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Effects of alkyl glycosides incorporated into liposomes prepared from synthetic amphiphiles on their tissue distribution in Ehrlich solid tumor-bearing mice

Yuko Daicho [a], Shoji Okada [b] and Rensuke Goto [a]

[a] *Laboratory of Radiation Chemistry, Graduate School of Nutritional and Environmental Sciences, University of Shizuoka, Shizuoka (Japan) and* [b] *Department of Radiobiochemistry, School of Pharmaceutical Sciences, University of Shizuoka, Shizuoka (Japan)*

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A study of the effects of alkyl glycosides incorporated into synthetic liposomes with respect to their stability, their in vivo distribution in Ehrlich solid tumor-bearing mice and their in vitro interaction with liver cells was undertaken. The synthetic liposomes were prepared from *N,N*-didodecyl-*N*$^{\alpha}$-[6-(trimethylammonio)hexanoyl]-L-alaninamide bromide ($N^+C_5Ala2C_{12}$) and labeled with ^{99m}Tc. *n*-Dodecyl glucoside (DG) and *n*-dodecyl sucrose (DS) were used as alkyl glycosides. The stability was hardly changed by incorporation of alkyl glycosides into the liposomes in saline and serum. The uptake of DG- and DS-modified $N^+C_5Ala2C_{12}$ liposomes decreased in liver and spleen compared with that of unmodified $N^+C_5Ala2C_{12}$ liposomes, resulting in an increase in blood and other tissues such as tumor, duodenum and kidney, where the DS-modified $N^+C_5Ala2C_{12}$ liposomes had a marked tendency. It was observed with electron micrographs that the size of $N^+C_5Ala2C_{12}$ liposomes became small by incorporation of alkyl glycoside. The smaller $N^+C_5Ala2C_{12}$ liposomes were found to result in the lower uptake in liver. The interaction of the liposomes with liver cells in vitro indicated that both DG- and DS-modified liposomes had a low affinity for liver cells compared with the unmodified liposomes and the extent of interaction of the DS-modified liposomes was weaker than that of the DG-modified liposomes.

Introduction

Many investigators have studied liposomes as a carrier for the delivery of the therapeutic or diagnostic agents. However, clinical applications of these liposomes have been limited by a rapid uptake into the reticuloendothelial system (RES). The role of charge, bilayer rigidity, and size of liposomes has been examined to increase uptake of liposomes into tissues other than liver and spleen [1–3]. Allen and Chonn [3] reported that the sphingomyelin/cholesterol (CH) unilamellar liposomes containing sphingomyelin which had a bilayer-rigidifying effect showed an increase in circulation time and a concomitant decrease in uptake into RES compared with phosphatidylcholine (PC)/CH liposomes. Furthermore, some attempts have been made to reduce the liver uptake of radioactive liposomes by pretreatment with a high dose of unlabeled liposomes [4,5] and with reticuloendothelial blockades such as dextran sulfate [6,7].

Another important approach to suppress the uptake of liposomes in RES and to give targeting toward specific tissues is to incorporate a glycolipid into liposomes because glycolipids are considered to play an important role on the cell surface in various biological recognition process. Liposomes having β-galactoside on their surface were preferentially taken up by parenchymal cells, whereas liposomes having α-mannoside were taken up by nonparenchymal cells [8,9], and liposomes containing mannosylated phospholipids were targeted selectively to macrophages [10]. It was found that liposomes contained sialylganglioside, especially, ganglioside G_{M1}, showed a reduced uptake by the RES, resulting in a prolonged circulation times [3,11,12].

On the other hand, the effect of poly(ethylene glycol) (PEG) compounds incorporated into liposomes on their tissue distribution has been recently studied. It was reported that the inclusion of amphipathic PEG in the lipid composition reduced effectively the uptake by

Correspondence: R. Goto, Laboratory of Radiation Chemistry, University of Shizuoka, 52-1, Yada, 422 Shizuoka, Japan.

the RES and resulted in prolonging the circulation time of liposomes [13–16].

Murakami et al. [17] synthesized some amphiphiles containing an amino acid residue in the hydrogen belt, which form stable single-walled vesicles upon sonication in an aqueous medium. Among their amphiphiles, we took up the liposomes formed by *N,N*-didodecyl-*N*$^{\alpha}$-[6-(trimethylammonio)hexanoyl]-L-alaninamide bromide ($N^+C_5Ala2C_{12}$) and labeled the liposomes with ^{99m}Tc by incorporating stearylamine-diethylenetriaminepentaacetic acid (SA-DTPA) as a chelator, where ^{99m}Tc having a short half-life was used for the application to radiopharmaceuticals [18]. The ^{99m}Tc-labeled $N^+C_5Ala2C_{12}$ liposomes were stable compared with liposomes prepared from PC and CH (1 : 1 molar ratio) and were similar in size to small unilamellar vesicles (SUVs) [18]. The liposomes were taken up in the liver and spleen of Ehrlich solid tumor-bearing mice to a large extent [19], while it was found that the liposomes bounded firmly to Ehrlich ascites tumor cells in vitro. Furthermore, it was observed that the pretreatment of dextran sulfate depressed the uptake of $N^+C_5Ala2C_{12}$ liposomes in liver [20].

