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Effect of Surface Modification of Liposomes with Sialoglycopeptide on Their Clearance from the Circulation

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The effect of covalent binding of sialoglycopeptide (GP) derived from fetuin to the liposome surface on the clearance of liposomes from the circulation of rats was investigated. Small unilamellar vesicles (SUV) and two types of multilamellar liposomes (MLV, LMLV) were prepared and coupled with GP without changing the size distribution of the liposomes. The leakage of carboxyfluorescein (CF) from these GP-modified liposomes in plasma was measured *in vitro*. The clearance of intravenously injected GP-modified liposomes was followed by monitoring entrapped CF and incorporated [^{3}H]cholesteryl oleate ([^{3}H]CHOL) in the liposomes.

It was shown that CF release from GP-modified SUV increased slightly with increasing amount of GP-modification. On the other hand, CF was released more rapidly from GP-modified MLV and LMLV. The circulation times of MLV and LMLV were reduced by the surface modification of the liposomes with GP. In contrast, the clearance of SUV was inhibited significantly by incorporation of GP on the surface of the liposomes. The clearance pattern of negatively charged liposomes containing dicetyl phosphate, which had equivalent ζ potential and similar size distribution to GP-modified SUV, suggests that the prolonged circulation time of GP-modified SUV is not attributable to the negative surface charge of liposomes. These results suggest that surface modification of SUV with sialoglycopeptide derived from fetuin may be a useful technique for designing liposomes with a long circulation time.

Keywords——liposome; surface modification; sialoglycopeptide; clearance; circulation time

The importance of prolonged presence of drug-entrapped liposomes in the circulation during targeting to specific tissues has been recognized for their successful use as a drug delivery system (DDS). Several attempts have succeeded to some extent in reducing sequestration of liposomes by the reticuloendothelial system (RES) through the manipulation of liposomal surface charge,[1,2] size,[3,4] cholesterol content[5-7] and blocking of RES.[1,8,9] However, for the further development of liposomes as a useful DDS, it seems necessary to reduce RES-uptake of liposomes carrying ligands which are incorporated on the liposomal surface to assist targeting to specific tissues. Earlier reports have shown that the behavior of erythrocytes *in vivo* is regulated by the amount of sialic acid present on their membrane, and desialylated erythrocytes are quickly removed from the circulation.[10-13] Bocci[14] has shown that cells which possess surface sialoglycoproteins escape from RES-uptake. Following these observations, it would seem that incorporation of sialoglycoside in liposomes may lead to the controlled clearance of liposomes from the circulation. To our knowledge, although a few workers have studied the effect of sialoglycolipid[15] or fetuin[16] on the hepatic uptake of liposomes, there has been no investigation of the effect of size distribution on the circulation time of sialoglycopeptide (GP)-modified liposomes.

The present report describes the effect of surface modification of size-defined liposomes by GP derived from fetuin on the clearance from the circulation of rats. Our results indicate that covalent surface modification of liposomes with GP inhibits the clearance of small

unilamellar vesicles (SUV) from the circulation, whereas it enhances the clearance of multilamellar liposomes (MLV and LMLV).

Materials and Methods

Materials——Egg yolk phosphatidylcholine (PC), synthetic dipalmitoyl phosphatidylethanolamine (PE), *N*-acetylneuramic acid (NeuNAc), cholesterol (CH), dicetylphosphate (DCP) and fetuin were purchased from Sigma Chemical Co. (St. Louis, MO). Carboxyfluorescein (CF) was obtained from Eastman (Rochester, NY). Pronase and Cica-705-Test PL Kit were purchased from Kaken Seiyaku Co. (Tokyo, Japan) and Kanto Chemicals Co. (Tokyo, Japan), respectively. Cholesteryl [1,2,6,7-^{3}H]oleate ([^{3}H]CHOL, 110 mCi/mg) and [^{3}H]inulin (308 μCi/mg) were obtained from New England Nuclear (Boston, MA), and Amersham Int. plc (Buckinghamshire, England), respectively. All other reagents were of analytical grade. CF was purified through a Sephadex LH-20 column (Pharmacia Fine Chemicals AB, Uppsala, Sweden) according to Ralston *et al.*[17] Phosphate-buffered saline (PBS, pH 7.4) contained 75.3 mM sodium chloride, 13.3 mM monobasic sodium phosphate and 53.4 mM dibasic sodium phosphate. HEPES buffer (pH 7.5) contained 50 mM HEPES, 23.6 mM NaOH and 76.4 mM sodium chloride.

