERENCES

1. PACK GT, ISLAMI AH. Tumors of the liver. *Recent Results Cancer Res* 1970, **26**, 1–3.
2. CHAN KT. The management of primary liver carcinoma. *Ann R Coll Surg Engl* 1967, **41**, 253–282.
3. VOGEL CL, LINSELL CA. Highlights, International Symposium on Hepatocellular Carcinoma—Kampala, Uganda (July 1971). *J. Natl Cancer Inst* 1972, **48**, 567–571.
4. GOLDMAN ID. Uptake of drugs and resistance. In: MIHICH E, ed. *Drug Resistance and Selectivity.* New York, Academic Press, 1973, Vol. 1, 299–358.
5. HERTZ R, LEWIS J, JR, LIPPSETT MB. Five years experience with the chemotherapy of metastatic choriocarcinoma and related trophoblastic tumors in women. *Am J Obstet Gynecol* 1961, **82**, 630–640.
6. LEONE LA, ALBALA MM, REGE VB. Treatment of carcinoma of the head and neck with intravenous methotrexate. *Cancer* 1968, **21**, 828–837.
7. FINKELSTEIN M, WEISSMANN G. The introduction of enzymes into cells by means of liposomes. *J Lipid Res* 1978, **19**, 289–303.
8. RAHMAN YE, ROSENTHAL MW, CERNY EA. Intracellular plutonium: removal by liposome-encapsulated chelating agent. *Science* 1973, **180**, 300–302.

9. PAPAHADJOPOULOS D, WILSON T, TABER R. Liposomes as vehicles for cellular incorporation of biologically active macromolecules. *In Vitro* 1980, **16**, 49–54.
10. KIMELBERG HH, ATCHISON ML. Effects of entrapment in liposomes on the distribution, degradation, and effectiveness of methotrexate *in vivo*. *Ann NY Acad Sci* 1978, **308**, 395–409.
11. RAHMAN YE, ROSENTHAL MW, CERNY EA, MORETTI ES. Preparation and prolonged tissue retention of liposome-encapsulated chelating agents. *J Lab Clin Med* 1974, **83**, 640–647.
12. JONAH MM, CERNY EA, RAHMAN YE. Tissue distribution of EDTA encapsulated within liposomes of varying surface properties. *Biochim Biophys Acta* 1975, **401**, 336–348.
13. RAHMAN YE, KISIELESKI WE, BUESS EM, CERNY EA. Liposomes containing ^{3}H-actinomycin D. Differential tissue distribution by varying the mode of drug incorporation. *Eur J Cancer* 1975, **11**, 883–889.
14. PAPAHADJOPOULOS D, POSTE G, VIAL WJ, BIEDLER J. Use of lipid vesicles as carriers to introduce actinomycin-D into resistant tumor cells. *Cancer Res* 1976, **36**, 2988–2994.
15. JONAH MM, CERNY EA, RAHMAN YE. Tissue distribution of EDTA encapsulated within liposomes containing glycolipids or brain phospholipid. *Biochim Biophys Acta* 1978, **541**, 321–333.
16. KIMELBERG HK, TRACY TF, JR, BIDDLECOME SM, BOURKE RS. The effect of entrapment in liposomes on the *in vivo* distribution of [^{3}H] methotrexate in a primate. *Cancer Res* 1976, **36**, 2949–2957.
17. FRY DW, WHITE JC, GOLDMAN ID. Rapid separation of low molecular weight solutes from liposomes without dilution. *Anal Biochem* 1978, **90**, 809–815.
18. PAGANO RE, TAKEICHI M. Adhesion of phospholipid vesicles to chinese hamster fibroblasts. *J Cell Biol* 1977, **74**, 531–546.
19. GERAN RI, GREENBERG NH, MACDONALD MM, SCHUMACHER AM, ABBOTT BJ. Protocols for screening chemical agents and natural products against animal tumors and other biological systems, 3rd edn. *Cancer Chemother Rep* 1972, **3**, 47–62.
20. DUNCAN DB. Multiple regression and multiple tests. *Biometrics* 1955, **11**, 1–42.
21. GOODNIGHT JH, SALL J. *Statistical Analysis Systems Users Guide.* Cary, NC, Statistical Analysis System Institute, Inc., 1979.
22. RAHMAN YE, CERNY EA, PATEL KR, LAU EH, WRIGHT BJ. Differential uptake of liposomes varying in size and lipid composition by parenchymal and Kupffer cells of mouse liver. *Life Sci* In press.
23. KAYE SB, BODEN JA, RYMAN BE. The effect of liposome (phospholipid vesicle) entrapment of Actinomycin D and methotrexate on the *in vivo* treatment of sensitive and resistant solid murine tumors. *Eur J Cancer* 1981, **17**, 279–289.
24. KIMELBERG HK. Differential distribution of liposome-entrapped ^{3}H-methotrexate and labelled lipids after intravenous injection in a primate. *Biochim Biophys Acta* 1976, **448**, 531–550.
25. RAHMAN YE, WRIGHT BJ. Liposomes containing chelating agents: cellular penetration and a possible mechanism of metal removal. *J Cell Biol* 1975, **65**, 112–122.
26. RAHMAN YE, TOLLAKSEN SL, NANCE SL *et al.* Cellular and subcellular distribution of drugs transported by liposomes. In: REID E, ed. *Methodological Surveys in Biochemistry.* Chichester, Harwood, 1977, Vol. 6, 401.
27. GOLDMAN ID, Effects of methotrexate on cellular metabolism: some critical elements in the drug-cell interaction. *Cancer Treat Rep* 1977, **61**, 549–558.
28. GOLDIE JH, HARRISON SI. Comparison of the cytotoxicity of methotrexate and aminopterin on methotrexate resistant murine lymphoma cells *in vitro*. *Eur J Cancer* 1978, **14**, 55–57.
29. CHELLO PL, SIROTNAK FM, DORRICK DM. Alterations in the kinetics of methotrexate transport during growth of L1210 murine leukemia cells in culture. *Mol Pharmacol* 1980, **18**, 274–280.
30. FRY DW, WHITE JC, GOLDMAN ID. Alteration of the carrier-mediated transport of an anionic solute, methotrexate, by charged liposomes in Ehrlich ascites tumor cells. *J Membr Biol* 1979, **50**, 123–140.
31. GOLDMAN ID, FYFE MJ. Free intracellular methotrexate (MTX) is required for maximum inhibition of DNA synthesis—augmentation by vincristine (VCR). *Proc Am Assoc Cancer Res* 1973, **14**, 199a.
32. COHEN M, BENDER RA, DONEHOWER R, MYERS CE, CHABNER BA. Reversibility of high-affinity binding of methotrexate in L1210 murine leukemia cells. *Cancer Res* 1978, **38**, 2866–2870.