In this paper, $N^+C_5Ala2C_{12}$ liposomes modified with alkyl glycosides were prepared to attempt the depression of the uptake in RES and the targeting toward specific tissues. Alkyl glycosides having monosaccharide and disaccharide, *n*-dodecyl glucoside (DG) and *n*-dodecyl sucrose (DS) were used to obtain the fundamental information with respect to the effect of glycolipid on the surface of $N^+C_5Ala2C_{12}$ liposomes on the tissue distribution. The stability of the modified $N^+C_5Ala2C_{12}$ liposomes in saline and serum and the tissue distribution in Ehrlich solid tumor-bearing mice were examined. In addition, the in vitro interaction with mouse liver cells was investigated.

Materials and Methods

Materials

Di-*n*-dodecylamine and Boc-L-alanine for the synthesis of amphiphilic compounds were purchased from Kanto Chemical Co., Japan and Peptide Institute Inc., Japan, respectively. *n*-Octyl glucoside (1-*O*-*n*-octyl β-D-glucopyranoside) and *n*-dodecyl glucoside (1-*O*-*n*-dodecyl β-D-glucopyranoside) were purchased from Boehringer Mannheim GmbH, Germany, and *n*-dodecyl sucrose (6-*n*-dodecyl β D-fructofuranosyl-α-D-glucopyranoside) from Mitsubishi Kasei Co., Japan. *n*-Stearyl glucoside (1-*O*-*n*-stearyl β-D-glucopyranoside) was donated through the courtesy of Dr. Hisashi Yoshioka, University of Shizuoka, Shizuoka, Japan. Minimum essential medium (MEM) and fetal bovine serum (FBS) were the products of Nissui Pharmaceutical Co., Japan and Hazleton Biologics Inc., USA, respectively. Bio-Rad protein assay was the product of Bio-Rad Laboratory Ltd., USA. A ^{99}Mo-^{99m}Tc generator was purchased from Daiichi Radioisotope Lab., Japan. Male mice (ddy, 20–25 g) were obtained from Japan SLC, Inc., Japan. Other chemicals used were guaranteed grade.

Preparation of ^{99m}Tc-labeled $N^+C_5Ala2C_{12}$ liposomes modified with alkyl glycoside

$N^+C_5Ala2C_{12}$ was synthesized by the procedure of Murakami et al. [17], and SA-DTPA as a chelator of ^{99m}Tc by the procedure of Hnatowich et al. [21]. The preparation of ^{99m}Tc-labeled $N^+C_5Ala2C_{12}$ liposomes has been previously reported [18]. $N^+C_5Ala2C_{12}$ (5 mM) and SA-DTPA (0.5 mM) were dispersed in 4 ml of saline by a vortex mixer. The suspension was sonicated for a total of 6 min (1 min sonication with 1 min cooling period, repeated six times) with a probe-type sonicator (Tomy, UR200P, Japan), giving clear solution, which was filtered through a 0.2 μm membrane filter (Toyo Roshi, i.d. 25 mm, Japan). A mixture of the liposome solution and stannous chloride solution was adjusted to pH 7.0 and after 10 min, $Na^{99m}TcO_4$ was added. After 40 min, the mixture was filtered through a 0.2 μm membrane filter. ^{99m}Tc-labeled $N^+C_5Ala2C_{12}$ liposomes were purified using a Sephadex G-75 column with saline. ^{99m}Tc-Labeled $N^+C_5Ala2C_{12}$ liposomes modified with alkyl glycoside ($N^+C_5Ala2C_{12}$/SA-DTPA/alkyl glycoside, 10 : 1 : 5 molar ratio) were prepared by the same method as that of ^{99m}Tc-labeled $N^+C_5Ala2C_{12}$ liposomes. The $N^+C_5Ala2C_{12}$ liposomes modified with DG and DS are abbreviated as DG-$N^+C_5Ala2C_{12}$ liposomes and DS-$N^+C_5Ala2C_{12}$ liposomes, respectively. $N^+C_5Ala2C_{12}$ was determined by the Orange II method [20]. The alkyl glycosides were assayed by anthrone-sulfuric acid method [21]. The diameters of liposomes were measured with an electron microscope according to the procedure described previously [16].