Preparation of Glycopeptide——The glycopeptide was prepared from calf serum fetuin according to the method of Warren and Fowler.[18] Briefly, 300 mg of calf serum fetuin was incubated in 20 ml of 50 mM HEPES buffer containing 10 mg of pronase and 0.2 ml of toluene at 37 °C for 72 h. After the digestion was terminated, insoluble material was removed by centrifugation and the supernate was fractionated on a Sephadex G-50 column. Each fraction was assayed for carbohydrate by the anthrone method. Absorbance at 225 nm was taken as a measure of peptide content. The faster-eluting carbohydrate peak was collected and lyophilized.

Preparation of Liposomes——Multilamellar liposomes were prepared from 20 μmol of PC, 15 μmol of CH, 5 μmol of PE, and when appropriate, various amounts of DCP. In some experiments 20 μCi (0.18 μg) of [^{3}H]CHOL was incorporated into the lipid mixture to label the lipid membrane of the liposomes.[2,6] After evaporation of the solvent $CHCl_3$, the dry lipid film was dispersed with 100 mM CF in 2 ml of PBS. The suspension was placed in a 25 mm diameter Millipore ultrafiltration cell (Bedford, MA) fitted with a 0.8 μm pore-size Nuclepore (Pleasanton, CA) membrane and extruded twice. The liposomes obtained by this procedure were designated as MLV. Multilamellar liposomes having an improved size-distribution (obtained by eliminating small liposomes) were prepared by washing and filtration of the MLV. PBS (2 ml) was added to 2 ml of MLV and the mixture was filtered in the same cell fitted with a 0.4 μm pore-size Nuclepore membrane until the filtrate reached a volume of 2 ml. This procedure was repeated at least 5 times. The liposomes remaining in the cell were designated as LMLV. Unentrapped CF was removed by gel chromatography on a Sepharose 4B column (1.5 × 40 cm). SUV were prepared from freshly prepared MLV by sonicating in a probe-type sonicator (Tomy Seiko, UR-200R) for a total of 60 min (30 s sonication with 30 s cooling periods) at 0 °C. The lipid suspension was extruded through a 0.1 μm pore-size Nuclepore membrane. Unentrapped CF was removed as described above.

Coupling of Glycopeptide to Liposomes——The glycopeptide was linked to free amino groups of liposomal PE using glutaraldehyde according to the method of Torchilin *et al.*[19] Liposome suspension (5 μmol of lipid/2 ml of PBS) was mixed with 4 mg of GP (contained in 0.5 ml of PBS). Glutaraldehyde was added slowly to the liposome suspension up to 15 mM final concentration and the mixture was incubated at 20 °C. After 10 min, 0.4 ml of 0.2 M lysine solution was added and the mixture was allowed to stand for an additional 2 h. Uncoupled glutaraldehyde, GP and lysine were removed by gel chromatography on a Sephadex 4B column (1.2 × 30 cm). After covalent coupling of the GP to liposomal PE, GP-modified liposomes (MLV, LMLV, SUV) were re-extruded through 0.8 μm, 0.8 μm, 0.1 μm pore-size Nuclepore membrane, respectively, to control the size distribution.

Estimation of Sialic Acid and Lipid——Liposome-bound glycopeptide content was estimated by determination of sialic acid using the method of Jourdian *et al.*[20] Three mol of sialic acid was considered to be equivalent to 1 mol of glycopeptide, assuming that the glycopeptide has three sialic acid residues. Phospholipid concentration in vesicle preparations was measured by enzymatic choline determination using a Cica-705-Test PL Kit. From the phospholipid concentration, the total lipid concentration was estimated and expressed as lipid concentration.

Size Distribution of Liposomes——A small volume of liposome suspension was mixed with equal volume of 2% phosphotungstate and then dialyzed. A drop of the liposomal suspension was placed on a collodion membrane-coated grid. The excess fluid was removed with filter paper and the grid was allowed to dry. The grids were examined under a JEOL JEM 100B electron microscope at 80 kV. The diameters of individual vesicles were measured. The size distribution of the liposomes was determined by counting at least 300 vesicles in three separate experiments. From the size distribution of the liposomes, the radius of the liposome with the average volume ($r_{\bar{v}}$), the radius of the liposome with the average surface area ($r_{\bar{s}}$) and η (ratio of $r_{\bar{v}}^3$ to $r_{\bar{s}}^2$) were calculated according to Pidgeon and Hunt.[21] The encapsulation ratio (E_p), which is defined as the ratio of the total volume of entrapped water (TVW) to the total volume of lipid (TVL), was obtained from an experiment using [^{3}H]inulin. The dry lipid film was dispersed with 2 ml of PBS containing about 0.2 μCi of [^{3}H]inulin. Unentrapped [^{3}H]inulin was removed by gel chromatography, and the radioactivity of liposomes was measured. The total volume of entrapped water was calculated from the value of

encapsulated radioactivity. *TVL*, the total number of liposomes (N_p) and the total surface area of liposomes (TSA_p) were calculated from Eqs. 5, 15, 18, in reference 21, respectively.

