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Liposomal Sustained-Release Delivery Systems for Intravenous Injection. III. Antitumor Activity of Lipophilic Mitomycin C Prodrug-Bearing Liposomes

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An intravenously injectable sustained-release delivery system for mitomycin C (MMC) was prepared by formulating a lipophilic MMC prodrug, *N*-(cholesteryloxycarbonyl)glycyl MMC (COCG-MMC), in unilamellar liposomes, and its blood disposition and antitumor activity were investigated in mice. Liposomes composed of egg phosphatidylcholine, egg sphingomyelin and COCG-MMC in a molar ratio of $7:3:X$ ($X=0$—2.7) were prepared by the combination of controlled dialysis or sonication and sequential extrusion. All the preparations prepared by controlled dialysis showed a fairly narrow size distribution. After intavenous injection of COCG-MMC-bearing liposomes at a dose of 20 μmol/kg as a prodrug, the blood levels of regenerated MMC were maintained for the first 7 h in the range of 1.16 to 0.48 μM. In contrast, when an equimolar amount of MMC was given in an aqueous solution, MMC was rapidly cleared with little remaining in the circulation after 2 h. COCG-MMC exhibited significant cytotoxicity against P 388 leukemia *in vitro* and its activity was approximately one-third that of the parent drug. Prodrug-bearing liposomes inhibited the growth of subcutaneously-implanted Colon 26 adenocarcinoma and human mammary carcinoma MX-1 xenograft. Compared with MMC aqueous solution, prodrug-bearing liposomes showed reduced antitumor activity and reduced toxicity. In each tumor system, the body weight change differences (test minus controls), indices of host toxicity, at the ID_{50}'s (the doses which inhibit tumor growth to 50% of controls) showed no significant difference between these dosage forms. The results indicate that COCG-MMC-bearing liposomes successfully maintain blood MMC levels over a prolonged period of time, but their therapeutic efficacy is almost equal to that of MMC aqueous solution.

Keywords——liposome; intravenous injection; mitomycin C; mitomycin C prodrug; blood disposition; sustained release; solid tumor; antitumor activity; toxicity; therapeutic efficacy

Mitomycin C (MMC) is an antitumor antibiotic that has been shown to have activity against a number of human neoplasms.[1] However, host toxicity, such as delayed cumulative myelosuppression, is one of the major problems in its clinical use.[2]

Barlogie and Drewinko have demonstrated that a given exposure dose of MMC induces equitoxic effects on cultured human colon adenocarcinoma (LoVo) regardless of the specific drug concentration and exposure time.[3] Furthermore, MMC has been found to show selective toxicity against hypoxic cells at a relatively low concentration *in vitro*.[4] On the basis of these findings, it is reasonable to assume that prolongation of blood MMC concentration at an optimal level might decrease its host toxicity and consequently improve its therapeutic efficacy.

Attempts have been made in our laboratory to develop intravenously injectable sustained-release delivery systems for antitumor agents by the use of liposomes as drug reservoirs.[5] In a previous study,[5a] we synthesized lipophilic derivatives of MMC in an attempt to improve the applicability of MMC to liposome carrier systems. Among the

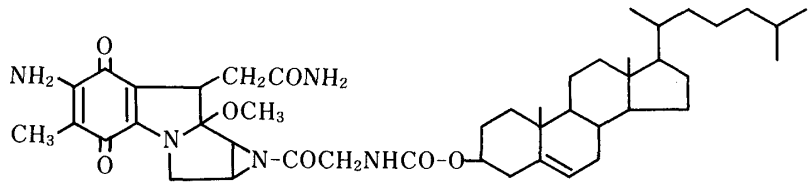

Fig. 1. Structure of COCG-MMC

derivatives synthesized, *N*-(cholesteryloxycarbonyl)glycyl MMC (COCG-MMC), shown in Fig. 1, showed the best properties as regards entrapment efficiency, lability and affinity for liposomes in the circulation.

In liposome disposition experiments,[5b] relatively small unilamellar liposomes composed of egg phosphatidylcholine (PC) and egg sphingomyelin (SM) had a long lifetime in the circulation and seem to be suitable as a carrier vehicle for sustained-release delivery systems to be applied by intravenous (i.v.) injection. In fact, these liposomes loaded with COCG-MMC successfully maintained blood MMC levels in rats after i.v. injection.[5b]

In the present study, the blood disposition of liposome-entrapped COCG-MMC and its antitumor activity against solid tumors were investigated in mice to determine whether this liposomal sustained-release delivery system will improve the therapeutic efficacy of MMC.