33. WHITE JC, GOLDMAN ID. Mechanism of action of methotrexate: IV. Free intracellular methotrexate required to suppress dihydrofolate reduction to tetrahydrofolate by Ehrlich ascites tumor cells *in vitro*. *Mol Pharmacol* 1976, **12**, 711–719.
34. HANKS JH, WALLACE JH. Determination of cell viability. *Proc Soc Exp Biol Med* 1958, **98**, 188–192.
35. TODD JA, LEVINE AM, TOKES ZA. Liposome-encapsulated methotrexate interacting with human chronic lymphocytic leukemia cells. *J. Natl Cancer Inst* 1980, **64**, 715–719.
36. POSTE G, PAPAHADJOPOULOS D. Lipid vesicles as carriers for introducing materials into cultured cells: influence of vesicle lipid composition on mechanism(s) of vesicle incorporation into cells. *Proc Natl Acad Sci USA* 1976, **73**, 1603–1607.
37. PAPAHADJOPOULOS D, POSTE G, VAIL WJ. Studies on membrane fusion with natural and model membranes. In: KORN ED, ed. *Methods in Membrane Biology*. New York, Plenum Press, 1979, Vol. 10, 1–121.
38. MARTIN FJ, MACDONALD RC. Phospholipid exchange between bilayer membrane vesicles. *Biochemistry* 1976, **15**, 321–327.
39. PAPAHADJOPOULOS D, MAYHEW DE, POSTE G, SMITH S. Incorporation of phospholipid vesicles by mammalian cells provides a potential method for modifying cell behavior. *Nature (Lond)* 1974, **252**, 163–166.
40. BATZRI S, KORN ED. Interaction of phospholipid vesicles with cells: endocytosis and fusion as alternate mechanisms for the uptake of lipid-soluble and water-soluble molecules. *J Cell Biol* 1975, **66**, 621–634.
41. CRITCHLEY DR, MACPHERSON I. Cell density dependent glycolipids in NIL_2 hamster cells, derived malignant, and transformed cell lines. *Biochim Biophys Acta* 1973, **296**, 145–159.
42. BRADY RO, FISHMAN PH. Biosynthesis of glycolipids in virus-transformed cells. *Biochim Biophys Acta* 1974, **355**, 121–148.
43. GAHMBERG CG, HAKOMORI S. Organization of glycoprotein and glycolipid in the plasma membrane of normal and transformed cells as revealed by galactose oxidase. In: MANSON LA, ed. *Biomembranes*. New York, Plenum Press, 1976, Vol. 8, 131–165.
44. SEGAL AW, GREGORIADIS G, LAVENDER JP, TARIN D, PETERS TJ. Tissue and hepatic subcellular distribution of liposomes containing bleomycin after intravenous administration to patients with neoplasms. *Clin Sci Mol Med* 1976, **51**, 421–425.
45. FREISE J, SCHMIDT FW, MAGERSTEDT P. Effect of liposome-entrapped methotrexate on Ehrlich ascites tumor cells and uptake in primary liver cell tumor. *J Cancer Res Clin Oncol* 1979, **94**, 21–28.
46. KOSLOSKI MJ, ROSEN F, MILHOLLAND RJ, PAPAHADJOPOULOS D. Effect of lipid vesicle (liposome) encapsulation of methotrexate on its chemotherapeutic efficacy in solid rodent tumors. *Cancer Res* 1978, **38**, 2848–2853.