Stability of $N^+C_5Ala2C_{12}$ liposomes modified with alkyl glycoside

A mixture of 3.5 ml of the ^{99m}Tc-labeled liposome solution and 3.5 ml of saline or FBS was incubated at 37°C. Aliquots of 1 ml taken periodically were chromatographed on a Sephadex G-75 column with saline. The radioactivity of each fraction was measured in a gamma counter (Packard Auto Gamma 5500). At the same time, the $N^+C_5Ala2C_{12}$ in each fraction was measured by the Orange II method. The stability at each incubation time was determined from the ratio of radioactivity (V) eluted in the void volume to the total radioactivity (T) eluted on a Sephadex G-75 gel filtration and represented as the ratio (%) of $(V/T)_t$ at time = t to $(V/T)_0$ at time = 0 in which the radioactivity in the void volume corresponded to that bounded with $N^+C_5Ala2C_{12}$ liposomes and that in the larger

retention volume corresponded to that released from $N^+C_5Ala2C_{12}$ liposomes, as described in the previous paper [16].

Retention of alkyl glycosides in modified $N^+C_5Ala2C_{12}$ liposomes

Aliquots of a modified $N^+C_5Ala2C_{12}$ liposome solution were chromatographed on a Sephadex G-75 column with saline at 2, 4 and 6 h after preparation on standing at room temperature. $N^+C_5Ala2C_{12}$ and alkyl glycosides in respective fractions in the void volume were determined by the methods mentioned above. The extent of retention of alkyl glycoside in $N^+C_5Ala2C_{12}$ liposomes was expressed as $(R_t/R_0)\times 100$ (%), where the molar ratio of alkyl glycoside to $N^+C_5Ala2C_{12}$ at zero time was R_0 and that at time t was R_t.

Tissue distribution of ^{99m}Tc-labeled $N^+C_5Ala2C_{12}$ liposomes with and without alkyl glycoside in Ehrlich solid tumor-bearing mice

Mice (dd/Y, 20–25 g) bearing Ehrlich solid tumor were obtained by a subcutaneous injection of 0.2 ml of Ehrlich ascites tumor cell suspension ($1\cdot 10^6$ cells/ml in saline) on the left hind leg 7 days before use. At this stage, tumors weighed between 0.5 g and 1.0 g. Tumor-bearing mice (five mice per group) were injected intravenously with 0.2 ml of ^{99m}Tc-labeled synthetic liposomes ($1\cdot 10^7$ cpm/ml in saline, 50 μg of amphiphile per ml in saline). After collecting blood from the corotid artery under etherization at a given time after injection, solid tumor and other organs were excised and weighed. The radioactivity was counted in a gamma counter.

Isolation and culture of liver cells

Mouse liver cells were isolated from male mice weighing 25–30 g essentially according to the collagenase liver perfusion technique described by Seglen [24]. Minor modifications to the method for rat liver were used. Within the anesthetized mouse, the liver was first perfused with Seglen's calcium free buffer and then with collagenase buffer containing Ca^{2+} (37°C, 8 ml/min). The softened liver was removed. The cells were suspended in Hank's buffer and filtered through a stainless filter (200 mesh) to remove cell debris. The cell suspension was centrifuged three times at $50\times g$ for 1 min. The liver cells were consisted of more than 95% parenchymal cells by morphological observation, because the size of parenchymal cells was larger than that of nonparenchymal cells. A portion of 2 ml of the cell suspension ($5\cdot 10^5$ cells/ml in MEM containing 5% FBS) was inoculated into 35 mm plastic culture dishes coated with 0.03% collagen solution and cultured at 37°C in 5% CO_2 incubator (humidity 90%).

Interaction of modified $N^+C_5Ala2C_{12}$ liposomes with cultured liver cells

After the culture of the liver cells for 4 h in 5% CO_2 incubator, the medium was removed from the dishes. A portion of 2 ml of 1 μM ^{99m}Tc-labeled synthetic liposomes ($1\cdot 10^5$ cpm/ml in MEM containing 5% FBS) was added immediately to the dishes. The dishes were incubated for a given time in 5% CO_2 incubator and then washed twice with phosphate-buffered saline. The cells were dissolved in 3 ml of 0.5 M NaOH. The radioactivity was counted in gamma counter. Protein was measured using the Bio-Rad protein assay.

