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Application of Synthetic Alkyl Glycoside Vesicles as Drug Carriers. III. Plasma Components Affecting Stability of the Vesicles[1)]

HIROSHI KIWADA,*,[a] IWAO NAKAJIMA,[a] HIROSHI MATSUURA,[a] MITSUKO TSUJI[b] and YURIKO KATO[b]

Faculty of Pharmaceutical Sciences, University of Tokushima,[a] 1–78–1, Shomachi, Tokushima 770, Japan, and Faculty of Pharmaceutical Sciences, Science University of Tokyo,[b] 12, Ichigaya Funagawaramachi, Shinjuku-ku, Tokyo 162, Japan

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Long-chain alkyl glycosides form liposome-like vesicles. However, they are unstable in plasma and thus are unsuitable as drug carriers. The mechanisms causing the instability of palmitoyl glucoside vesicles (Glu-liposomes) in plasma were investigated in this study. They very rapidly released about 70% of their aqueous content at the start of incubation with fresh rat plasma at 37 °C. On the other hand, phosphatidylcholine liposomes (PC-liposomes) released about 30% of their content, though the release pattern was very similar. Two components were suspected to be involved in destabilizing the Glu-liposomes in plasma from a plasma dilution experiment, and their effects seemed to depend on the type or size of the vesicles. The activity disappeared on pre-heating of the plasma at 56 °C for 30 min in the case of PC-liposomes, but not Glu-liposomes, and about 35% of the contents of the latter was still released on incubation even with pre-heated plasma. This result indicates that the activity destabilizing glycoside vesicles in plasma was composed of two factors, one heat-stable and the other heat-labile. The heat-stable one was consumed by incubation with empty glycoside vesicles, regardless of the sugar moiety or size of vesicles, but not by PC-liposomes. Therefore, the heat-stable factor seemed to be specific to vesicles covered with sugar moieties. By fractionation of plasma protein by the salting-out technique, the activity was found in the albumin fraction.

Keywords——liposome; drug carrier; alkyl glycoside vesicle; palmitoyl glucoside; stability; release; plasma protein

Introduction

Alkyl glycosides form liposome-like vesicles (AGV). Recently, several types of synthetic liposomes based on non-phospholipid amphiphiles have been reported.[2)] We have studied the application of alkyl glycoside vesicles as drug carriers, and the preparation and physical properties of the vesicles were reported previously.[3)] Their surface carries sugar moieties, and therefore they are expected to be useful as targetable drug carriers having carbohydrate as an affinity probe. The tissue distribution and pharmacokinetics of the targeting efficacy of these vesicles were studied. The superior accumulation of galactoside vesicles in the liver as compared with conventional phosphatidylcholine liposomes was reported.[4)]

For the application of the vesicles as drug carriers, stability in the plasma is a very important property. If vesicles are unstable in the plasma, the water-soluble contents will be immediately released into the blood and excreted after the injection, and the vesicles will not be useful as drug carriers. Though the AGV showed higher stability than conventional phosphatidylcholine liposomes, a part of their contents was rapidly released on incubation with fresh rat plasma at 37 °C, as shown in the previous paper.[3)]

Liposomes are candidates for effective drug carriers *in vivo*.[5] However, there are problems, such as uptake by the reticuloendothelial system (RES), and the stability in the blood. (A part of intravenously injected liposomes rapidly releases the contents into the blood.) In order to overcome the stability problem, some attempts were made to polymerize liposomal lipids[6] or to form a polycarbohydrate coating.[7] These attempts gave good results *in vitro*, but it is not clear whether these modifications affect the biological fate of the liposomes *i.e.* interaction with RES and/or other biological systems. On the other hand, there have been many investigations on the mechanisms of the instability of liposomes in the blood[8]: exchange of lipids between liposomes and high-density lipoprotein (HDL)[9] and extraction of lipids by albumin[10] are possibilities. Hydrolysis of phospholipid by phospholipase A[11] or participation of lecithin-cholesterol acyltransferase was also considered.[12] The existence of a transfer-stimulating factor of liposomal phospholipid to HDL in plasma was suggested.[13] Recently, the mechanisms were investigated in detail and it has become apparent that the hydrolysis of phospholipids in the outermost layer by phospholipase does not cause the release of the contents, whereas the extraction of the lipid by HDL does cause it.[14] Nagpurkar *et al.* reported the binding on liposomes of the phosphocholine-binding protein in rat serum.[15] A role of complement-activating factor or opsonin of this protein such as C-reactive protein (CRP)[16] is also suspected. On the other hand, it was reported that serum components rather stabilize liposomes below the gel-liquid transition temperature of phospholipids.[17] It is still not clear what components cause the very rapid destabilization of liposomes in the blood.