Experimental

Chemicals——MMC was purchased from Wako Pure Chemical Industries. COCG-MMC was prepared according to the method previously described.[5a] PC and SM were obtained from Sigma Chemical Co. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from the same source. All other chemicals were of reagent grade or better.

Preparation of Unilamellar Liposomes——Liposomes used in this study were composed of PC, SM and COCG-MMC in a molar ratio of 7 : 3 : X (X=0—2.7). Liposome preparations except at the highest dose (168 μmol/kg) were prepared by controlled dialysis as described previously,[5] their final phospholipid concentration being about 25 mM (blood disposition study) or 35 mM (antitumor activity study). Since the liposome preparation for the highest dose could not be prepared by controlled dialysis, it was prepared by sonication[6] at 4 °C for 30 min with a probe sonifier (Tomy Seiko Co., BH-200P) and its final phospholipid concentration was about 60 mM. All preparations were subjected to sequential extrusion[7] through 0.2 and 0.1 μm pore-sized polycarbonate membranes (Nuclepore) and subsequently sterilized by filtration through a Millex GV filter (0.22 μm, Millipore). The concentration of liposome-entrapped COCG-MMC was determined by high performance liquid chromatography (HPLC) as described previously.[5] The liposome preparations were diluted with sterile pH 7.4 phosphate-buffered saline (PBS) to obtain the desired drug concentration and kept under nitrogen at 4 °C until use.

Vesicle Size Measurement——The diameters of liposomes were determined by a dynamic laser light scattering system (Coulter, model N4).

Blood Disposition Study——Female ICR mice weighing 22—24 g were used. The animals were injected with 0.01 mg/g of MMC aqueous solution or COCG-MMC liposome suspension at a dose of 20 μmol drug/kg into the tail vein. At various times after injection, mice were anesthetized with ether and blood samples were obtained by cardiac puncture. For analysis of COCG-MMC, an aliquot (0.25 ml) of the blood was immediately frozen in liquid nitrogen to avoid postcollection degradation of the prodrug, and lyophilized. Residual blood samples were centrifuged to obtain plasma for determination of MMC. To avoid postcollection regeneration of MMC, an aliquot (0.2 ml) of the plasma was immediately frozen in liquid nitrogen and stored in a freezer until assay.

Analysis——MMC and COCG-MMC were extracted from biological samples and determined by HPLC as described previously.[5b]

***In Vitro* Cytotoxicity Assay**——*In vitro* cytotoxicity was evaluated using primary cultured P 388 leukemia cells. The culture medium used was Dulbecco's modified Eagle's medium containing fetal calf serum (10%, v/v), 2-mercaptoethanol (50 μM), penicillin G (100 μunits/ml) and streptomycin (100 μg/ml). P 388 ascites cells harvested from the peritoneal cavity of a P 388-bearing DBA/2 mouse were plated into flat-bottomed cell wells (Corning) at a concentration of 1.25×10^4 cells/well (38 mm^2 surface area) in 50 μl of the culture medium. Various concentrations of test compound dissolved in 50 μl of saline containing HCO-60 (2%, w/v) were added to the wells. After culture for 48 h at 37 °C in a CO_2 incubator, the cytotoxicity was estimated on the basis of cell viability detected by MTT colorimetric assay, as described by Green *et al.*[8]

Antitumor Activity Evaluation——Colon 26 adenocarcinoma and human mammary carcinoma MX-1 were maintained by serial subcutaneous passage of tumor fragments (2 × 2 × 2 mm) through female BALB/c mice and male BALB/c-nu/nu mice, respectively. The chemotherapy study was performed according to the National Cancer Institute protocols for screening[9] with slight modifications. Colon 26 adenocarcinoma (2 × 2 × 2 mm tumor fragment) was implanted subcutaneously into the axillary region of female BALB/c mice (16—22 g) using a 13-guage trocar. Human mammary carcinoma MX-1 (2 × 2 × 2 mm tumor fragment) was implanted into the axillary region of male BALB/c-nu/nu mice (19—25 g) in a similar manner. Mice were treated intravenously with COCG-MMC-bearing liposomes or MMC aqueous solution in a volume of 0.01 ml/g body weight. In the Colon 26 adenocarcinoma system, chemotherapy was carried out on days 7 and 10, and an evaluation was performed on day 14. In the case of human mammary carcinoma MX-1 xenograft, the treatment days were days 11, 15 and 19, and the evaluation day was day 27. In each experiment, mice were weighed and caliper measurements of the tumor were made on the treatment days and evaluation day. The tumor volume was calculated from the following formula.[9]

