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Effects of blood on the uptake of charged liposomes by perfused rat liver: cationic glucosamine-modified liposomes interact with erythrocyte and escape phagocytosis by macrophages

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Abstract

The uptake of liposomes by rat liver was investigated by perfusion. Positively and negatively charged liposomes were taken up to a greater extent than neutral liposomes in Hanks' buffer. However, the uptake of cationic glucosamine-modified (PGlcN) liposomes was suppressed when blood was added to the perfusate, while the uptake of negatively charged phosphatidylserine (PS) and neutral liposomes was enhanced, probably due to the action of opsonins. The uptake of cationic PGlcN-liposomes by the liver was suppressed when red blood cells were added to the perfusate, while uptake was little affected in the presence of serum. These results revealed that rat erythrocytes play an important role in the capacity of cationic liposomes to avoid uptake by the reticuloendothelial system (RES). Cationic PGlcN-liposomes bound to rat erythrocytes weakly; however, the zeta potential of erythrocytes increased in the presence of cationic PGlcN-liposomes and showed homogeneous distribution. Cationic PGlcN-liposomes may surround the erythrocytes with an ionic atmosphere. Cationic PGlcN-liposomes would interact with erythrocytes through electrostatic interaction and could thus escape phagocytosis by macrophages. © 1997 Elsevier Science B.V.

Keywords: Liposome; Liver perfusion; Positive charge; Reticuloendothelial system (RES); Erythrocyte; Blood cell

1. Introduction

In the past 30 years, liposomes have been studied as both biomembrane models and as drug carriers. Liposomes have various advantages as drug carriers, as they are biodegradable, have low

Abbreviations: EPC, egg yolk phosphatidylcholine; Chol, cholesterol; PS, bovine brain phosphatidylserine; PGlcN, methyl-2-amino-2-deoxy-6-*O*-palmitoyl-D-glucoside; PGlc, methyl-6-*O*-palmitoyl-D-glucopyranoside; SA, stearylamine; RES, reticuloendothelial system.

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toxicity, and can encapsulate hydrophilic drugs within the aqueous phase, lipophilic drugs within the lipid phase. Furthermore, they can encapsulate amphipathic drugs. However, there are inevitable drawbacks to their use in vivo. The liver and spleen are largely responsible for the clearance of injected liposomes from the blood circulation, and uptake by the reticuloendothelial system (RES) is one of the major barriers to the use of liposomes for drug delivery (Gregoriadis, 1988; Gregoriadis and Allison, 1980). One approach taken to avoid the phagocytosis of liposomes by macrophages is to coat the liposomes with polyethylene glycol (PEG) chains, which prolongs the circulation time of the liposomes due to steric hindrance or to an increase of liposomal surface hydrophilicity (Illum et al., 1986; Allen et al., 1991; Papahadjopoulos et al., 1991; Blume and Cevc, 1993). Cationic stearylamine (SA) liposomes also remain in the blood longer than neutral or acidic liposomes (containing phosphatidylserine or phosphatidic acid) (Gregoriadis and Neerunjun, 1974). Recently, cationic Lipofectin™ liposomes, containing dioleoyltrimethylammonium chloride (DOTMA), have been studied as tools for the delivery of plasmid DNA and RNA into cells (Felgner et al., 1987; Felgner and Ringold, 1989; Smith et al., 1993).

Many researchers have reported that the interactions between liposomes and blood components, especially serum components, play a mediating role in liposome uptake (Hoekstra and Scherphof, 1979; Chonn et al., 1992; Moghimi and Patel, 1992; Patel, 1992; Liu et al., 1995). It is important to understand these interactions so that the fate of liposome-entrapped drugs can be controlled for effective therapy. However, information about these interactions is rather sparse.

We reported that amino sugar modification prolongs the lifetime of liposomes in vivo (Aoki et al., 1995). In this study, we investigated the effects of blood components on the uptake of liposomes in a rat liver perfusion system to understand the mechanisms whereby amino sugar-modified cationic liposomes avoid uptake by the RES.