In this study, the factor causing rapid lysis of cetylglucoside vesicles in the plasma was investigated and the effect was compared with that on conventional phosphatidylcholine liposomes.

Experimental

Materials——Palmitoyl glycosides were synthesized as described in the previous paper.[3] Hydrogenated egg-phosphatidylcholine (PC) was a gift from Nippon Fine Chem. Co., (Osaka). Dicetyl phosphate (DCP) and cholesterol (CH) were purchased from Nakarai Chem. Co. (Kyoto) and Kanto Chem. Co. (Tokyo), respectively. 5(6)-Carboxyfluorescein (Eastman Kodak, N.Y.) was used without further purification. All other chemicals and reagents were obtained and used as described in the previous papers.[3,4]

Preparation of Liposomes——Liposomal types used in this experiment were MLV (multilamellar vesicles), REV (reverse-phase evaporation vesicles) and SUV (small unilamellar vesicles). They were prepared by the methods described previously.[3] Sonication for SUV preparation was carried out with a probe-type sonicator (Tomy Seiko, UR-200P) or a bath-type sonicator (Tocho, IUC-2811) until the suspension become semi-clear. 5(6)-Carboxyfluorescein (CF) was used as an aqueous marker.[18] The marker solution was prepared by dissolving 25 mmol of CF in 1.4 ml of 5 N NaOH solution and adjusting the pH and osmotic pressure to 7.4 and 280 mOsm/Kg, respectively, by dilution with distilled water. Lipid composition of the liposomes was liposomal base (cetylglycoside or PC), DCP and CH in a molar ratio of 4 : 1 : 4 and the lipid concentration in the preparation was 100 μmol/5 ml (total lipids). Unencapsulated marker was removed by dialysis in cellulose dialysis tubing.[3] When required, liposomes were sized by the extrusion and dialysis method described in the previous paper.[4]

Procedure——Whole blood of a Wistar male rat (body weight 250 g) was drained through polyethylene tubing inserted in the carotid artery into a heparinized test tube. Fresh plasma was obtained after centrifugation at 3000 rpm for 10 min. The plasma was treated as described later.

A 0.1 ml aliquot of liposomal suspension (2 μmol as total lipids) was incubated with 0.9 ml of the plasma at 37 °C. At 1.5, 3.0, 6.0, 12.0, 18.0, 30.0, 60.0, 90.0, and 120.0 min after the start of the incubation, a 10 μl aliquot was transferred into 5 ml of cold phosphate-buffered saline (PBS). A 1 ml aliquot of the diluted mixture was well mixed with 1 ml of 5% Triton X-100 solution and then 2 ml of water was added. The latency of CF in the liposomes was calculated from the fluorescence intensities with and without Triton X-100 treatment and expressed as a percentage of the initial latency.[18]

Fractionation of Plasma Protein——Plasma proteins were fractioned by the salting-out technique with ammonium sulfate according to Cohn *et al.*[19] Powdered ammonium sulfate was added to the pre-heated plasma at the concentration of 33% saturation and the precipitate (0—33% fraction) was washed and dialyzed. Ammonium sulfate was successively added to the supernatant to obtain higher saturation fractions.