Results and Discussion

Stability of modified $N^+C_5Ala2C_{12}$ liposomes

We reported the labeling of vesicles prepared from $N^+C_5Ala2C_{12}$ by using SA-DTPA as a chelator for ^{99m}Tc and the stability in saline and serum on the basis of release of ^{99m}Tc-labeled SA-DTPA [18]. $N^+C_5Ala2C_{12}$ liposomes were more stable than small unilamellar PC/CH liposomes which were composed of PC and CH(1:1, molar ratio) [18]. In this study, alkyl glycosides such as DG and DS were incorporated into $N^+C_5Ala2C_{12}$ liposomes at a molar ratio of alkyl glycoside to $N^+C_5Ala2C_{12}$ of 0.5. The effect of the incorporated alkyl glycosides on the stability of $N^+C_5Ala2C_{12}$ liposomes in saline was studied. The stability at each incubation time was measured from the ratio of radioactivity (V) eluted in the void volume to the total radioactivity (T) eluted on Sephadex G-75 column and represented as the ratio (%) of $(V/T)_t$ at time $= t$ to $(V/T)_0$ at time $= 0$. In the measurement, the radioactivity of ^{99m}Tc in the void volume was mainly bound to the SA-DTPA incorporated into $N^+C_5Ala2C_{12}$ liposomes because the amphiphiles were detected in the void volume proportional to the radioactivity. The radioactivity in the larger retention volume corresponded to that of ^{99m}Tc-SA-DTPA [18]. It was found that $N^+C_5Ala2C_{12}$ liposomes were kept stable in saline at 37°C after 24 h, and neither DG nor DS affected the stability of the liposomes in saline even after 24 h, as shown in Fig. 1A. However, the stability of the modified $N^+C_5Ala2C_{12}$ liposomes might be apparent because alkyl glycoside was readily released from the liposomes on standing. The extent of release of alkyl glycoside from the modified $N^+C_5Ala2C_{12}$ liposomes was examined by gel filtration on Sephadex G-75 because the alkyl glycosides, eluted in the void volume and in the larger retention volume, were found to correspond to that incorporated into $N^+C_5Ala2C_{12}$ liposomes and to that released from the liposomes, respectively. The extent of retention of alkyl glycoside in $N^+C_5Ala2C_{12}$ liposomes in saline was represented as $(R_t/R_0)\times 100$ (%) when molar ratio of alkyl glycoside to $N^+C_5Ala2C_{12}$ at zero time was R_0 and that at

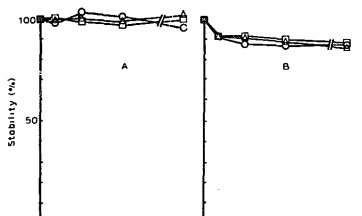

Fig. 1. Stability of DG- and DS-modified $N^+C_5Ala2C_{12}$ liposomes in saline (A) and serum (B). ^{99m}Tc-labeled liposomes were incubated in saline or FBS, and chromatographed on Sephadex G-75. The stability at each incubation time was determined from the ratio of radioactivity (V) in the void volume to the total radioactivity (T) eluted on a Sephadex G-75 gel filtration and represented as the ratio (%) of $(V/T)_t$ at time = t to $(V/T)_0$ at time = 0. ○, $N^+C_5Ala2C_{12}$ liposomes; △, DG-$N^+C_5Ala2C_{12}$ liposomes; □, DS-$N^+C_5Ala2C_{12}$ liposomes.

each time of 2, 4, and 6 h was R_t (Table I). It was obvious that no octyl glucoside could be incorporated into $N^+C_5Ala2C_{12}$ liposomes, but alkyl glycoside having a longer alkyl chain such as stearyl glucoside compared with DG tended to retain more stable in the liposomes than DG. The aikyl glycosides in DG- and DS-$N^+C_5Ala2C_{12}$ liposomes were gradually released from the liposomes with time, but the alkyl glycosides were retained more than 50% at least up to 6 h in the liposomes.

TABLE I

Retention of alkyl glycoside in $N^+C_5Ala2C_{12}$ liposomes in saline

Aliquots of modified $N^+C_5Ala2C_{12}$ liposome solution were chromatographed on a Sephadex G-75 with saline at 2, 4 and 6 h after preparation. $N^+C_5Ala2C_{12}$ and alkyl glycosides in the fraction eluted in the void volume were determined by the Orange II method [21] and the anthrone-sulfuric acid method [22], respectively. The extent of retention of alkyl glycosides in $N^+C_5Ala2C_{12}$ liposomes was expressed as $(R_t/R_0)\times 100(\%)$, where the molar ratio of alkyl glycoside to $N^+C_5Ala2C_{12}$ at zero time was R_0 and that at each time was R_t.