Measurement of ζ Potential——The ζ potentials of liposomal suspensions were measured in a PEN KEM Model 500 Laser Zee meter at 10 V/cm, 20 °C.

***In Vitro* Experiments**——Pooled rat plasma (0.6 ml) was mixed with CF containing liposomes (0.05 μmol lipid) and PBS to yield a final volume of 1.0 ml. This mixture was incubated at 37 °C. The lipid concentration and volume ratio of plasma to liposomes were adjusted to be similar to those expected upon intravenous injection into rats (1.0 μmol of lipid). The volumes of blood and plasma in a rat weighing about 200 g were assumed to be 20 and 12 ml, respectively. At set time intervals, duplicate 50 μl samples were taken and rapidly mixed with 4.95 ml of cold PBS. The diluted samples were centrifuged at 3000 rpm for 5 min and the supernate was assayed in the absence (free CF, C_f) and presence (total CF, C_t) of deoxycholic acid (0.4% final concentration) on a Shimadzu fluorimeter, by using excitation and emission wavelengths of 490 and 520 nm, respectively. The amount of entrapped CF (D_e) was calculated from $C_e \cdot V$, where C_e is determined from $C_e = C_t - C_f$ and V is the volume of the incubation mixture. The percentage of entrapped CF after the incubation (D_e) with respect to the initial entrapped CF prior to the incubation (*DE*) was expressed as latency.

$$\text{latency } (\%) = D_e/DE \times 100 \tag{1}$$

***In Vivo* Experiments**——Male Wistar rats weighing 200 ± 30 g were used in all experiments. Each rat was injected intravenously (tail vein) with 0.5 ml of liposome (1.0 μmol of lipid) suspension entrapping 100 mM CF. At set time intervals, 50 μl of blood was collected from the jugular vein and assayed for CF as described for the experiments *in vitro*. The total entrapped CF in the circulation was calculated from $C_e \cdot V_d$, where V_d is the apparent volume of distribution. V_d was estimated from Eq. 2.

$$V_d = DB/C_e(t=0) \tag{2}$$

DB is the injected dose of entrapped CF and C_e ($t=0$) is the C_e value obtained by extrapolation of the initial linear portion of a log C_e *versus* time plot to the concentration axis. Total entrapped CF in the circulation was expressed as a percentage of the injected dose.

$$CF\ (\%\ \text{of dose}) = C_e \cdot V_d/DB \times 100 \tag{3}$$

To estimate the clearance of liposomes, the values of *CF* were corrected as follows:

$$CF'\ (\%\ \text{of dose}) = CF/\text{latency} \times 100 \tag{4}$$

The ^3H radioactivities were measured by decolorizing 0.2 ml of blood with 30% H_2O_2 and 2 N KOH in isopropanol. The mixture was allowed to stand overnight and Sintisol EX-H was added. The samples were neutralized with 10% CH_3COOH, and the radioactivities (C_a) were measured in a scintillation counter (Aloka, LSC-673). The apparent volume of distribution V_d' was estimated as described for entrapped CF. Total radioactivities in the circulation were expressed as a percentage of the injected radioactivities.

$$^3H\ (\%\ \text{of dose}) = C_a \cdot V_d'/DA \times 100 \tag{5}$$

DA is the injected radioactivity of [^{3}H]CHOL.

Results

In order to study the effect of GP-modification of the liposomal surface on liposome clearance from the circulation, other factors such as lipid composition, dose, size and surface charge were kept constant. Throughout the experiments, liposomes were prepared with PC, CH and PE at a molar ratio of 4 : 3 : 1. CH and PE were used to enhance the stability of the liposomes and to bind GP covalently to the liposomal surface, respectively. It is generally accepted that the uptake of liposomes by the RES is the major cause of the elimination of liposomes from the circulation and that RES uptake of liposomes is a saturable process.[22,23] Therefore, in this study we used 1 μmol of PC per 200 g rat body weight as a nonsaturable dose.

Size Distribution of Liposomes

It was reported that the rate of clearance of liposomes from the circulation and the tissue distribution depend on the size distribution of liposomes.[3,4,24-27] In the present study three types of liposomes differing in size distribution were used. Figures 1A and 1a show the vesicle