2. Materials and methods

2.1. Materials

Egg yolk L-α-phosphatidylcholine (EPC) was provided by Asahi Kasei Co. Ltd. (iodine value 65; Japan). Bovine brain L-α-phosphatidylserine (PS) was purchased from Sigma (USA). [1,2(*n*)-^{3}H]Cholesteryl hexadecyl ether (^{3}H-CHE) and [1-^{14}C]palmitic acid were purchased from Daiichi Pure Chemical Co. Ltd. (Japan). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (WSC) was obtained from Dojindo Laboratories (Japan). Soluene-350® was purchased from Packard Instrument Co. Inc. (USA). Clear-sol I was obtained from Nacalai Tesque Inc. (Japan). All other chemicals, which were purchased from Wako Pure Chemical Industry Ltd., were of special grade, and cholesterol (Chol) was recrystallized from ethanol. Water was glass distilled twice.

2.2. Synthesis of methyl-2-amino 6-palmitoyl glucoside

Methyl - 2 - amino - 6 - palmitoyl - D - glucoside (PGlcN) and methyl - 6 - palmitoyl - D - glucoside (PGlc) were synthesized in a manner similar to that described (Miyajima et al., 1993). Radiolabeled PGlcN was synthesized in a similar manner with some modification. A mixture of methyl-2-(*N*-benzyloxycarbonyl)amino-2-deoxyl-D-glucoside (2 mmol), palmitic acid (2 mmol) and WSC (3 mmol) in pyridine was stirred overnight. After adding of water (10 ml), the reaction mixture was filtered and purified by column chromatography over silica gel with chloroform/ethyl acetate (1:1). The purified compound was reduced in a same manner.

2.3. Preparation of liposomes

Liposomes were basically prepared as described (Aoki et al., 1995). Multilamellar vesicles (MLVs) were prepared by extrusion (Olson et al., 1979). Liposomes consisting of EPC and Chol (8:2,

molar ratio), were used as controls and those consisting of EPC, Chol, and test lipid (PS, PGlc, or PGlcN) (6:2:2, molar ratio) were termed modified liposomes. The lipid mixtures in chloroform were evaporated to form thin lipid films. To prepare lipid-labeled liposomes, ^{3}H-CHE, a non-exchangeable, non-degradable marker, was added to the lipid mixture. The thin lipid film was dried overnight in vacuo, then hydrated with phosphate-buffered saline (PBS, pH 7.4). The suspensions were successively extruded through polycarbonate filters of various pore sizes (0.6 and 0.2 μm pore size 5 times; vesicles with a diameter of 200 nm; VET_{200} were prepared by extrusion). The size of the liposomes was measured by dynamic light scattering on a Photal laser particle analyzer (LPA-3100; Otsuka Electronics Co. Ltd., Japan) connected to a photon correlator (LPA-3000). According to the dynamic light scattering, the mean diameter of the liposomes was approximately 200 nm, with homogeneous distribution as described (Aoki et al., 1995). The zeta potential of liposomes with a diameter of approximately 200 nm was calculated using Smoluchowski's equation (Adamson, 1967) from their electrophoretic mobility in PBS (pH 7.4) at 25°C, using an electrophoretic light scattering spectrophotometer (Zetasizer 4; Malvern Instruments, UK) (Aoki et al., 1995).

2.4. Perfusion of rat liver

The livers of male Wistar rats, weighing from 180 to 200 g, were perfused by the method of Mortimore et al. (1959) with some modification. The rats were anesthetized by an intraperitoneal injection of nembutal. The abdomen was opened wide, the common bile duct was cannulated with a polyethylene tube (PE-10) and the hepatic portal vein was cannulated with a polyethylene tube (PE-160). The chest was opened and the inferior vena cava was cannulated through the right atrium with a polyethylene tube (Orion AWG-12). The inferior vena cava immediately above the renal vein was then ligated to prevent leakage of the perfusate. Liposome uptake was measured by recirculating (i) and continuous flow methods (ii).