Results and Discussion

Release Profiles of CF from Liposomes on Incubation with Fresh Plasma

Time courses of latency of CF contained in MLV (not sized) during incubation with fresh rat plasma at 37 °C are shown in Fig. 1. Palmitoyl glucoside liposomes (Glu-liposomes) very rapidly released the marker at the start of the incubation. About 70% of the contents was released, followed by a slow release phase. On incubation with PBS very slight release was observed. Therefore, it is considered that Glu-liposomes are lyses immediately on incubation with plasma, but only to the extent of 70%. This release profile from Glu-liposomes in plasma raised the following possibilities. (1) The destabilizing factor(s) is very potent but exists in a small amount in the plasma and does not recover its activity after acting. (2) It is inactivated immediately after the interaction with liposomes or on incubation at 37 °C. (3) It cannot affect the inner layers of the liposomes and the contents in the inner compartments do not leak into the medium. (4) It can affect only a part of the liposomal preparation, *e.g.* larger liposomes.

PC-liposomes showed similar release profiles to Glu-liposomes. However, the released percentage was very much smaller than that of Glu-liposomes (about 30%). This result suggests that PC-liposomes are more stable than Glu-liposomes, possibly because PC has two hydrocarbon anchors for forming a lipid layer though alkyl glucoside has one as discussed in a previous paper.[3] It is also considered that the destabilizing factor(s) or mechanism(s) is different between PC-liposomes and Glu-liposomes, as suggested in a previous paper.[4]

The following experiments were carried out to elucidate the factor(s) and mechanism(s) of destabilization of the alkyl glycoside vesicles in plasma.

Effect of Dilution of Plasma on the Destabilization of Glu-Liposomes

The release of CF from Glu-liposomes in plasma was limited to 70% of total CF content as mentioned above. If the limitation was caused by the small amount of a factor in the plasma, the release amount of CF should be proportional to the amount of the plasma. The release in 1 h with diluted plasma samples is shown in Fig. 2. The release did not decrease with increasing dilution at lower dilution ratios (1/2—1/8), but decreased drastically at high dilution ratios (1/16<). The results indicate that the amount of the destabilizing factor in plasma was sufficient but the activity was saturated or masked under normal conditions.

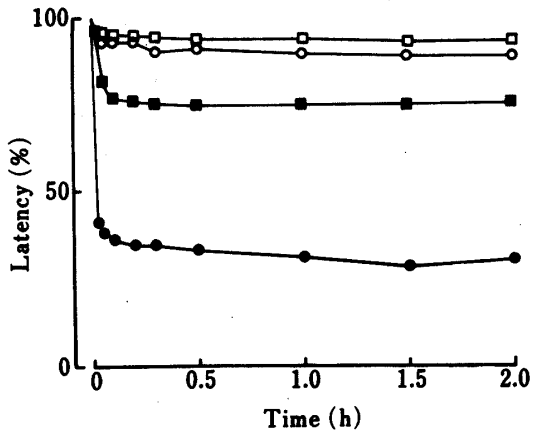

Fig. 1. Release Profiles of CF from Liposomes Incubated at 37 °C

Liposomes containing CF were incubated with fresh rat plasma (●, Glu-MLV; ■, PC-MLV) or PBS (○, Glu-MLV; □, PC-MLV) at 37 °C. CF latency values were obtained by taking 10 μl aliquots periodically. They are expressed as percentages of the latencies in the respective liposomal preparations. Points are mean ± S.D. of three experiments.

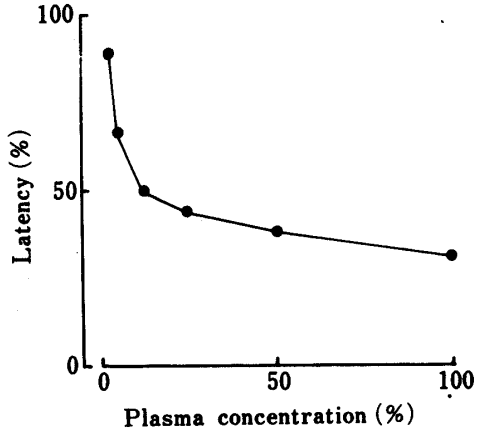

Fig. 2. Effects of the Plasma Concentration on the Latency of CF after Incubation at 37 °C for 1 h

Liposomes (Glu-MLV) containing CF were incubated with fresh rat plasma at various concentrations at 37 °C for 1 h. Latency values were obtained in the same manner as in Fig. 1.