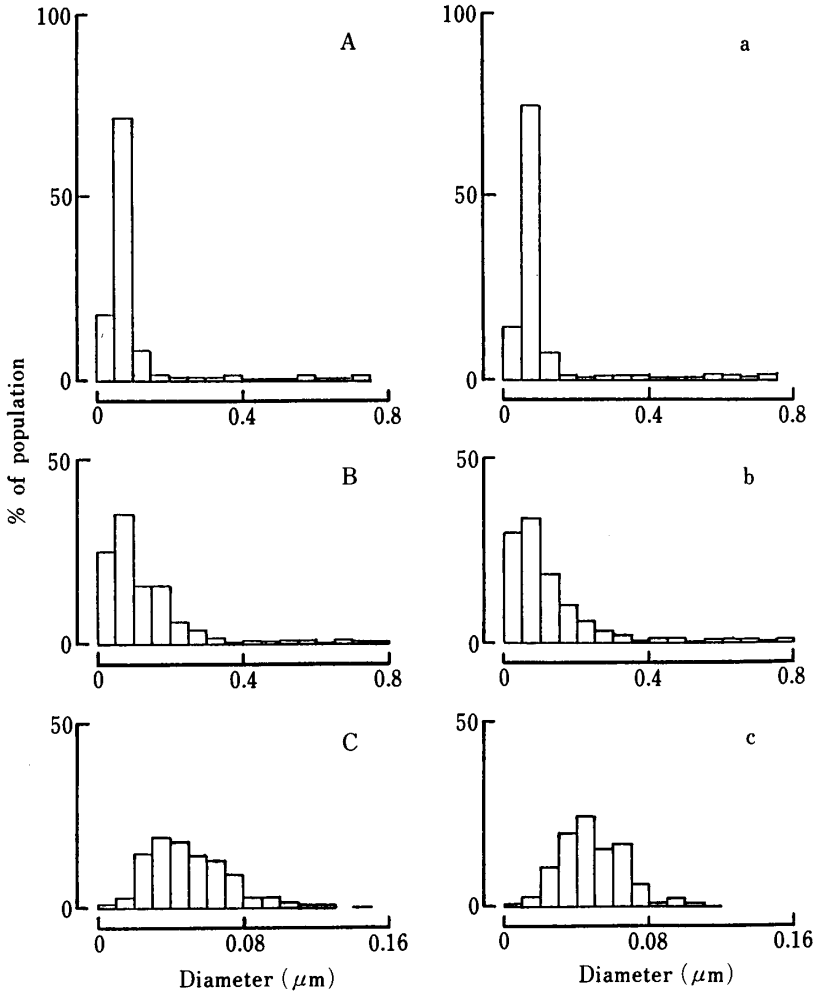

Fig. 1. Size Distribution Determined by Negative-Stain Electron Microscopy of PC/CH/PE (4 : 3 : 1) Liposomes

Vesicles were prepared as detailed in Materials and Methods. A, MLV extruded through a 0.8 μm membrane; a, G-MLV re-extruded through a 0.8 μm membrane after GP-modification; B, LMLV extruded through a 0.8 μm membrane and washed on a 0.4 μm membrane; b, G-LMLV re-extruded through a 0.8 μm membrane after GP-modification; C, SUV extruded through a 0.1 μm membrane; c, G-SUV re-extruded through a 0.1 μm membrane after PG modification.

size distribution of MLV before (A) and after (a) GP-modification, measured from negative stain electron micrographs. There were no vesicles which had a diameter in excess of 0.8 μm. The mean diameters ($\bar{d}$) of MLV and G-MLV were 0.0852 and 0.0848 μm, respectively. These values indicated that the mean diameter of liposomes before extrusion was smaller than the pore diameter of the membrane (0.8 μm). The size distribution of G-MLV presented a very similar pattern to that of MLV, indicating that GP-modification did not change the size distribution. Figures 1B and 1b show the size distributions of LMLV and G-LMLV obtained after extrusion through the 0.8 μm membrane, followed by washing on the 0.4 μm membrane. The mean diameters of LMLV and G-LMLV were 0.109 and 0.106 μm, respectively. SUV and G-SUV exhibited relatively homogeneous distributions of vesicles with mean diameters of 0.0498 and 0.0483 μm, respectively (Fig. 1C and 1c). The radius of the liposome with the average volume ($r_{\bar{v}}$), the radius of the liposome with the average surface area ($r_{\bar{s}}$) and η for G-SUV, G-MLV and G-LMLV, estimated from these vesicle size distributions, are listed in Table I. The encapsulation ratio (E_p), measured as the ratio of TVW to TVL, the total number of liposomes (N_p) and the total surface area of the liposomes (TSA_p) are also given in Table I.

TABLE I. Values of Mean Radius of GP-Modified Liposomes and Calculated Parameters

	$\bar{r}$ (μm)	$r_{\bar{s}}$ (μm)	$r_{\bar{v}}$ (μm)	η (μm)	E_p	N_p [a]	TSA_p [b] (μm^2)
G-SUV	0.0241	0.0265	0.0274	0.0292	0.60	1.14×10^{13}	1.01×10^{11}
G-MLV	0.0424	0.0544	0.0718	0.1250	1.70	1.07×10^{12}	3.99×10^{10}
G-LMLV	0.0529	0.0696	0.1090	0.2680	2.46	3.93×10^{11}	2.38×10^{10}

a) Calculated from Eq. 15 in ref. 21 assuming that *TVL* was 6.155×10^8 ($\mu m^3/\mu mol$). *b*) Calculated from Eq. 18 in ref. 21.

