Antibody Dependent, Complement Mediated Liver Uptake of Liposomes Containing GM_1

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Purpose. We have previously reported that GM_1 exhibits an opposite effect on regulating liposome circulation time in mice and rats (Liu et al. Pharm. Res. Vol. 12:508-512 (1995)). Inclusion of GM_1 into liposomes significantly prolongs liposome circulation time in mice, while it dramatically decreases the blood half life and increases liver uptake of liposomes in rats. The purpose of this study was to elucidate the mechanism that underlies this phenomenon.

Methods. Single-pass liver perfusion in vitro and complement mediated liposome lysis assay was used.

Results. Serum appeared to play an important role in determining the liver uptake of GM_1 liposomes. Specifically, rat serum enhanced the uptake of GM_1-containing liposomes by the perfused liver. Such activity was also found in human and bovine serum, but not in mouse serum. Taking human serum as an example, we demonstrated that such serum activity can be blocked by EDTA and EGTA/Mg^{2+}. Antibodies against human IgM and the third component of complement system (C3) also inhibited serum activity.

Conclusions. The presence of naturally occurring anti-GM_1 antibodies in rats, through the activation of the classic pathway of complement system, is likely the cause of rapid blood clearance of GM_1-liposomes. The third component of complement is likely to serve as the opsonin that is directly involved in mediating liposome clearance.

KEY WORDS: liposomes; drug delivery; GM_1; opsonin; liver perfusion.

INTRODUCTION

Liposomes have been extensively tested in experimental animals and in humans as carriers for drug delivery (1). Of the major progress made in the last few years is the development of liposomes with prolonged circulation time in blood, commonly called long-circulating or sterically stabilized liposomes. In addition to the basic composition of phosphatidylcholine and cholesterol for conventional liposomes, these long-circulating liposomes usually contain a small percentage (5-10, mole%) of amphipathic molecules containing a bulky hydrophilic head groups. The molecules found to be active in prolonging the liposome circulation time include ganglioside GM_1 (2, 3), phosphatidylinositol (2), polyethylene glycol derivatives of phosphatidylethanolamine (PEG-PE) (4) and vinyl polymer derivatives (5). Pharmacokinetic analysis and therapeutic studies with tumor bearing mice revealed that these long-circulating liposomes containing either PEG-PE (2, 6) or GM_1 (2, 7, 8) have considerable potential as drug carrier for cancer therapy. Elevated liposome accumulation has been found in the tumor using tumor bearing mice model system (2, 6–8). Results from preclinical studies with doxorubicin encapsulated into sterically stabilized liposomes revealed an increased therapeutic efficacy compared to free drug or drug encapsulated in the conventional liposomes (for review, see 6). With the ability of extravasating from the leaky vessels in tumor and accumulating at the tumor site, these newly discovered long-circulating liposomes have sparked the interests for future applications of liposomes as a drug carrier for cancer treatment.

While it is evident that the prolonged circulation time in blood is important for tumor targeting, we have recently reported that the circulation time of GM_1-containing liposomes is dependent on animal species (9). In mice, GM_1 prolongs the liposome circulation time and reduces liposome uptake by the liver and spleen. In rats, however, inclusion of GM_1 into liposomes dramatically decreases liposome circulation time and increases their uptake by the RES.

To elucidate the mechanisms underling the converse activity of GM_1 in regulating liposome circulation time in different animal species, we have systematically investigated the effect of serum on the liver uptake of GM_1-containing liposomes. Using a liver perfusion technique, we demonstrated that the rapid clearance of GM_1-containing liposomes in rats is likely to be mediated by the naturally occurring anti-GM_1 antibodies in blood. Comparing to the level of anti-GM_1 antibodies among different animal species including human, bovine, rat and mouse, mouse serum is unique in absence of anti-GM_1 antibodies. The high uptake of GM_1-liposomes by the perfused liver of both rats and mice in the presence of human serum suggests that it may be less likely that such liposome formulation will have long circulation time in humans and therefore may not be useful for cancer therapy.