(i) The liver was perfused at a rate of 25 ml/min. in the physiological direction, using a peristaltic pump (ATTO SJ-1215) with a combined bubble trap-depulser located between the pump and the portal cannula. The reservoir was 80 ml of liposomal suspension containing 5 μmol of total lipid in 10 mM HEPES/Hanks' buffer (pH 7.4). This was stirred gently, maintained at 37°C, and continuously bubbled with 95% oxygen/5% carbon dioxide. To prepare perfusate containing erythrocytes, blood was collected into a heparinized syringe and washed three times with PBS, after which packed erythrocytes were added to the perfusate. The liver was initially flushed with 200 ml of buffer before being perfused with vesicle suspension in a closed loop. The perfusion was continued for 1 h, during which 200 μl aliquots of liposomal suspension were taken from the reservoir at 5-min intervals. When the reservoir contained blood or erythrocytes, the samples were decolored by 100 μl of 30% H_2O_2. The liposome concentration in the reservoir was determined by using ^{3}H-CHE as the lipid marker. Finally, the liver was flushed with 150 ml of buffer and collected. To determine the amounts of the liposomes in liver, around 50 mg of tissue was dissolved in Soluene-350 and neutralized with HCl, then Clear-sol I was added. The tissue samples were examined in triplicate. The radioactivity levels in the samples were determined in a scintillation counter (LS5000TA; Beckman USA).

(ii) The liver was perfused at a rate of 25 ml/min. The reservoir was a vesicle suspension containing 50 μM of total lipid in 10 mM HEPES/Hanks' buffer (pH 7.4), maintained at 37°C, and continuously bubbled with 95% oxygen/5% carbon dioxide. The liver was initially flushed with 150 ml of buffer before being perfused. Samples of the outflow passed through the liver were taken at regular intervals. The extract ratio (E) was calculated from Eq. (1),

$$E\,(\%) = \frac{C_{in} - C_{out}}{C_{in}} \quad (1)$$

where, C_{in} and C_{out} are the lipid concentration of the inflow and the outflow, respectively. We confirmed that the liposomes did not bind to the polyethylene tube. The viability of the liver was checked by bile flow (> 4 μl/min).

2.5. Binding of liposomes to erythrocytes

Rat erythrocytes were washed three times with 10 mM HEPES/Hanks' buffer (pH 7.4) and dispersed in the same buffer. Erythrocyte suspensions were warmed at 37°C, then mixed with the suspension of liposomes labeled with ^{3}H-CHE (hematocrit, 1%). After a 5-min incubation at 37°C, the suspension was centrifuged ($300 \times g$ for 1 min) and the radioactivity levels in the supernatants was determined in the Beckman LS5000TA scintillation counter. Erythrocyte suspensions with a hematocrit of 1% contained 1×10^8 erythrocytes/ml.

2.6. Zeta potential of erythrocytes

Rat erythrocytes were washed three times with 10 mM HEPES/150 mM NaCl (HBS, pH 7.4) and dispersed in the same buffer. The EPC/Chol/PGlcN (6:2:2) liposomes were prepared in HBS by extrusion as described above and the lipid concentration was determined by Bartlett's method (Bartlett, 1959). The zeta potential of the erythrocytes with or without liposomes was calculated with Smoluchowski's equation (Adamson, 1967) from their electrophoretic mobility in HBS (pH 7.4) at 25°C, using an electrophoretic light scattering spectrophotometer (Zetasizer 4; Malvern Instruments, UK). PGlcN transferred rat erythrocytes were prepared by a incubation with EPC/Chol/PGlcN (6:2:2) liposomes for 15 min at 37°C. After a 15-min incubation, erythrocytes were washed three times with HBS (pH 7.4) and dispersed in the same buffer.