$$\text{tumor volume } (\text{mm}^3) = a \times b^2/2$$

where a and b represent the largest and smallest diameters of the tumor mass, respectively. Antitumor activity was expressed in terms of the tumor growth inhibition (G.I.), calculated by means of the following formula.

$$\text{G.I. } (\%) = [1 - \{\text{mean of } (T_n/T_0)/\text{mean of } (C_n/C_0)\}] \times 100$$

where C_0 and C_n represent the tumor volumes of each mouse in control groups (treated with PBS or plain liposomes) on the first treatment day and on the day of evaluation, respectively. T_0 and T_n represent the tumor volumes of each mouse in the treatment groups on the first treatment day and on the evaluation day, respectively.

Results

Characterization of Liposomes

The diameters of the liposomes used in this study are summarized in Table I. Compared with the plain liposomes, prodrug-bearing liposomes prepared by controlled dialysis showed a slight increase in size, but still maintained size homogeneity. Under storage at 4 °C, liposome size and homogeneity did not alter significantly during 3 months. On the other hand, the liposomes prepared by sonication (preparation H) had a relatively small mean diameter, but were rather heterogeneous in size. Under the same storage conditions, precipitation of the vesicles was observed within a week.

TABLE I. Size of Liposomes Used in the Blood Disposition (I) and Antitumor Activity (II) Studies

Study	Preparation[a]	COCG-MMC conc. (mM)	Diameter[b] (nm)
I	A[c]	2.00	127 ± 28
II	B	0	124 ± 24
	B′	0	122 ± 23
	C′	0.81	128 ± 23
	D	1.68	175 ± 35
	D′	1.59	164 ± 36
	E	2.99	198 ± 39
	E′	3.23	200 ± 39
	F	5.39	171 ± 34
	F′	5.47	189 ± 22
	G	9.58	175 ± 29
	G′	7.90	165 ± 32
	H[d]	16.77	90 ± 65

a) Preparations B, D, E, F, G and H were used for the chemotherapy of Colon 26, and B′, C′, D′, E′, F′ and G′ for MX-1 xenograft. *b*) Liposome size was determined by dynamic laser light scattering and expressed as the mean ± S.D. *c*) Lipid concentration was about 25 mM. *d*) Preparation H was prepared by sonication and its lipid concentration was about 60 mM. Others were prepared by controlled dialysis and their lipid concentration was about 35 mM.

Blood Disposition of Liposome-Entrapped COCG-MMC

Figure 2 shows the blood levels of COCG-MMC and its parent drug regenerated in mice after i.v. injection of liposome-entrapped prodrug. The blood clearance profile of MMC administered intravenously in an aqueous solution is also illustrated for comparison. MMC

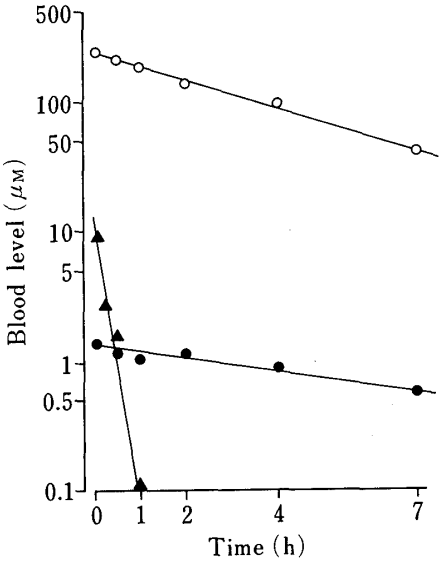

Fig. 2. Blood Levels of COCG-MMC and Regenerated MMC in Mice after i.v. Injection of COCG-MMC-Bearing Liposomes

○, COCG-MMC; ●, regenerated MMC; ▲, MMC (after i.v. injection in an aqueous solution). Closed and open symbols mean plasma and blood concentrations, respectively. Each point represents the mean of three mice. Each S.D. value was less than 8%.