MATERIALS AND METHODS

Materials

Egg phosphatidylcholine (PC) was purchased from Avanti Polar Lipids. Cholesterol (Chol), antibody fractions of goat anti-human IgM and anti-human C3 serum were from Sigma. ^{3}H-cholestanylether (^{3}H-CE) was purchased from DuPont NEN. Monosialoganglioside (GM_1) was from Supelco. ^{111}In-diethylenetriamine pentaacetic acid distearylamine complex (^{111}In DTPA-SA) was prepared according to the protocol described in the previous report (7). Purification of IgG using Protein A column was performed according to the instructions from the manufacturer. IgM fraction was purified following Johnstone and Thorpe (10). Animals were from Harlan Sprague Dawley Inc.

Liposome Preparation

Liposomes composed of PC/Chol/GM_1 (10:5:1, molar ratio) were prepared by extrusion (11). Lipids with a trace amount of ^{111}In-DTPA-SA, which is an nontransferable, nondegradable and widely used lipid marker for liposome studies in vivo, were dried and vacuum desiccated for 1 hour to remove the residual organic solvent. Lipid film was hydrated

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in PBS (pH 7.5) overnight at room temperature. Lipid suspension was then extruded 20 times through two layers of polycarbonate filters (Nucleopore) with pore size of 1 μm in diameter using LiposoFast extruder (Avestin Inc.). For liposome lysis assay, lipid film was hydrated in PBS containing calcein (50 mM) and extruded through filters with pore size of 100 nm to obtain unilamellar liposomes. Free calcein was removed from liposomes by gel filtration chromatography on a Bio-Gel A1.5 column. Liposome concentration in lysis assay was determined by using either ^{111}In-DTPA-SA or ^{3}H-CE as the lipid marker. The average diameter of liposomes was measured by a submicron particle analyzer (Coulter N4SD).

Single-pass Perfusion Studies of Liposome Uptake by Liver

Depending on the animal species used for the experiments, the amount of multilamellar liposomes and the volume of the perfusates were adjusted based on animal weight and blood volume. For rat liver, GM_1-containing liposomes (0.6 μmole total lipids in 60 μl) were incubated with 0.4 ml of Krebs Henseleit buffer (pH 7.4) or freshly collected serum for 10 min at 37°C and the mixture was then diluted to 24 ml using Krebs Henseleit buffer. Twenty ml of the mixture were perfused via the portal vein through the liver prewashed with 20 ml of buffer to remove blood contaminant. The perfusion rate was kept at 7 ml/min. For mouse liver, the same batch of liposomes (0.12 μmole total lipids in 12 μl) was incubated with 0.2 ml of either buffer or serum under the same conditions. The mixture was then diluted to 2.4 ml and 2 ml of the diluted mixture was perfused through the liver pre-washed with 3 ml of buffer. The perfusion rate was about 1.7 ml/min. The inferior vena cava of the anesthetized animals (using ether vapor) was cut at the beginning of the perfusion to drain the blood and the subsequent perfusate. Liposomes were only allowed to pass through the liver once. Unbound liposomes in the liver were removed by washing the perfused liver by 50 ml (for rats) and 5 ml (for mice) of buffer via the same route. Solutions used in perfusion were all prewarmed to 37 °C and the process was performed at room temperature and finished in less than 15 min. The amount of liposome uptake by the perfused liver was calculated based on the total ^{111}In radioactivity in the perfused liver. Liver viability in perfusion was monitored by glutamic oxaloactic transaminase (S-GOT) activity in the perfusate. The activity of glutamic oxaloacetic transaminase in the perfusate was found below 3 units/ml (n = 30).

Liposome Content Release Assay

Unilamellar liposomes with an average diameter of about 100 nm were used for our leakage assay. Liposomes (25 nmole total lipids in 25 μl) were added to 225 μl of buffer (PBS, pH 7.5) containing different amount of serum with or without other testing agents, and the mixture was then incubated for 30 min at 37°C. A fraction of the mixture was then added to 2.9 ml of HEPES buffer (pH 7.5) containing 2 mM EDTA to check calcein release from liposomes using a spectrofluorometer with λex = 490 nm and λem = 520 nm. The total release of calcein from liposomes was obtained by adding 50 μl of 5% deoxycholate to lyse the liposomes. The percentage of calcein release under different conditions was calculated using the method as previously described (12).