3. Results and discussion

The time courses of liposomes in the perfusate during recirculating liver perfusion are shown in Fig. 1. The radioactivity of control liposomes, PGlc-liposomes, and negatively charged PS-liposomes in the reservoir decreased gradually. However, that of cationic PGlcN-liposomes decreased rapidly over 10–20 min, then gradually increased. This is discussed later. Cationic PGlcN-liposomes were more accumulated than neutral liposomes (control and PGlc-liposomes) in the liver after 1 h of recirculation in buffer (Table 1). However, these results are contrary to those found in vivo (Aoki et al., 1995), suggesting that interactions between liposomes and blood components play a role in liposomal uptake.

Therefore, we studied the effects of blood. In the presence of 2.5% v/v rat whole blood, the lipid concentration of PGlcN-liposomes in the reservoir hardly decreased (Fig. 2). In the presence of rat blood, the uptake of cationic PGlcN-liposomes in the liver was suppressed, while that of neutral and anionic PS-liposomes was enhanced (Table 1). We believe that the enhancement of neutral or anionic liposomal uptake is due to the presence of opsonins (Moghimi and Patel, 1988, 1989; Chonn et al., 1992; Patel, 1992; Liu et al., 1995). To examine the effect of the blood on the uptake of PGlcN-liposomes in more detail, we added rat serum and erythrocytes to the perfusate separately. Adding 1.25% v/v rat serum (the same volume as that used in the above experiment) to the perfusate led to changes in the lipid concentration in the reservoir that differed from those found in buffer (Fig. 2). In buffer containing serum, more cationic than neutral liposomes were uptaken (Table 1). Adding 5% v/v rat serum caused a gradual decrease in the lipid concentration in the reservoir (data not shown) and the

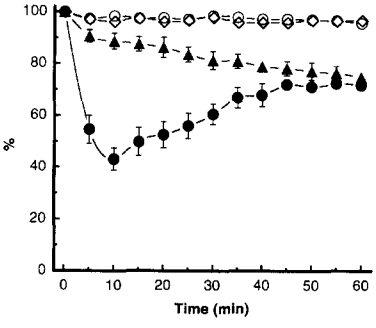

Fig. 1. Time course of the lipid concentration of the reservoir in 10 mM HEPES/Hanks' buffer during the recirculating perfusion of the rat liver with liposomes. The liposomes were labeled with ^{3}H-CHE. The initial lipid concentrations were 62.5 μM and each value is expressed as a percentage $\pm$ S.D. of the initial lipid concentration; $n = 3$. Liposomes: (○), EPC/Chol (8:2); (◇), EPC/Chol/PGlc (6:2:2); (●), EPC/Chol/PGlcN (6:2:2); (▲), EPC/Chol/PS (6:2:2).

Table 1
Uptake of liposomes in the liver after 1 h recirculating perfusion

Lipid composition (molar ratio)	% of dose			
		Added		
	In buffer	Blood	Serum	Erythrocytes
EPC/Chol (8:2)	1.5 ± 0.2	6.4 ± 0.1*	N.D.	N.D.
EPC/Chol/PGlc (6:2:2)	1.4 ± 0.3	7.9 ± 1.1*	N.D.	N.D.
EPC/Chol/PGlcN (6:2:2)	24.4 ± 3.1	4.6 ± 0.7*	18.8 ± 6.1	2.3 ± 0.1*
EPC/Chol/PS (6:2:2)	26.0 ± 0.8	34.9 ± 3.2*	N.D.	N.D.

The rat liver was perfused by recirculating for 1 h at a dose of 5 μmol of total lipid per liver. Values for the uptake of liposomes in the liver are expressed as % of dose ± S.D.
* Significant difference from the value in buffer ($P<0.01$).
N.D., not done.