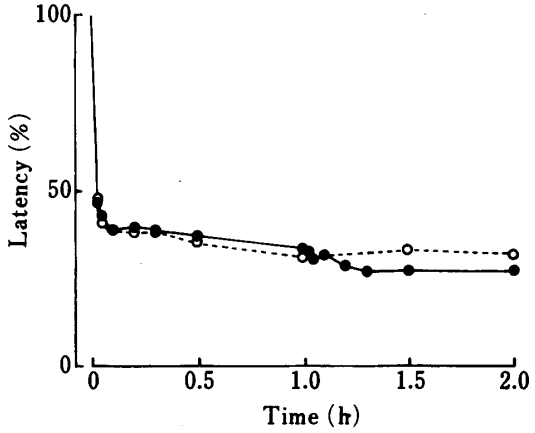

Fig. 3. Effect of Addition of Fresh Plasma on the Release Profiles

●: Liposomes (Glu-MLV) were incubated under standard conditions (0.1 ml of liposomal suspension +0.9 ml of fresh plasma). After 1 h of incubation, 0.9 ml of fresh plasma was added to the incubation mixture. ○: Liposomes were incubated in two volumes of fresh plasma (0.1 ml of liposomal suspension + 1.8 ml of fresh plasma) from the initiation of the incubation. Sample treatments were the same as in Fig. 1.

TABLE I. Latency of CF Encapsulated in Various Types of Glu-Liposomes after Incubation with Fresh Rat Plasma for 1 h

Type of liposomes	Latency (%)
Glu-MLV	37.6±1.3
Glu-REV	55.9±1.5
Glu-REV (extruded)	66.2±2.4
Glu-SUV	87.1±1.7

Values are expressed as means±S.D. of three experiments.

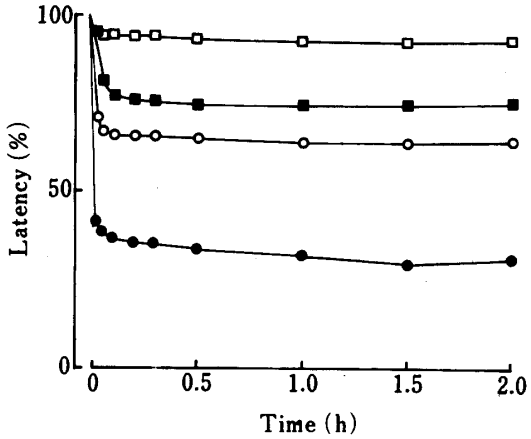

Fig. 4. Effects of Pre-Heating of Plasma at 56 °C for 30 min

Liposomes were incubated with fresh plasma (●, Glu-MLV; ■, PC-MLV) or pre-heated plasma at 56 °C for 30 min (○, Glu-MLV; □, PC-MLV). Samples were treated in the same manner as in Fig. 1.

The possibility that the factor was inactivated immediately on incubation by denaturation or consumption was also examined. Figure 3 shows the effects of adding fresh plasma to the incubation mixture during the incubation. Even after the addition of fresh plasma, very little marker was released. The results suggested that the limited destabilization arises from a property of the liposomes themselves, such as size distribution and/or others.

Effects of Size or Type of Liposomes

Various types of liposomes were prepared and their release profiles in plasma were examined. The results are listed in Table I as latent percent of CF after a 1 h incubation. Sonicated liposomes (SUV) were most stable. Liposomes prepared by the reverse-phase evaporation method (REV) were more stable than conventional MLV. Extruded REVs (0.4 μm) were more stable. These results show that smaller liposomes are more resistant to lysis. Therefore, the limited lysis may be due to the presence of small liposomes in the MLV preparation. However, it is not reasonable that 30% of the encapsulated volume is accounted for by liposomes small enough to resist the factor in the conventional MLV preparation.