RESULTS AND DISCUSSION

Liposome clearance from blood is generally determined by liposome uptake by the macrophages of the RES, primarily in liver and spleen (1). Liposomes with low affinity to the RES circulate longer in blood than those with higher affinity. In the case of mice, GM_1-containing liposomes seem to have low affinity to the RES and therefore exhibit a prolonged blood circulation time (2, 3, 7), while the same liposomes in rats show high affinity to the liver and exhibit a very short half-life in blood (9).

To explore the mechanism by which GM_1 exhibits the opposite effect on liposome affinity to the liver in these two animal species, we have employed a single-pass liver perfusion technique which has been previously used in similar studies (13, 14). In the absence of serum (Table 1), about 7% of the total liposome perfusate was taken up by rat liver in comparison to an approximate 3% by mouse liver. However, different levels of liposome uptake by the perfused liver were obtained in the presence of serum. Depending on the animal species, serum activity in enhancing liver uptake of liposomes varies. For example, for rat liver, serum from rat (31%), human (32%) and bovine (38%) showed a 4 to 5 fold increase in liposome uptake compared to the basal level (7%), which is lower than the level of increase (6 to 9 fold) by the same serum when mouse liver was used. In experiments where mouse liver was used, up to about 17% of the perfusate was recovered from the perfused liver in the presence of rat serum, in comparison to 28% in the presence of human serum. In both liver systems, however, mouse serum did not show any activity. Increasing mouse serum concentration in the preincubation mixture did not increase liposome uptake (data not shown), suggesting that mouse serum does not contain the components existing in serum of rat, human and bovine.

It is known from our previous experiments that serum activity in enhancing the liver uptake of GM_1-containing liposomes is temperature sensitive. Treatment of serum at

Table 1. Effect of Serum on Liver Uptake of GM_1-Containing Liposomes[a]

Liposome Treatment	Uptake (%)					
	Rat Liver			Mouse Liver		
	1	2	Average	1	2	Average
Buffer	8.7	5.4	7.0	2.8	3.1	3.0
Mouse serum	6.6	7.2	6.9	3.4	3.6	3.5
Rat serum	31.3	29.6	30.5	16.8	17.2	17.0
Human serum	32.8	30.5	31.7	28.5	26.7	27.6
Bovine serum	35.2	41.4	38.3	ND	ND	—

[a] Multilamellar liposomes composed of PC/Chol/GM_1 (10:5:1, molar ratio) were incubated with buffer or freshly collected serum at 37°C for 10 min and then perfused through the liver as described in methods section. The amount of liposomes taken up by the perfused liver was presented as percentage of the total liposomes perfused (ND, not determined).

56°C for 30 min abolished the serum activity (9). Among many serum components that are heat labile under these conditions are the components of the complement system (10). If complement components are the opsonins for GM_1-liposomes, it is likely that they work through the activation of complement system. To test this possibility, we prepared liposomes that contain calcein as the content marker and examined the lytic activity of serum under different conditions. Due to the fact that human serum shows high activity in enhancing liposome uptake by both rat and mouse liver (Table 1), we choose human serum for our experiments. Figure 1 shows the results of liposome leakage assay using freshly collected serum from two individuals. As observed in this figure, variations of serum activity between the individuals were evident. For example, over 90% of calcein release was observed at 40% serum from individual A, compared to only 40% calcein release when the same concentration of serum from individual B was used. The highest calcein release from liposomes in serum B was only about 60% which was approximately 30% less than that in serum A at the serum concentration up to 90%. More importantly, pretreatment of serum at 56°C for 30 min completely abolished the lytic activity of serum, supporting the notion that complement system is directly involved.

To demonstrate whether serum induced clacein release was due to the change of liposome permeability or membrane solubilization, we have performed a chromatographic analysis on serum (from individual A, 80% serum) treated GM_1 liposomes (^{111}In-DTPA-SA labeled) using Biogel A1.5 column (1.5 x 70 cm). It was found that serum treated liposomes were eluted from the column at the same volume fractions as those without serum treatment, suggesting that the size of liposomes did not change upon the serum treatment. If liposomes were solublized by the serum, one would predict that ^{111}In DTPA-SA should be eluted from the column at later fractions since the size of liposomes is generally larger than the micelles.