amount of PGlcN-liposomes in the liver was 19.4 ± 1.3% of the dose. Serum components therefore interacted with the cationic PGlcN-liposomes. Tyrrell et al. (1977) reported that serum components (albumin and globulin) prevented the electrostatic attraction of cationic liposomes between cells and caused a decrease in the uptake of the liposomes. Serum components can bind to the surface of the cationic liposomes and change the charge density on the liposomes. However, these components cannot be factors in the avoidance of the RES by cationic PGlcN-liposomes. In the presence of rat erythrocytes (hematocrit, 1%), the lipid concentration of PGlcN-liposomes in the reservoir hardly decreased and the liposomal uptake in the liver was similar to that in the presence of rat blood (Table 1). That is, the addition of erythrocytes had an effect on the uptake of cationic liposomes similar to that shown upon the addition of blood. These results show that erythrocytes play an important role in the avoidance of the RES by cationic PGlcN-liposomes. The erythrocyte membrane has a negative charge arising mainly from sialic acid, and the zeta potential of erythrocytes is about −20 mV in phosphate buffer (Walter et al., 1983). Therefore, cationic liposomes can electrostatically interact with erythrocytes. The RES may not recognize the liposomes adsorbed onto the surface of blood cells as foreign substances. Nicholas and Jones (1991) reported that dipalmitoylphosphatidylcholine-based liposomes containing stearylamine (SA) also bound to erythrocytes and suppressed liver uptake in the presence of blood in rat liver perfusion.

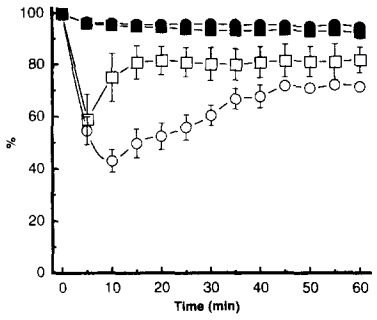

Fig. 2. Effects of rat whole blood, serum, and erythrocytes on changes in the lipid concentration of the reservoir during recirculating perfusion of rat liver with EPC/Chol/PGlcN (6:2:2)-liposomes. The liposomes were labeled with ^{3}H-CHE. The initial lipid concentrations were 62.5 μM and each value is expressed as a percentage ± S.D. of the initial lipid concentration; $n = 3$. (○), 10 mM HEPES/Hanks' buffer. In the same buffer containing: (■), 2.5% v/v rat whole blood; (□), 1.25% v/v rat serum; (●), rat erythrocytes (hematocrit, 1%).

The results of the continuous flow method are shown in Fig. 3. The results of the continuous flow method and recirculating methods were similar. The uptake of control and PGlcN-liposomes in buffer reached a steady state within about 60 and 200 s, respectively. In the steady state, the extract ratio (E) of control and PGlcN-liposomes in buffer was 2.5 ± 0.4% and 17.1 ± 0.1%, respectively. In buffer containing blood, the extract

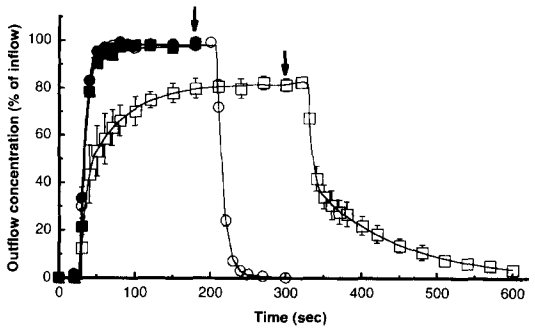

Fig. 3. Initial uptake of liposomes by constant flow perfusion of rat liver. The arrows show where the perfusate containing liposomes was replaced with 10 mM HEPES/Hanks' buffer. The liposomes were labeled with ^{3}H-CHE. The initial lipid concentrations were 50 μM and each value is expressed as a percentage $\pm$ S.D. of the initial lipid concentration; $n = 3$. (○), EPC/Chol (8:2)-liposomes in 10 mM HEPES/Hanks' buffer; (□), EPC/Chol/PGlcN (6:2:2)-liposomes in the same buffer; (●), EPC/Chol/PGlcN (6:2:2)-liposomes in the same buffer containing 2.5% v/v rat blood (■), EPC/Chol/PGlcN (6:2:2)-liposomes in the same buffer containing rat erythrocytes (hematocrit = 1%).

ratio (E) of control liposomes was $5.0 \pm 0.9\%$. The uptake of control-liposomes was enhanced by blood. In buffer containing blood or erythrocytes, the uptake of PGlcN-liposomes reached a steady state within about 60 s and the extract ratios (E) were $1.6 \pm 0.1\%$ or $2.2 \pm 0.3\%$, respectively.