Time after preparation (h)	Extent of retention (%)			
	n-octyl glucoside	*n*-dodecyl glucoside	*n*-stearyl glucoside	*n*-dodecyl sucrose
0	0	100	100	100
2	0	91	100	71
4	0	71	100	50
6	0	50	77	67

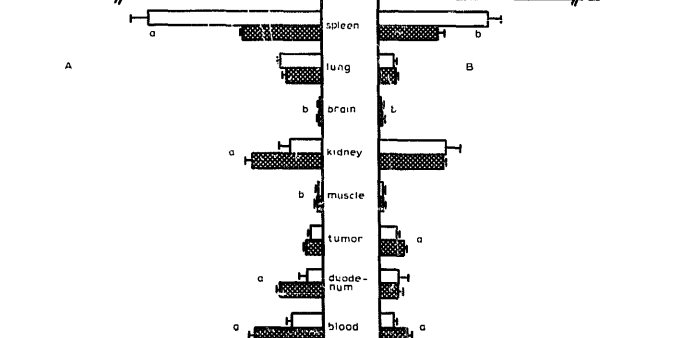

Fig. 2. Distribution of ^{99m}Tc-labeled $N^+C_5Ala2C_{12}$ liposomes with and without DG in Ehrlich solid tumor-bearing mice. Ehrlich solid tumor-bearing mice were i.v. injected with 0.2 ml of ^{99m}Tc-labeled liposomes ($2\cdot10^6$ cpm/10 μg per 0.2 ml), and then treated as described in the text. (A) 1 h after injection; (B) 6 h after injection. □, $N^+C_5Ala2C_{12}$ liposomes; ■, DG-$N^+C_5Ala2C_{12}$ liposomes. Each value is normalized to a body weight of 25 g. Results are expressed as means ± S.D. ($n = 5$). [a] Significantly different from control liposomes, $P < 0.01$; [b] significantly different from control liposomes, $P < 0.05$.

Furthermore, the stability of the modified $N^+C_5Ala2C_{12}$ liposomes in serum was studied in order to examine the effect of high density lipoprotein (HDL) on the liposomes. Fig. 1B shows that the modified $N^+C_5Ala2C_{12}$ liposomes became unstable with time, but the extent of stability was kept at 87%, even after 24 h. When ^{99m}Tc-labelled $N^+C_5Ala2C_{12}$ liposomes were destroyed in the presence of serum, it was assumed that ^{99m}Tc-labeled SA-DTPA incorporated into the liposomes was released and bound with some high molecular components in the serum, and then, consequently, the complexes eluted in the void volume on Sephadex G-75 gel filtration. In the previous paper [18], in order to examine this point, SA-DTPA labeled with ^{99m}Tc was incubated in 50% FBS at 38°C and the mixture was gel filtrated. The peak of radioactivity appeared separately from the void volume, which corresponded to that of radioactive SA-DTPA itself. Therefore, the results in Fig. 1B show the stability of the modified $N^+C_5Ala2C_{12}$ liposomes themselves.

When synthetic liposomes such as $N^+C_5Ala2C_{12}$ liposomes are used as a drug carrier, the toxicity becomes a major problem. However, in the case where synthetic liposomes are used as radiopharmaceuticals, the toxicity becomes less important because it is administered at a minute dose. The stability was hardly affected by incorporation of alkyl glycosides. This shows that $N^+C_5Ala2C_{12}$ liposomes may be useful as a basic structure for surface modification with various compounds such as glycolipids and glycoproteins.

Tissue distribution of modified $N^+C_5Ala2C_{12}$ liposomes in Ehrlich solid tumor-bearing mice

The effects of surface modification of $N^+C_5Ala2C_{12}$ liposomes with alkyl glycoside on the tissue distribution were examined, where Ehrlich solid tumor-bearing mice were used because it had been observed that $N^+C_5Ala2C_{12}$ liposomes had a high interaction with Ehrlich ascites tumor cells in vitro, although the tumor uptake in vivo was low (about 1% dose/g tissue) [19]. Figs. 2 and 3 show distributions of ^{99m}Tc-labeled $N^+C_5Ala2C_{12}$ liposomes modified with DG and DS, respectively. $N^+C_5Ala2C_{12}$ liposomes without alkyl glycoside were used as control liposomes. The results are represented as a percentage of the dose per gram of tissue 1 h and 6 h after injection. The results represent the means ± S.D. for five mice. The uptake of DG-$N^+C_5Ala2C_{12}$ liposomes 1 h after injection