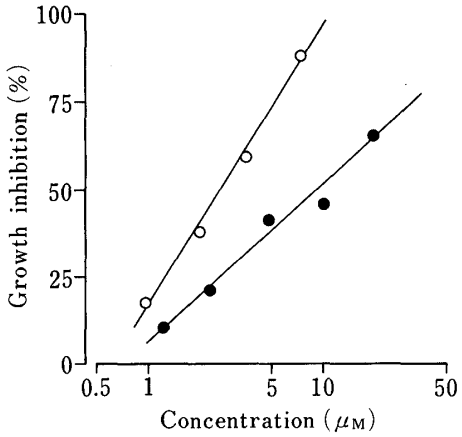

Fig. 3. Growth-Inhibitory Effect of COCG-MMC and MMC on Primary Cultured P 388 Leukemia Cells

○, MMC; ●, COCG-MMC. Each point represents the mean of three tests. Each S.D. value was less than 10%.

TABLE II. Antitumor Activity of COCG-MMC-Bearing Liposomes and MMC Aqueous Solution against Colon 26 Adenocarcinoma[a)]

Preparation	Dose (μmol/kg/d)	Body weight change (g) (Day 14−7)	Day 7 Tumor volume (mm^3)	Day 14 Tumor volume (mm^3)	G.I.[b)] (%)
PBS	0	−3.5±0.3	224±11	1416± 64 (8/8)[c)]	0
MMC aq. soln.	4.8	−2.3±0.2	220±19	1027± 79 (8/8)	26.3
	7.5	−2.8±0.4	216±13	1086± 39 (8/8)	19.9
	12.0	−4.0±0.3	225±29	746± 55 (8/8)	44.5
	19.2	−4.5±0.4	221±17	279± 28 (8/8)	79.8
	29.9	−6.2	216±17	145± (2/8)	84.8
Plain liposomes	0	−3.7±0.3	224±12	1466± 93 (8/8)	0
COCG-MMC-liposomes	16.8	−3.4±0.4	216±17	1264± 57 (8/8)	9.0
	29.9	−3.0±0.5	214±25	974±154 (8/8)	29.3
	53.9	−3.8±0.4	210±14	826± 34 (8/8)	39.0
	95.8	−4.7±0.2	223±14	496± 45 (8/8)	66.3
	168.0	−4.2±0.4	225±23	293± 82 (8/8)	82.1

a) Colon 26 fragments (2×2×2 mm) were implanted subcutaneously into female BALB/c mice. The drugs were given intravenously on days 7 and 10 after inoculation. Evaluation was carried out on day 14. *b*) Growth inhibition, see Experimental. *c*) The figures in parenthesis are the number of survivors on day 14/the number of animals used. Each result is the mean±S.E.

was rapidly cleared and little remained in the circulation 2 h after injection of its aqueous solution. The elimination curve of MMC showed a monoexponential pattern having a half-life of 9 min. On the other hand, when an equimolar amount of COCG-MMC was given in the liposome-entrapped form, the prodrug remained in the circulation over a prolonged period of time and sustained blood levels of regenerated MMC were observed. Blood MMC levels decreased slowly with a half-life of 4.7 h and were maintained for the first 7 h in the range of 1.16 to 0.48 μM at a dose of 20 μmol/kg.

In Vitro Cytotoxicity of COCG-MMC

In vitro cytoxicity assay was performed using primary cultured P 388 leukemia cells, and the results are shown in Fig. 3. The concentrations of drugs required to inhibit cell growth to 50% of the controls (IC_{50}'s) were determined to be 2.6 μM for MMC and 9.0 μM for COCG-MMC. In this culture system, the activity of the prodrug was approximately one-third that of the parent drug.