On the other hand, Hunt carried out a kinetic analysis of liposomal stability in plasma and reported that the outer layer of PC-liposomes was more permeable that the inner one during incubation with plasma.[20] Therefore the limited release may be due to release from the outer compartment but not from the inner compartment. However, REVs, which are considered as uni- or oligo-lamellar vesicles, showed lower release than MLV as presented

above. This is not consistent with the hypothesis that the release occurs from the outer compartment. The stability of SUV and REV seems to be due to some structural feature formed during sonication or the REV procedure. On the other hand, the outer layer of large MLV may contain structural defects.

Effect of Pre-heating of Plasma

The complement system is a self defense mechanism which is inactivated by heating at 56 °C for 30 min.[21] The alkyl glycoside vesicles used in this study are foreign particles and showed be affected by this system. The release profiles were examined on incubation with plasma heated at 56 °C for 30 min. In heated plasma, the release from Glu-MLV was 35%, significantly lower than in fresh plasma (Fig. 4). This result suggests that the vesicles are partially lysed in plasma by a factor which is inactivated by heating at 56 °C for 30 min, though it is uncertain whether the factor is the complement system. Apolipoprotein (Apo A-1) has been reported to be heat-labile,[22] so participation of this protein in another possibility. The released amount was still different from that in PBS, and interaction with a factor which is not inactivated by heating may also be involved. On the other hand, release from PC-liposomes in heated plasma was less than 10%, and was not different from that in PBS as shown in Fig. 4. Thus, Glu-MLV are lysed by both heat-stable and heat-labile factors in plasma and PC-MLV only by the heat-labile one.

Effects of Pretreatment with Empty Liposomes

The release profiles of Glu-MLV in plasma pre-incubated with various types of liposomes at 37 °C for 10 min after pre-heating at 56 °C for 30 min were examined. The results are given in Table II. The glucoside liposomes (MLV) retained 91.0% of their contents after incubation at 37 °C for 1 h with the plasma pre-incubated with empty glucoside liposomes (same type and same amount). This indicates that the destabilizing activity was consumed by the empty glucoside liposomes. It is not clear whether the consumption was caused by binding on the liposomal surface, degradation, transformation of the factor, *etc.* The consumption of the activity was also observed on pre-incubation with other glycoside liposomes (galactoside and mannoside). On the other hand, pre-incubation with PC-liposomes did not affect the release of CF from Glu-liposomes. These observations suggest that the heat-stable destabilizing factor specifically interacts with the liposomal surface covered with sugar moieties and is consumed, regardless of sugar type. The factor did not lyse PC-liposomes as mentioned above, and therefore it seems not to be consumed by interaction with PC-liposomes or not to interact with them. Interestingly, even though the sonicated glucoside liposomes resisted the plasma destabilizing factor as presented above, the activity disappeared after the incubation of pre-heated plasma with them. The factor seems to be consumed by interaction with small

TABLE II. Latency of CF after Incubation with Plasma Pre-heated and Pre-incubated with Various Types of Empty Liposomes

Types of liposomes	Latency (%)
PBS	64.2±0.4
Glu-MLV	91.0±0.4[a]
PC-MLV	66.1±0.5[b]
Gal-MLV	91.9±0.6
Man-MLV	92.6±0.5
Glu-SUV	93.5±0.4

Values are expressed as means±S.D. of three experiments. *a*) Significantly different from PBS ($p<0.01$). *b*) Significantly different from Glu-MLV ($p<0.01$).

TABLE III. Latency of CF after Incubation with Subfractions of Pre-heated Rat Plasma

Fraction	Latency (%)
Whole plasma	64.7±0.5
0—33%	88.9±0.9
33—50%	86.0±0.4
50—68%	77.2±1.5[a]
68—	86.4±0.7

Values are expressed as means±S.D. of three experiments. *a*) Significantly different from other fractions ($p<0.05$).

glycoside liposomes but is unable to lyse them.

Activity of Plasma Subfractions

The activity of the plasma protein fractionated by the salting-out technique with ammonium sulfate was examined. Protein concentration was adjusted to that in native plasma by dilution of the fractionated protein. The results are shown in Table III. The activity was found in the fraction of 50—68% saturation (albumin fraction). Since this factor selectively interacts with vesicles covered with sugar moieties and lyses them, it may play a role in self-defence mechanisms, considering that the surface of bacteria is covered with polysaccharides.

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