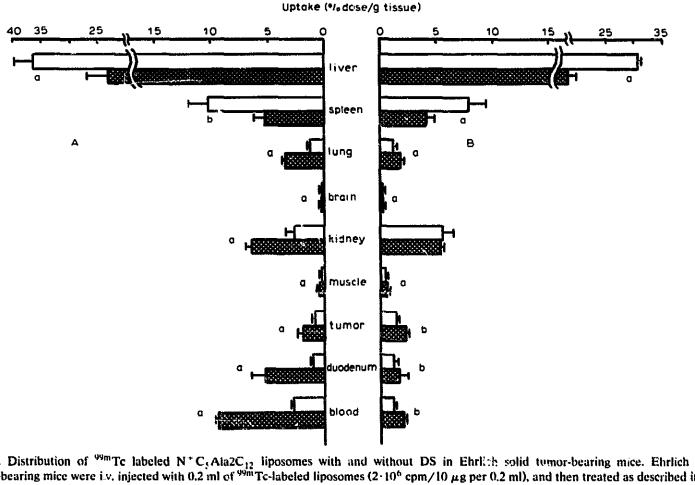

Fig. 3. Distribution of ^{99m}Tc labeled $N^+C_5Ala2C_{12}$ liposomes with and without DS in Ehrlich solid tumor-bearing mice. Ehrlich solid tumor-bearing mice were i.v. injected with 0.2 ml of ^{99m}Tc-labeled liposomes ($2 \cdot 10^6$ cpm/10 μg per 0.2 ml), and then treated as described in the text. (A) 1 h after injection; (B) 6 h after injection. □, $N^+C_5Ala2C_{12}$ liposomes; ■, DS-$N^+C_5Ala2C_{12}$ liposomes. Each value is normalized to a body weight of 25 g. Results are expressed as means ± S.D. ($n = 5$). [a] Significantly different from control liposomes. $P < 0.01$; [b] significantly different from control liposomes, $P < 0.05$.

decreased by about 30% in liver and about 50% in spleen compared with that of control liposomes, and that of DS-N$^+$C$_5$Ala2C$_{12}$ liposomes decreased about 40% in liver and about 50% in spleen. The uptake in both organs 6 h after injection was also decreased. The values of RES/blood for DG- and DS-N$^+$C$_5$Ala2C$_{12}$ liposomes 1 h after injection were 5.3 ± 0.5 (S.D.) and 2.5 ± 0.8 (S.D.), respectively, and they were reduced to about one-third and about one-sixth, respectively, compared with that of the control liposomes. The ratio of %dose in liver plus spleen to %dose in blood is expressed as RES/blood ratio, in which blood volume is taken to be 7% of body weight. These extents were moderately near to that of ganglioside G_{M1} in PC/CH liposomes and to that in sphingomyelin/PC/CH liposomes [3,11], but were significantly high compared with that by G_{M1} in distearoylphosphatidylcholine/CH liposomes 24 h after injection [11]. It was reported that the presence of sialic acid in G_{M1} was clearly a determining factor in the avoidance of RES uptake, and the molecular conformation, the location of the negative charge relative to the phospholipid bilayer, and the packing characteristics of G_{M1} in phospholipid bilayers might be closely related with their avoidance [3,11,12]. Although respective uptakes into RES might significantly depend on used lipids, glycolipid and labeling agents, it was of interest that alkyl glycoside with a simple monosaccharide or disaccharide decreased the ratio of RES/blood.

The decrease of the ratio of RES/blood of both modified N$^+$C$_5$Ala2C$_{12}$ liposomes resulted in increase in other tissues such as tumor, duodenum and kidney 1 h after injection as shown in Figs. 2 and 3, although the uptake in tumor increased to a small extent. On the other hand, a remarkable increase in tumor uptake (up to 25-fold) was observed in G_{M1}-incorporated liposomes labeled using ^{67}Ga-labeled deferoxamine in mice bearing J6456 tumors [11]. Ogihara et al. [2] reported that PC/CH liposomes carrying ^{67}Ga-nitrilotriacetic acid in the tumor of Ehrlich solid tumor-bearing mice were taken up higher than in the liver. Accordingly, it appears that such differences in the labeling agent and in the tumor model make it difficult to compare directly the tumor uptake, as described by Gabizon et al. [11]. However, it was of interest that the uptake in tumor 6 h after injection increased in comparison with that 1 h after injection, in spite of decreases in other tissues.