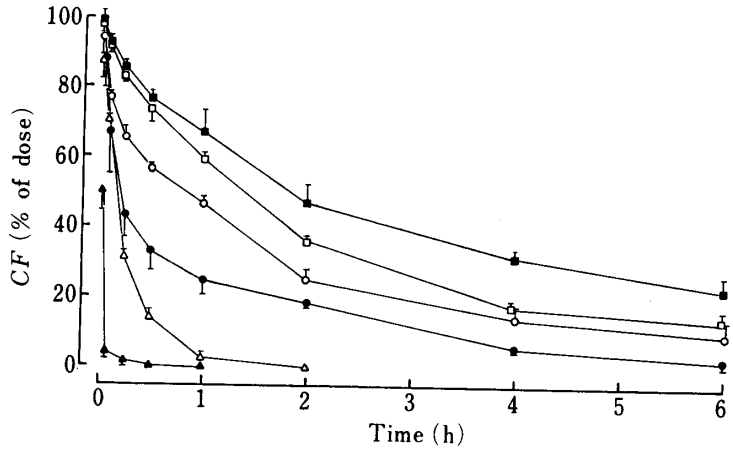

Fig. 2. The Effect of Surface Modification of Liposomes on Their Clearance from the Circulation

Rats were injected intravenously with control MLV (○), G-MLV (●; molar ratio of GP to PE, 0.053), control LMLV (△), G-LMLV (▲; molar ratio of GP to PE, 0.049), control SUV (□) or G-SUV (■; molar ratio of GP to PE, 0.028). Each animal received 0.5 ml of liposome suspension (1.0 μmol of lipid) entrapping 100 mM CF. Total quenched CF in the circulation was measured at various time intervals and the results are expressed as percentage of the injected dose. Each point represents the average of three animals ± S.D.

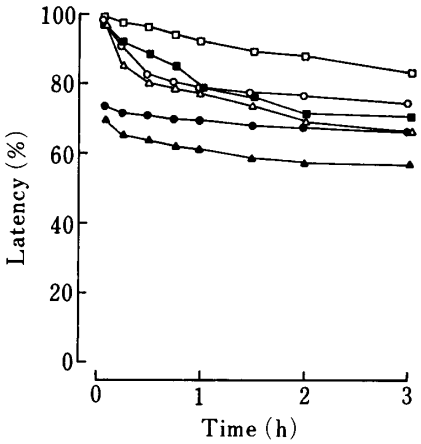

Fig. 3. The Effect of Surface Modification of Liposomes on the Latency in Pooled Plasma

Control MLV (○), G-MLV (●; molar ratio of GP to PE, 0.053), control LMLV (△), G-LMLV (▲; molar ratio of GP to PE, 0.049), control SUV (□) and G-SUV (■; molar ratio of GP to PE, 0.028) were each mixed with pooled rat plasma and incubated at 37 °C. The percentage of entrapped CF with respect to the initially entrapped CF prior to incubation was expressed as latency according to Eq. 1. Each point is the mean of three experiments (S.D. <2%).

Elimination of Liposomes from the Circulation

The effect of surface modification on the elimination of MLV, LMLV and SUV from the circulation is shown in Fig. 2. The ordinate indicates the intact liposomes expressed as entrapped CF with respect to the intravenously administered dose, as determined by means of Eq. 3. The period of time required for the initial dose to be reduced to half (t_{50}) decreased with increase of the diameter of the liposomes. The t_{50} values for control SUV, MLV and LMLV were 1.4, 0.8 and 0.2 h, respectively. As is evident from Fig. 2, the rates of clearance of MLV

TABLE II. *In Vivo* Clearance and *in Vitro* Release of Liposomal CF

	Liposome	GP/PE (Molar ratio)	GP/TSA_p[a] (mol/μm^2)	t_{50}[b] (h)	Latency[c] (%)	CF'[d] (% of dose)
SUV	Control	0	0	1.4	90	56
	G-SUV	0.028	3.46×10^{-20}	1.9	77	78
MLV	Control	0	0	0.8	80	63
	G-MLV	0.053	1.66×10^{-19}	0.2	70	40
LMLV	Control	0	0	0.2	90	56
	G-LMLV	0.049	2.57×10^{-19}	0.02	67	4

a) Values from Table I. *b*) t_{50} was estimated from Fig. 2. *c*) Estimated from Fig. 3 for periods of time equivalent to t_{50} of the respective control liposomes. *d*) Estimated by Eq. 4 at times equivalent to t_{50} of the respective control liposomes.