In an attempt to identify the serum components that are involved in liposome lysis and in mediating liposome uptake by the liver macrophages, we decided to examine the effect of EDTA and EGTA/Mg^{2+} on lytic activity of serum to determine which pathway of the complement activation was responsible for the serum activity. Both EDTA (which is known to shut off the entire complement system) and EGTA/Mg^{2+} (which only block the classical pathway) abolished the lytic activity of serum (data not shown), suggesting that only the classical pathway is involved.

If the classical pathway is involved, anti-GM_1 antibodies are expected to exist in the serum. We have examined this possibility using purified antibodies from the serum of individual A (Figure 1). IgG fraction obtained through a Protein A column or IgM fraction obtained through the method of Johnstone and Thorpe (10) was added to the serum of individual B to see whether these antibodies will increase the lytic activity of serum from the individual B. As shown in Figure 2, addition of IgM significantly increased the lytic activity of the serum. Over 60% increase in liposome lysis was obtained when the added concentration of IgM reached 400 μg/ml. In contrast, addition of IgG did not change the serum lytic activity. To confirm the apparent conclusion that it is IgM not IgG that is responsible for lysing GM_1 liposomes, we tested the lytic activity of serum that were preabsorbed by the Protein A beads. No loss of the lytic activity from pretreatment of serum by the protein A beads was observed (data not shown). Liposomes composed of PC and cholesterol without GM_1 were not sensitive to serum (data

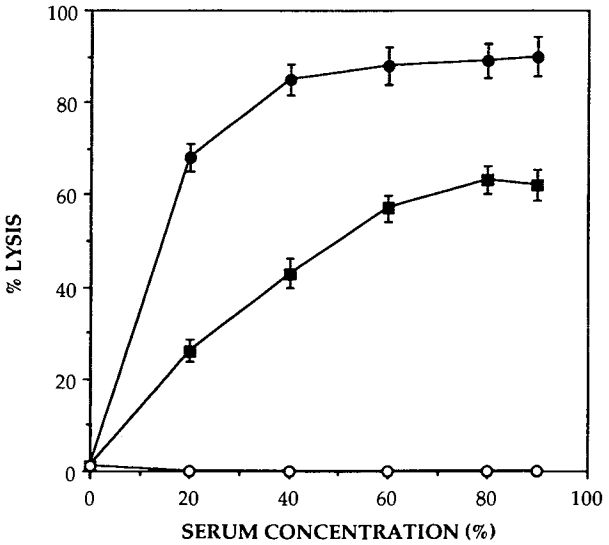

Figure 1. Serum concentration dependent liposome lysis. Calcein containing liposomes (25 nmole lipids in 25 μl) were incubated in buffer (PBS, pH 7.4) or buffer containing different amount of human serum from individual A (●), B (■) or serum treated at 56°C for 30 min (○). The final volume of the mixture was 250 μl for all the experiments. The mixture was then incubated at 37°C for 30 min. A fraction of the mixture was used to check the calcein release using a spectrofluorometer (λex = 490 nm, λem = 520 nm). The percent calcein release was calculated according to method previously described (16). Data represent the average (SD) of four measurements.

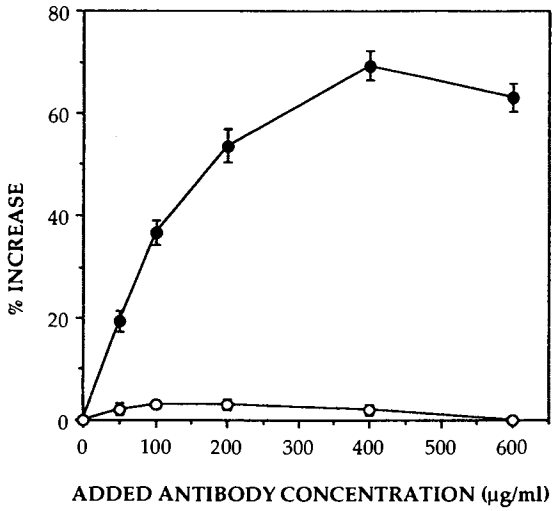

Figure 2. Antibody enhanced liposome lysis. Antibody fractions either IgM (●) or IgG (○) from serum of individual A were mixed with serum of individual B (Figure 1). The final volume of the mixture was 250 μl and the serum concentration was 40%. Error bars represent standard deviation of four measurements of two independent experiments.