The reduced uptake of alkyl glycoside-modified synthetic liposomes by RES may be attributed mainly to two factors: (1) the formation of smaller liposomes by incorporation of alkyl glycosides and (2) the reduced affinity of the liposomes for the liver by incorporation of alkyl glycoside. It is well known that the reduction of liposomal size diminishes the uptake of liposomes by RES. It was observed that multilamellar vesicles

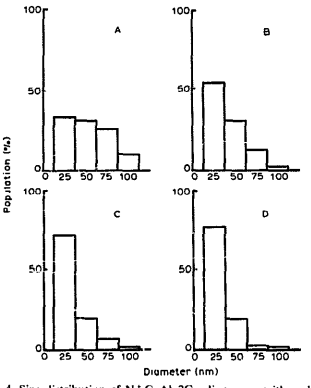

Fig. 4. Size distribution of N$^+$C$_5$Ala2C$_{12}$ liposomes with and without DG. N$^+$C$_5$Ala2C$_{12}$ liposomes with and without DG were sonicated for 10 and 30 min. Size distribution was measured from electron micrographs (Japan Electron Optics Lab. Co., JEM 100S) after negative staining with uranyl acetate. (A) N$^+$C$_5$Ala2C$_{12}$ liposomes sonicated for 10 min; (B) DG-N$^+$C$_5$Ala2C$_{12}$ liposomes sonicated for 10 min; (C) N$^+$C$_5$Ala2C$_{12}$ liposomes sonicated for 30 min; (D) DG-N$^+$C$_5$Ala2C$_{12}$ liposomes sonicated for 30 min.

(MLVs) were predominantly accumulated in RES, but SUVs exhibited a broader tissue distribution than MLVs [1,2]. Furthermore, it was reported that the uptake of liposomes in Kupffer cells decreased and, on the contrary, that in parenchymal cells increased as the liposome size became small [25]. Fig. 4 shows the size distribution of synthetic liposomes prepared by sonication for 10 and 30 min, where the size was measured from electron micrographs negatively stained by uranyl acetate. The size of DG-N$^+$C$_5$Ala2C$_{12}$ liposomes sonicated for 10 min was smaller than that of control liposomes. Sonication for 30 min resulted in decrease in the size of both DG-N$^+$C$_5$Ala2C$_{12}$ liposomes and control liposomes compared with those prepared by sonication for 10 min, and the size of DG-N$^+$C$_5$Ala2C$_{12}$ liposomes was nearly similar to that of control liposomes. The liposomes sonicated for 30 min were taken up less by the liver than those sonicated for 10 min, indicating that the liver uptake decreased with decreasing liposome size (Fig. 5). On the other hand, among the liposomes sonicated for 30 min, the uptake of DS-N$^+$C$_5$Ala2C$_{12}$ liposomes by the liver was significantly decreased compared with that of control and DG-N$^+$C$_5$Ala2C$_{12}$ liposomes, suggesting that the sucrose on the surface of the liposomes took part in diminishing the uptake in liver.

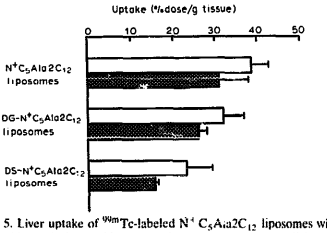

Fig. 5. Liver uptake of ^{99m}Tc-labeled $N^+C_5Ala2C_{12}$ liposomes with and without alkyl glycoside sonicated for 10 and 30 min. Ehrlich solid tumor-bearing mice were i.v. injected with ^{99m}Tc-labeled liposomes ($2 \cdot 10^6$ cpm/10 μg per 0.2 ml) and then treated 1 h after injection as described in the text. □, 10 min sonication; ■, 30 min sonication. Results are expressed as means ± S.D. ($n = 5$).

The uptake of $N^+C_5Ala2C_{12}$ liposomes with alkyl glycoside increased significantly in duodenum 1 h after injection compared with the control liposomes, as shown in Figs. 2 and 3. It was considered that the modified $N^+C_5Ala2C_{12}$ liposomes tended to migrate with bile from liver to duodenum because the contents in the duodenum were not removed when the radioactivity of the tissue was determined. Therefore, the migration of DG-$N^+C_5Ala2C_{12}$ liposomes in the intestine was examined (Fig. 6). The radioactivity of DG-$N^+C_5Ala2C_{12}$ liposomes was observed to be several times higher in small intestine than that of control liposomes 1 h after injection and to migrate to cecum and large intestine 3 h after injection. DS-$N^+C_5Ala2C_{12}$ liposomes also indicated the similar distribution in intestine (data not shown). This might be explained as follows: the size of DG- and DS-$N^+C_5Ala2C_{12}$ liposomes was small compared with that of control liposomes and their modified liposomes had a low affinity for liver, resulting in transferring rapidly into intestine from liver (parenchymal cells) via bile duct.