TABLE III. Clearance of G-SUV from the Circulation Carrying Various Amounts of Sialoglycopeptide

Liposome	GP/PE (Molar ratio)	GP/TSA_p (mol/μm^2)	Time (min)	Liposomal ^3H (% of dose)[a]	Entrapped CF (% of dose)[b]	$\frac{^3H}{CF}$	Latency (%)[c] *in vitro*	Corrected entrapped CF CF' (% of dose)[d]	$\frac{^3H}{CF'}$
Control SUV	0		5	93.3±2.05	91.2±2.90	1.02	99.2	91.9	0.99
			15	90.1±2.16	82.5±3.54	1.09	97.2	84.9	0.97
			30	73.5±5.61	72.9±3.42	1.00	95.9	76.0	0.96
			60	59.7±0.64	58.7±3.30	1.02	92.3	63.6	0.92
			120	31.2±2.59	36.0±2.20	0.87	87.8	41.0	0.88
	0.012	1.48×10^{-20}	5	96.7±4.50	89.8±4.98	1.07	98.2	91.4	1.06
			15	85.8±0.57	81.8±1.28	1.05	93.5	86.7	0.99
			30	79.3±2.97	71.7±0.80	1.11	91.0	78.8	1.01
			60	71.4±1.01	62.6±2.48	1.14	88.0	71.1	1.00
			120	59.5±4.64	48.5±3.60	1.22	79.8	60.8	0.98
G-SUV	0.028	3.46×10^{-20}	5	99.1±1.54	90.7±1.41	1.09	96.6	93.9	1.06
			15	87.1±1.37	84.6±1.64	1.02	92.0	92.0	0.95
			30	83.8±3.64	71.0±2.53	1.18	88.7	80.0	1.05
			60	72.0±7.10	57.8±5.38	1.25	78.6	73.5	0.98
			120	59.6±7.93	47.2±4.60	1.26	72.8	64.8	0.92
	0.137	1.69×10^{-19}	5	95.3±4.35	95.1±0.70	1.00	95.8	99.3	0.96
			15	89.6±3.49	82.2±2.40	1.09	91.7	89.6	1.00
			30	84.5±3.57	75.1±2.87	1.13	86.1	87.2	0.97
			60	83.1±2.76	64.7±0.68	1.28	79.6	81.2	1.02
			120	68.8±6.03	49.4±3.04	1.39	72.9	67.8	1.01

Total radioactivities and entrapped CF in the circulation were measured at various time intervals and the results are expressed as percentages of the injected dose (mean ± S.D.). Entrapped CF in the circulation was corrected by using the *in vitro* latency according to Eq. 4 and is listed as CF'. *a*) Calculated from Eq. 5. *b*) Calculated from Eq. 3. *c*) Calculated from Eq. 1. *d*) Calculated from Eq. 4.

and LMLV from the circulation increased markedly after GP-modification (t_{50} for G-MLV was 10 min, t_{50} for G-LMLV was 1 min). G-LMLV was removed completely within 15 min of injection. In contrast, the rate of clearance of SUV decreased significantly after GP-modification of the liposomal surface (t_{50} was 1.9 h for G-SUV). Since the amount of entrapped CF in the circulation depends on both liposome clearance and CF release through the liposomal membrane, it is necessary to correct for the effect of CF leakage in order to estimate the liposome clearance. It was shown[6] that rat whole blood affects liposomal permeability even less than plasma, so it is probable that the CF release caused by plasma *in*

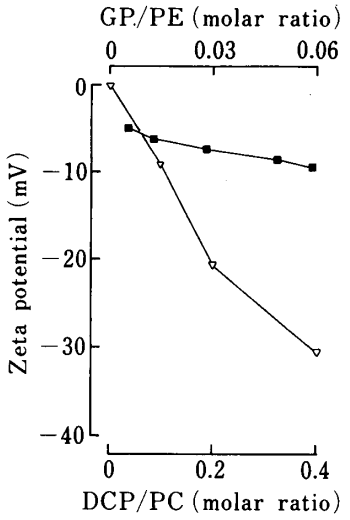

Fig. 4. Zeta Potentials of SUV

The ζ potentials of G-SUV (■; with varying molar ratio of GP/PE) and the ζ potentials of negatively charged SUV (▽; PC/CH/DCP=4:3:0—1.6) were measured at 10 V/cm, at 20 °C.