When the liver was flushed with buffer after the continuous flow perfusion of cationic PGlcN-liposomes in buffer, a large amount of liposomes (about 0.8 μmol lipid) was obtained from the outflow (Fig. 3). This indicates that the cationic PGlcN-liposomes bind to the liver cell surface and that they were flushed with the buffer. This phenomenon may explain the initial drop in the concentration of cationic liposomes in the reservoir in the recirculating method. In other words, cationic liposomes initially bound to the liver cell surface. Thereafter, the bound liposomes are flushed with buffer containing a low concentration of liposomal lipid and the concentration in the reservoir increases. The serum component weakens the electrostatic attraction of cationic liposomes between cells (Tyrrell et al., 1977) and hence, the initial drop in the presence of serum is smaller that in buffer (Fig. 2). Similarly, the binding of cationic liposomes to the liver cell surface would explain the long time required to reach the steady-state in the continuous flow method.

However, it is possible that PGlcN, which is a single-chain acyl compound like a fatty acid, leaves the liposomal membrane, the electrostatic interaction between PGlcN-liposomes and cell surface decreases, and the liposomes then dissociate from the cell surface (Margolis et al., 1984; Kleinfeld and Storch, 1993; Horber et al., 1995). Therefore, we examined the behavior of cationic lipid PGlcN using liposomes labeled with ^{3}H-CHE and ^{14}C-PGlcN. The time courses of the PGlcN concentration in the liposomal membrane in the perfusate during recirculating liver perfusion are shown in Fig. 4. The PGlcN in liposomal membrane decreased to 50% within 10 min, then gradually decreased in Hanks' buffer, suggesting that PGlcN leaves the liposomal membrane and is distributed over liver cells. When the perfusate contained rat erythrocytes, it was separated by centrifugation into supernatant and erythrocytes to determine the PGlcN distribution. The PGlcN concentration in the perfusate containing the rat erythrocytes determined by ^{14}C/^{3}H gradually decreased. The PGlcN concentration in the liposo-

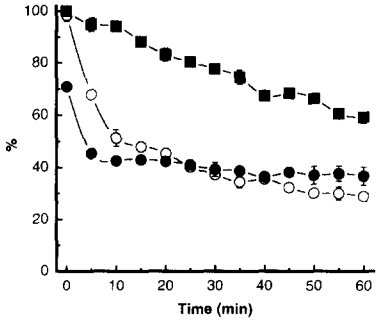

Fig. 4. Time course of the PGlcN concentration of liposomal membranes in the reservoir in 10 mM HEPES/Hanks' buffer during recirculating perfusion of the rat liver with liposomes labeled with ^{3}H-CHE and ^{14}C-PGlcN. The initial lipid concentrations were 62.5 μM and each value is expressed as a percentage $\pm$ S.D. of the initial PGlcN concentration in the membrane determined by the ratio of ^{14}C/^{3}H; $n = 3$. (○), EPC/Chol/PGlcN (6:2:2)-liposomes in 10 mM HEPES/Hanks' buffer; (■), EPC/Chol/PGlcN (6:2:2)-liposomes in the same buffer containing rat erythrocytes (hematocrit = 1%), (●), EPC/Chol/PGlcN (6:2:2)-liposomes in the supernatant separated by centrifugation in the same buffer containing rat erythrocytes (hematocrit = 1%).