Interaction of modified $N^+C_5Ala2C_{12}$ liposomes with liver cells in vitro

In order to examine the lower uptake of $N^+C_5Ala2C_{12}$ liposomes with alkyl glycosides in liver in vivo than that of control liposomes, the in vitro interaction of these liposomes with the liver cells consisting mostly of parenchymal cells was investigated (Fig. 7), where the interaction meant both the uptake into the cells and the adsorption on the cell surface. The interaction at 37°C was inhibited significantly by incorporation of DG or DS into the liposomes, and DS-$N^+C_5Ala2C_{12}$ liposomes were inhibited more strongly than DG-$N^+C_5Ala2C_{12}$ liposomes, in agreement with the results of in vivo experiments. Their interactions at 4°C were remarkably decreased compared with those at 37°C, indicating that a metabolic energy was required to incorporate the liposomes into the liver cells.

It has been predicted that surface hydrophilicity might be a key in the phagocytosis of particulate matter [12,13,15]. Illum et al. [26] have shown that hydrophilic coating decreased the uptake of colloidal particles by the liver and by peritoneal macrophages. The prolonged circulation half-lives of liposomes con-

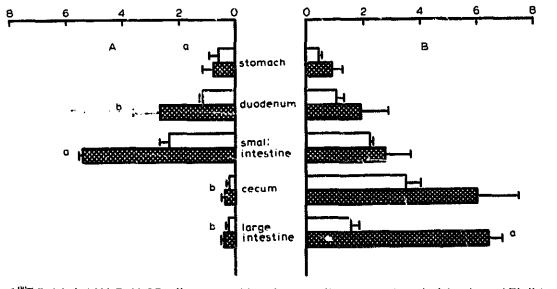

Fig. 6. Distribution of ^{99m}Tc-labeled $N^+C_5Ala2C_{12}$ liposomes with and without DG in gastrointestinal fractions of Ehrlich solid tumor-bearing mice. Experimental procedure was the same as described in the legend of Fig. 2. (A), 1 h after injection; (B) 3 h after injection. □, $N^+C_5Ala2C_{12}$ liposomes; ■, DG-$N^+C_5Ala2C_{12}$ liposomes. Results are expressed as means ± S.D. ($n = 3$). [a] Significantly different from control liposomes, $P < 0.01$; [b] significantly different from control liposomes, $P < 0.05$.

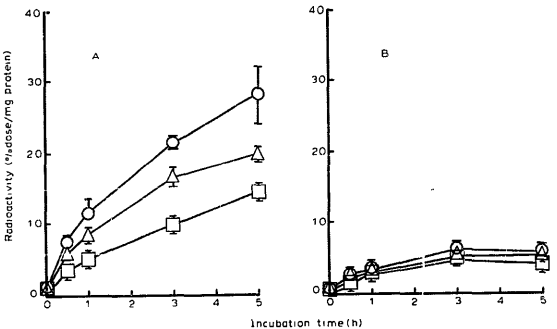

Fig. 7. Interaction of modified $N^+C_5Ala2C_{12}$ liposomes with cultured liver cells in vitro. A portion of 2 ml of 1 μM ^{99m}Tc-labeled synthetic liposomes ($1 \cdot 10^5$ cpm/ml) was added to the dish containing liver cells cultured for 4 h, and then treated as described in the text. (A) Incubated at 37°C; (B) incubated at 4°C; ○, $N^+C_5Ala2C_{12}$ liposomes; △, DG-$N^+C_5Ala2C_{12}$ liposomes; □, DS-$N^+C_5Ala2C_{12}$ liposomes. Results are expressed as means ± S.D. ($n = 3$).

taining G_{M1} has also been ascribed to the increase surface hydrophilicity by incorporation of G_{M1} to the liposomes [12]. A similar mechanism has been proposed for the effect of PEG on PEG-modified liposomes [13,15]. Therefore, it was considered that the reduction of uptake of the modified $N^+C_5Ala2C_{12}$ liposomes by RES was partly due to the increase in the surface hydrophilicity by modifying alkyl glycoside. This was also supported by the result that the extent of interaction of DS-$N^+C_5Ala2C_{12}$ liposomes with liver cells was lower than that of DG-$N^+C_5Ala2C_{12}$ liposomes because DS having two sugar groups might be more hydrophilic compared with DG.

In conclusion, alkyl glycosides such as DG and DS could be incorporated into $N^+C_5Ala2C_{12}$ liposomes, and their modified liposomes were found to be stable in saline and serum. The uptake of the liposomes into the RES was reduced by the modification with alkyl glycosides, especially DS. These results show that these modification of the liposomes with alkyl glycosides may be a useful method for designing liposomes with a long retention time in blood.

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