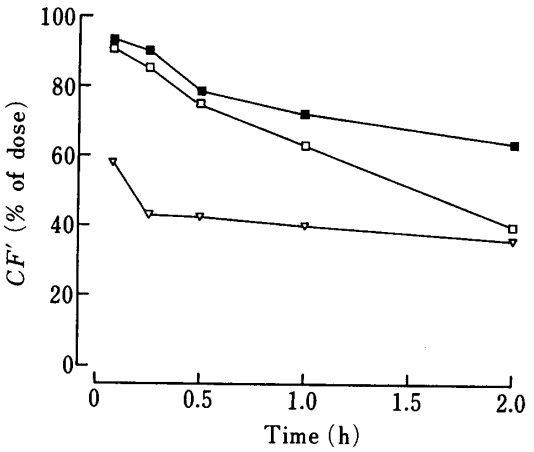

Fig. 5. Comparison of the Clearance of G-SUV with the Clearance of Negatively Charged SUV from the Circulation

Rats were injected intravenously with control SUV (□), G-SUV (■; molar ratio of GP to PE, 0.028; ζ potential, −8 mV) or negatively charged SUV (▽; molar ratio of DCP to PC, 0.1). The percentage of entrapped CF in the negatively charged SUV to administered dose was corrected by using *in vitro* latency according to Eq. 4 and expressed as corrected % of dose. Values for control SUV and G-SUV were taken from the data in Table III.

vitro is comparable to that occurring *in vivo*. The time course of CF release in plasma from SUV, MLV and LMLV and also the effect of GP-modification on the latency of liposomes are shown in Fig. 3. The latency for these three types of liposomes was reduced by GP-modification. Latencies of G-MLV and G-LMLV decreased rapidly immediately after incubation (to about 70% within 5 min), and thereafter decreased slowly. On the other hand, the latency of SUV was reduced at an even lower rate. The values of t_{50} for respective preparations, the latencies at the time equivalent to t_{50} of the respective control liposomes, and the values of *CF* (% of dose) estimated from Eq. 4 are summarized in Table II. About 63% of CF entrapped in MLV still remained at 0.8 h after injection, whereas only 40% of CF remained in G-MLV at the same period of time. Entrapped CF in G-LMLV was removed more rapidly than that in G-MLV, with less than 4% of the dose remaining in the blood at 0.2 h after injection. In contrast, entrapped CF in G-SUV exhibited a slow clearance, with about 78% of the dose being present in the blood at 1.4 h, suggesting that the clearance of SUV was inhibited significantly by the incorporation of GP on the liposomal surface. These results clearly indicate that the effect of GP-modification on the clearance of liposomes depends on the size of the liposomes.

GP-Dependent Clearance of SUV

The relationship between the rate of clearance of SUV and the amount of GP-modification was studied by using CF and [^{3}H]CHOL as a nonexchangeable membrane radioactive marker. Control SUV and G-SUV differing in the amount of GP-modification (the molar ratio of GP to PE was 0.012, 0.028 or 0.137) were injected intravenously into rats, and entrapped CF and radioactivity of [^{3}H]CHOL in the circulation were measured at set time intervals. The disappearance of entrapped CF and [^{3}H]CHOL from the circulation was reduced with increasing amount of GP-modification. In contrast to the clearance of [^{3}H]CHOL, there was no significant difference of entrapped CF between G-SUVs. This

different behavior of the two markers appears to result from CF leakage through the liposomal membrane. The values of $^3H/CF$ in Table III, which show the ratio of liposomal [^{3}H]CHOL to entrapped CF, became larger than 1 with increasing amount of GP-modification, suggesting that G-SUV became more permeable with increase of GP-modification. In order to estimate the clearance of the liposomes from the circulation, the *in vitro* CF-leakage from each preparation in plasma was measured and is listed as latency in Table III. Entrapped CF in the circulation was corrected with this latency according to Eq. 4 and is listed as CF' (corrected entrapped CF). The values of $^3H/CF$ were very close to 1, indicating that CF' reflects the *in vivo* behavior of the liposomes.

Effect of Surface Charge

It is well known that the clearance of liposomes from the circulation is influenced by the surface charge of the liposomes.[1,3] A possible explanation for the diminished clearance of GP-modified SUV from the circulation is that negative surface charges imparted to the liposomes by sialic acid might be involved in the elimination mechanism of liposomes. This possibility was tested with PC/CH/DCP liposomes which had equivalent ζ potential to G-SUV. Figure 4 shows the changes of ζ potentials of liposomes with GP-modification and DCP content. The ζ potential of G-SUV at GP/PE equal to 0.028 (about -8 mV) corresponded to the potential of negatively charged SUV at DCP/PE equal to 0.1 (about -9 mV). Accordingly, *in vitro* latency and *in vivo* clearance of negatively charged SUV were examined (at a molar ratio of DCP to PC was 0.1). The size distribution of negatively charged SUV was similar to that of G-SUV (GP/PE was 0.028; data not shown). The percentage of entrapped CF in negatively charged SUV with respect to administered dose was corrected by using the *in vitro* latency (data not shown). The time course of CF' (% of dose) is shown in Fig. 5. As is evident from Fig. 5, negatively charged SUV were cleared more rapidly than G-SUV or control SUV. Thus, the negative charge of sialoglycolipid may not account for the decreased clearance rate of G-SUV.