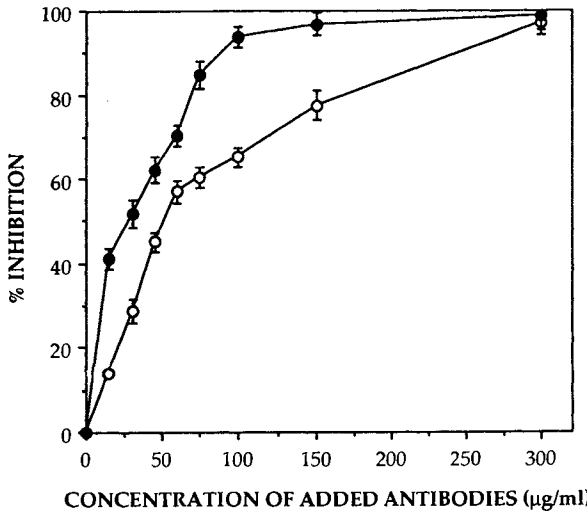

Figure 3. Inhibition of liposome lysis by goat anti-human IgM and anti-C3 antibodies. Different amount of goat anti-human IgM (●) or goat anti-human C3 (○) antibodies were incubated for 30 min at 37°C before liposomes were added. The serum concentration (from individual A) used in this experiment was 40%. Other assay conditions were same as those described in Figure I. Percentage of inhibition was calculated using the percent lysis in the absence of goat anti-human antibodies as 100%. Error bars represent standard deviation of four individual measurements from two independent experiments.

not shown). suggesting that IgM in the serum are specific to GM_1.

The results presented so far indicate that both IgM and the complement system are involved. To provide direct evidence for such hypothesis, we depleted IgM and C3 from serum and examined whether the lytic activity in the serum can be inhibited. In these experiments, lytic activity was examined in the presence of different concentrations of goat anti-human IgM or anti-human C3 antibodies. The result (Figure 3) was a dramatic inhibition of liposome lysis. Over 90% of the lysis was inhibited at the concentrations of 100 μg/ml and 300 μg/ml for goat anti-human C3 and goat anti-human IgM antibodies, respectively.

It appeared evident from the liposome leakage assay that the complement system is activated through the classical pathway and thus lysis is initiated by the anti-GM_1 antibodies (IgM as the isotype). To examine if the same mechanism can be applied to the liposome uptake by the perfused liver, we treated human serum under different conditions and examined the effects on the total liposome uptake by the perfused liver. As summarized in Table 2, addition of EGTA/Mg^{2+} completely inhibits the serum mediated liposome uptake. Pretreatment of serum by goat anti-human IgM or C3 antibodies also blocks the serum mediated liposome uptake by the liver.

The fact that serum mediated liposome uptake can be blocked by treatments of EGTA/Mg^{2+} and goat anti-human C3 antibodies suggests that it is unlikely that IgM plays any direct role in mediating liposome recognition by the liver macrophages, as the binding properties of IgM to liposomes should not change under such conditions. Inhibition of serum-mediated liposome uptake by the liver by goat anti-human C3 antibodies strongly supports the hypothesis that C3 serves as serum opsonin and promotes the liposome uptake by the liver. Increase in liposome uptake by addition of human IgM to mouse serum (Table 2) suggests that mouse complement system is also active. The only components missing in mouse serum, compared to others tested so far, are the anti-GM_1 antibodies.

The mechanism of GM_1-mediated liposome uptake by rat liver, likely through the Kupffer cells, is proposed and schematically presented in Figure 4. Intravenously injected GM_1-liposomes are rapidly bound by the anti-GM_1 antibodies when the liposomes are exposed to the blood. Subsequently, the complement system is activated by the antibody-liposome complex through the classical pathway. Some of the activated complement components deposit on the surface of liposomes. Those bound components serve as opsonin(s) that promote the uptake of liposomes by the liver

Table 2. Effect of Serum Treatment on Liver Uptake of GM_1-Liposomes[a]