Antitumor Activity of COCG-MMC-Bearing Liposomes against Solid Tumors

The antitumor activity of intravenously-injected COCG-MMC-bearing liposomes was investigated in subcutaneously-implanted Colon 26 adenocarcinoma and human mammary carcinoma MX-1 xenograft systems, and the results are summarized in Tables II and III, respectively. The antitumor activity of MMC aqueous solution is included for comparison. Both preparations significantly inhibited the growth of these solid tumors at optimal doses. Figure 4A, B illustrates the dose-growth inhibition curves in Colon 26 (A) and MX-1 xenograft (B) systems. Dose-dependent tumor growth inhibition by COCG-MMC-bearing liposomes was observed in both tumor systems. Compared with MMC aqueous solution, prodrug-bearing liposomes exhibited similar patterns of growth inhibition curves, but shifted to the high dose region. In the Colon 26 system, the doses required to inhibit tumor growth to 50% of the controls (ID_{50}'s) were 63.4 μmol/kg/d for COCG-MMC-bearing liposomes and 12.3 μmol/kg/d for MMC aqueous solution. On the other hand, ID_{50} values in the MX-1

TABLE III. Antitumor Activity of COCG-MMC-Bearing Liposomes and MMC Aqueous Solution against Human Mammary Carcinoma MX-1 Xenograft[a]

Preparation	Dose (μmol/kg/d)	Body weight change (g) (day 19−11)	Day 11 Tumor volume (mm^3)	Day 27 Tumor volume (mm^3)	G.I.[b] (%)
PBS	0	1.8±0.2	124±16	3111±243 (7/7)[c]	0
MMC aq. soln.	1.0	1.8±0.2	109±18	1612±238 (7/7)	44.1
	1.7	1.7±0.2	129±17	447±203 (7/7)	88.1
	3.0	1.9±0.2	110±23	17± 4 (7/7)	99.4
	5.4	0.9±0.3	117±15	7± 1 (7/7)	99.8
	9.6	−0.7±0.3	122±18	8± 2 (7/7)	99.8
	16.8	−4.7±0.4	127±17	13 (2/7)	99.7
Plain liposomes	0	2.8±0.2	110±13	2854±184 (6/6)	0
COCG-MMC-liposomes	8.1	2.4±0.6	111±13	2329±228 (6/6)	22.9
	15.9	1.6±0.2	105±17	492± 98 (6/6)	83.0
	32.3	1.6±0.2	124±21	8± 1 (6/6)	99.7
	54.8	0.6±0.3	105± 9	4± 1 (6/6)	99.9
	79.0	−0.3±0.4	103±13	3± 1 (6/6)	99.9

a) MX-1 fragments (2×2×2 mm) were implanted subcutaneously into male BALB/c-nu/nu mice. The drugs were given intravenously on days 11, 15 and 19 after tumor inoculation. Evaluation was performed on day 27. *b*) Growth inhibition, see Experimental. *c*) The figures in parenthesis are the number of survivors on day 27/the number of animals used. Each result is the mean±S.E.

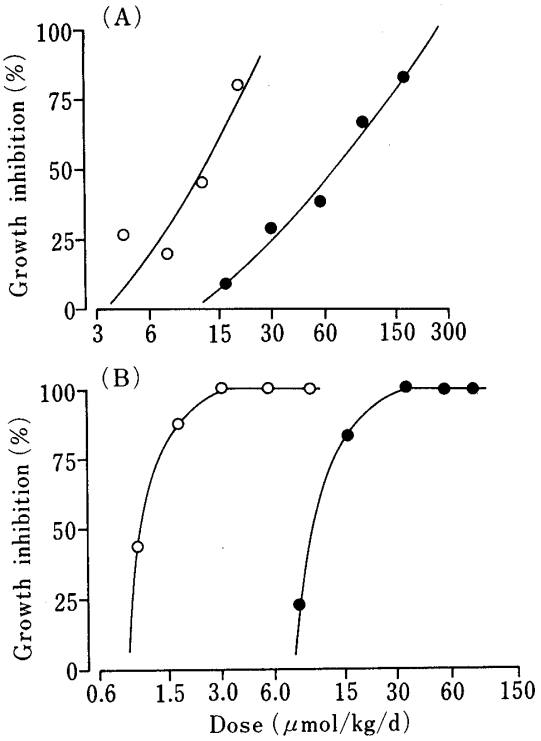

Fig. 4. Dose-Growth Inhibition Curves of COCG-MMC-Bearing Liposomes and MMC Aqueous Solution in Colon 26 (A) and MX-1 Xenograft (B) Systems

○, MMC aqueous solution; ●, COCG-MMC-bearing liposomes. The numbers of animals used in systems (A) and (B) are given in Tables II and III, respectively.