Discussion

In the present work we studied the effect of GP-modification of three types of liposomes differing in size (SUV, MLV, LMLV) on their clearance from the circulation in rats. G-MLV and G-LMLV were cleared more rapidly than the respective control liposomes. The rate of clearance of liposomes increased with increase of the diameter of the liposomes. In contrast, the rate of clearance of G-SUV decreased significantly with increasing amount of GP-modification. In order to compare the effects of surface modification of SUV to MLV or LMLV, surface density must be considered, since at the same lipid concentration SUV has about four times as much surface area as LMLV, as can be seen in Table I. Based on the data shown in Table II, the surface density of G-MLV was 4.8 times greater than that of SUV. However, if one compares the behavior of G-MLV ($GP/TSA_p = 1.661 \times 10^{-19}$ mol/μm^2) with that of G-SUV ($GP/TSA_p = 1.692 \times 10^{-19}$ mol/μm^2 in Table III), both of which have almost the same surface density, then one sees that G-MLV were cleared more rapidly and G-SUV were cleared more slowly than the respective control liposomes. Thus, the effect of GP-modification on the clearance of liposomes from the circulation depends on the size distribution of the liposomes.

There are two possible explanations for the decreased clearance of G-SUV from the circulation: (1) inhibition of the uptake of SUV by liver, spleen or other organs. (2) increase of the affinity for blood components as a result of GP-modification. Lelkes and Tandeter[28] have recently suggested that the association between cholesterol-rich liposomes and red blood cells may be responsible for their slow clearance from the circulation. However, the latter possibility

seems unlikely, as the interactions between liposomes and blood components should decrease after GP-modification, considering the negative surface charge of G-SUV. Many studies[29-31] have shown that the uptake of liposomes by the reticuloendothelial system is the major cause of the elimination of liposomes from the circulation. Rahman *et al.*[27] suggested that large multilamellar liposomes are mainly taken up by the Kupffer cells, whereas small unilamellar liposomes (diameter about 0.08 μm) are taken up by parenchymal cells. This is presumed to be due to the inability of larger particles to penetrate the gaps in the hepatic sinusoids. It has also been reported that the phagocytosis of liposomes by macrophages was diminished significantly by binding of the sialoglycoprotein, fetuin, to the liposome surface.[16] Utsumi *et al.*[32] have shown that the phagocytic reaction of human PMN cells was markedly suppressed when sialoglycoprotein of human erythrocytes was incorporated into the target liposomes. Our present results suggest that in the case of G-SUV, the uptake of liposomes by Kupffer cells or parenchymal cells is inhibited by GP-modification. However, in the case of MLV or LMLV, another factor, which increases the clearance of liposomes, such as adsorption of opsonin may be predominant. Further studies are under way to investigate the tissue distribution of G-SUV and the effect of GP-modification on the uptake by reticuloendothelial tissues such as liver and spleen.

Khaw and his co-workers[16] have shown that liposomes covalently bound with fetuin alone or both IgG and fetuin were rapidly removed from the circulation, but liposomes hydrophobically bound with fetuin were cleared from the circulation more slowly. Our preliminary study using SUV, MLV and LMLV, which were covalently bound with fetuin has shown that only fetuin-modified SUV were cleared more slowly than control SUV (data not shown). Although the reason for the difference between the two studies is not apparent, it is conceivable that the size distribution of liposomes did not change when fetuin was coupled by hydrophobic interaction, but when fetuin was bound covalently by glutaraldehyde, liposomes were linked with each other to form aggregates. The aggregated liposomes would be cleared from the circulation rapidly. In the present study, glycopeptide was covalently bound to liposomes, which were then filtered with membrane filters to control the size distribution.

It is of interest that the negatively charged SUV, which had equivalent ζ potential and similar size distribution to G-SUV, were cleared more rapidly than G-SUV. Therefore, it could be attributed to the glycoside residues bound to the liposomal surface that the circulation time of G-SUV was prolonged in spite of the negative charge of the liposomes.

In order to target liposomes to specific tissues, it is desirable that the liposomes should have a long circulation time with reduced affinity for the reticuloendothelial system. The present results have shown that, although it is necessary to improve the latency, the circulation time of SUV could be controlled by GP-modification. It is of interest in designing liposomes with a long circulation time that the clearance of SUV was inhibited by sialoglycopeptide derived from fetuin.

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