Treatment	Uptake (%)			Inhibition (%)[b]
	1	2	Average	
None	32.8	31.3	32.1	0
EGTA/Mg^{2+} (1.5 mM final concentration)	4.3	4.4	4.4	100
Goat anti-human-IgM antibodies (400 μg)	8.5	8.6	8.6	94
Goat anti-human C3 antibodies (100 μg)	11.1	12.9	12.0	80
Mouse serum + human IgM[c]	28.5	27.1	27.8	

[a] Human serum (400 μl) was incubated at 37°C for 30 min in the presence of the added reagents in a total volume of 0.6 ml. Liposomes were then added and the mixture was incubated for an additional 10 min at 37°C followed by dilution and perfusion as described in the method sections. Rat liver was used in this experiment. The amount of liposome uptake by the perfused liver is presented as the percentage of the total perfusate.

[b] The percentage of inhibition was calculated using the following formula:

$$\text{Inhibition (\%)} = \frac{\text{Uptake with treated serum \% } - \text{ 7.0\% (uptake in the absence of serum)}}{\text{Uptake with untreated serum \% } - \text{ 7.0\% (uptake in the absence of serum)}} \times 100$$

[c] Liposomes (0.6 μmole lipids, 60 μl) were incubated with mixture of mouse serum (400 μl) and IgM (150 μl, 2.43 mg/ml in PBS) purified from the serum of individual A (Figure 1). The time of incubation, conditions for perfusion and data analysis were the same as the rest of the experiments.

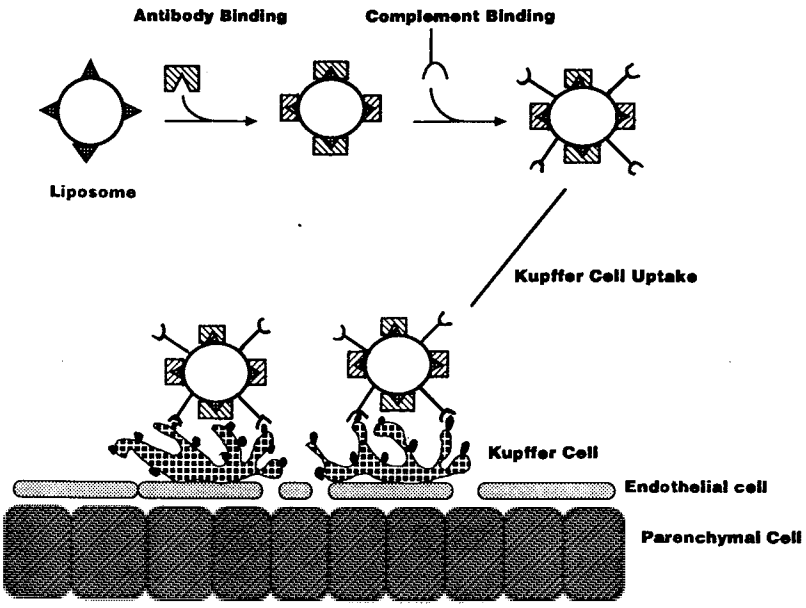

Figure 4. Schematic presentation of the mechanism of GM_1-mediated liposome uptake by liver.

Kupffer cells. The entire process resembles the clearance process of invading bacteria by our immune system.

It is evident that anti-GM_1 antibodies (IgM) in rat, human and bovine serum initiate the cascade events of complement activation that subsequently results in a rapid liposome uptake by the liver macrophages. It is not clear, however, what the physiological or immunological implications are for the presence of those antibodies. As matter of fact, naturally-occurring antibodies against phospholipids, cholesterol and glycolipids have also been found in many other animal species (15-17). Specificity of antibodies to various liposome lipids differ characteristically among different species, and titers vary among different individuals. For example, anti-cholesterol antibodies have been found in pigs, but such antibodies appear to be absent in guinea pigs (17). Nevertheless, this is the first time that linkage of the naturally occurring anti-lipid antibodies to liposome circulatory behavior in different animal species has been demonstrated.

Finally, it is our observation that GM_1 may not be an appropriate lipid to be used in liposome formulations that are intended for clinical use. Different levels of anti-GM_1 antibodies (as described in Figure 1) are likely to result in different pharmacokinetics of liposome entrapped drugs among the individuals. The subsequent immune reactions caused by binding of antibodies to GM_1 in liposome membranes and activation of the complement system may further complicate the therapy.

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