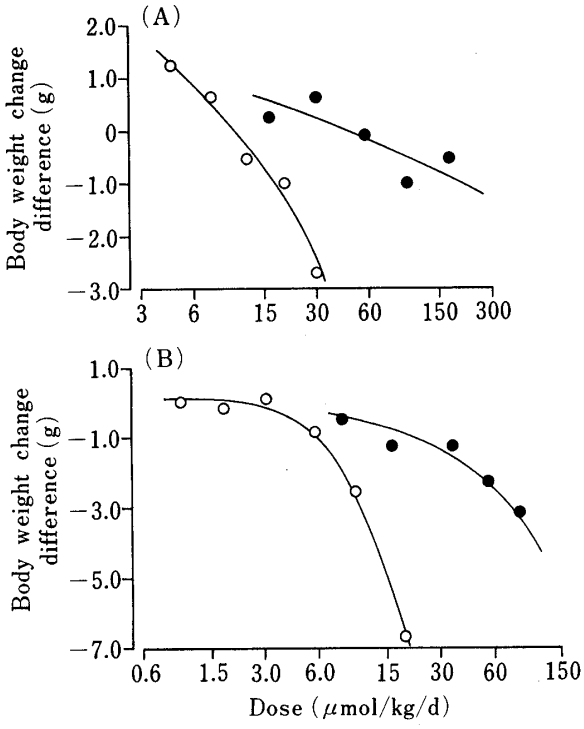

Fig. 5. Dose-Body Weight Change Difference Profiles of COCG-MMC-Bearing Liposomes and MMC Aqueous Solution in Colon 26 (A) and MX-1 Xenograft (B) Systems

○, MMC aqueous solution; ●, COCG-MMC-bearing liposomes. The numbers of animals used in systems (A) and (B) are given in Tables II and III, respectively.

TABLE IV. Therapeutic Index (T.I.) Values of COCG-MMC-Bearing Liposomes and MMC Aqueous Solution in Colon 26 and MX-1 Xenograft Systems

Tumor system	Preparation	ID_{50} (μmol/kg/d)	T.I.[a] (g)
Colon 26	COCG-MMC-liposomes	63.4	−0.1
	MMC aq. soln.	12.3	−0.4
MX-1 xenograft	COCG-MMC-liposomes	10.9	−0.6
	MMC aq. soln.	1.0	0.1

a) Each T.I. value is expressed as the body weight change difference (test minus control) at ID_{50}.

xenograft system were 10.9 μmol/kg/d for prodrug-bearing liposomes and 1.0 μmol/kg/d for MMC aqueous solution. Prodrug-bearing liposomes apparently exhibited lower antitumor activity against both solid tumors than MMC aqueous solution.

The host toxicity of these two preparations was estimated on the basis of the body weight change difference (test minus controls). Figure 5A, B shows the dose-body weight change difference profiles in the Colon 26 (A) and MX-1 xenograft (B) systems. In each tumor system, the profile for COCG-MMC-bearing liposomes was shifted to a higher dose region than that for MMC aqueous solution. These results suggest decreased host toxicity of the liposome preparations. Expressing the therapeutic index (T.I.) as the body weight change difference at ID_{50}, the slight difference in T.I. values between the two preparations (Table IV) indicates no

significant overall change in therapeutic efficacy for both tumor systems.

Discussion

Many attempts have been made to improve the therapeutic efficacy of antitumor agents by encapsulation within liposomes.[10] However, no consistent pattern has emerged from previous studies; some researchers have claimed major success in overcoming drug resistance,[11] reducing toxicity[12] and so on,[13] while others have reported disappointing results.[14] This variation in findings can be explained by at least three factors. 1) The quality of liposomes (size, homogeneity, stability *etc.*) was not defined or controlled. 2) Evaluation systems (tumor models, implantation route, therapeutic dosage regimen *etc.*) were different among experimenters. 3) In some cases, the investigators considered the therapeutic and toxic effects in isolation.

Quality of liposomes has proved to affect the *in vivo* kinetics,[15] which may be closely related to the antitumor activity of liposome-encapsulated drugs. As a result, it is essential to control the quality of liposomes for proper evaluation of the antitumor effects of liposomal drugs. As to the quality of liposomes used in the present study, prodrug-bearing liposomes showed slightly increased mean diameters compared with the plain liposomes (preparations B and B′), but size homogeneity was maintained in all the preparations with the exception of preparation H (Table I). Reduced physicochemical stability of preparation H during storage may be due to its size heterogeneity.

As shown in Fig. 2, intravenously-injected COCG-MMC-bearing liposomes maintained the blood levels of regenerated MMC in mice. This finding is consistent with the previous observation[5b] in rats. Prodrug-bearing liposomes seem to act as slow-release reservoirs releasing the parent drug gradually.

In vitro cytotoxicity of COCG-MMC was evaluated using primary cultured P 388 leukemia cells. It showed reduced activity relative to MMC (Fig. 3). This can be explained by the fact that COCG-MMC is a latent form of MMC.[5a] Similar reduction of antimicrobial activity by chemical modification of MMC to lipophilic 1a-*N*-substituted derivatives has been demonstrated by Sasaki *et al.*[16]

Sasaki *et al.* have also carried out fundamental studies on the *in vivo* antitumor effects of lipophilic MMC derivatives in the free and liposome-entrapped forms.[16b,17] However, the experimental tumors studied were limited to L 1210 leukemia[16b,17] and P 388 leukemia,[16b] grown as an ascites tumor, and drugs were administered intraperitoneally (i.p.). Although the i.p.–i.p. system, where direct contact between drugs and target cells may occur, is convenient for screening active compounds, it seems to be impractical from the viewpoint of clinical application. Not only leukemias but also solid tumors are targets of chemotherapy and drugs are usually given *via* the oral or i.v. route.

In the present study, the chemotherapeutic effects of COCG-MMC-bearing liposomes were examined using subcutaneously-implanted solid tumors, which had been proved to show different sensitivities to MMC,[18] and treatments were performed by i.v. injection. In fact, on the basis of ID_{50} values for MMC aqueous solution, MX-1 was twelve times more sensitive to MMC than Colon 26. In both tumor systems (differing in drug sensitivity), COCG-MMC-bearing liposomes exhibited lower antitumor activity and lower host toxicity than MMC aqueous solution (Figs. 4 and 5).

Kaye has pointed out that the effects of liposomal encapsulation on the therapeutic efficacy of antitumor agents should be evaluated in terms of the ratio of their therapeutic/toxic effects (*i.e.*, T.I.).[19] In the present study, the T.I. was expressed as the body weight change difference (an index of host toxicity) at ID_{50}, and the therapeutic efficacy of the preparations was estimated. As shown in Table IV, significant differences in T.I. were not observed between

COCG-MMC-bearing liposomes and MMC aqueous solution. Similar results were obtained for intravenously- or intraperitoneally-implanted L 1210 leukemia systems (unpublished data). It is apparent, in contrast to our hopes, that MMC prodrug-bearing liposomes could not alter the therapeutic efficacy of MMC. As previously predicted by Skipper *et al.*,[20] such modification in pharmacokinetics by sustained-release preparations would result in reduction of the cytotoxicity of phase non-specific drugs including MMC. The results so far seem to confirm their prediction.

Although, in the present case, prodrug-bearing liposomes showed therapeutic efficacy almost equal to that of MMC aqueous solution, the results of the blood disposition study indicate the possibility that sustained blood levels of a drug would be achieved by combined use of a lipophilic prodrug possessing the cholesteryl promoiety and liposome carriers.

For a successful approach in liposomal carrier systems, a drug possessing an action mechanism favorable to the pharmacokinetic properties of the systems should be chosen. In contrast to phase non-specific drugs, the activity of phase-specific antitumor drugs such as 1-β-D-arabinofuranosylcytosine (Ara-C) is markedly dependent upon the schedule of drug administration.[21] Furthermore, there is already clinical evidence that prolonged drug exposure produces optimal therapeutic effects.[22] Judging from these findings, Ara-C seems to be a good candidate for our liposomal delivery systems. Further studies are under way to develop Ara-C prodrug-bearing liposomes for enhancement of its therapeutic efficacy.

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