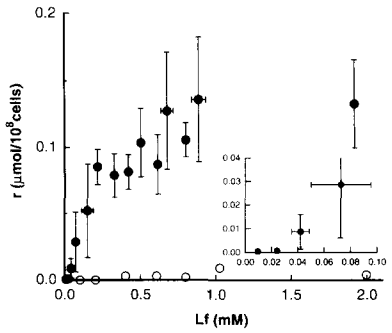

Fig. 5. Binding isotherm of liposomes to rat erythrocytes at 37°C in 10 mM HEPES/Hanks' buffer. (○), EPC/Chol (8:2) and (●), EPC/Chol/PGlcN (6:2:2)-liposomes.

mal membrane in the supernatant obtained by centrifugation decreased to 40% within 5 min, then remained unchanged. These results show the distribution of PGlcN throughout the erythrocytes and the liver cells in the presence of rat erythrocytes in the perfusate. PGlcN was immediately distributed throughout erythrocytes, however, about 40% of PGlcN remained associated in the liposomal membrane after 1 h liver perfusion. The liposomes retained a positive charge and interacted with the erythrocytes.

The binding isotherms of the neutral and cationic PGlcN-liposomes are shown in Fig. 5. Cationic, but not neutral liposomes bound to the erythrocytes. The binding isotherms of the cationic PGlcN-liposomes assumed a sigmoidal curve. This is because the cationic lipid PGlcN was distributed throughout the erythrocytes and the liposomal surface charge decreased largely at a low lipid concentration, whereas the liposomal surface charge decreased little at a high lipid concentration (data not shown). When the suspension was dispersed in a medium with low ion strength using glucose, the binding increased and all cationic liposomes bound to the erythrocytes (data not shown). This finding shows that the binding depends mainly on electrostatic interaction. The binding isotherm shows that about 30% of the cationic PGlcN-liposomes bind to the rat erythrocytes and that 70% of liposomes remain free under in situ liver perfusion conditions ([L] = 62.5 μM, Ht = 1%). Therefore, the RES avoiding mechanisms of PGlcN-liposomes cannot be explained by only the binding to rat erythrocytes.

The zeta potential of rat erythrocytes in the presence of cationic PGlcN-liposomes is shown in Fig. 6. The zeta potential of rat erythrocytes was about -20 mV. Adding cationic liposomes with a zeta potential of about $+15$ mV to the erythrocytes increased the zeta potential. The zeta potential of the mixture showed homogeneous distribution despite the exsistence of unbound cationic liposomes (data not shown). The zeta potential of cationic liposomes alone was not observed in the presence of rat erythrocytes. The zeta potential of rat erythrocytes was affected by basic lipid PGlcN which leaves the liposomal membrane (Fig. 6). However, the zeta potential of mixture was higher than that of PGlcN transferred rat erythrocytes. Therefore, the phenomenon that the zeta potential of erythrocytes increased in the presence of cationic PGlcN-liposomes was not only due to transferred basic lipid PGlcN. This finding suggests that cationic liposomes surround the erythrocytes with an ionic atmosphere to decrease the surface charge density of the erythrocytes.

In conclusion, our liver perfusion experiments showed that the uptake of cationic liposomes in the liver is suppressed in the presence of erythrocytes or blood. The interaction of cationic liposomes with erythrocytes is not still unclear, however, that the mechanisms of the RES-avoiding nature of the cationic liposomes involve an interaction with blood cells. Cationic PGlcN-lipo-

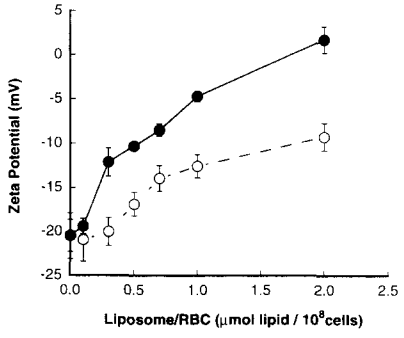

Fig. 6. The zeta potential of the rat erythrocytes. (●) The zeta potential of the rat erythrocytes in the presence of EPC/Chol/PGlcN (6:2:2)-liposomes. (○) The zeta potential of the PGlcN transferred rat erythrocytes prepared by incubation with EPC/Chol/PGlcN (6:2:2)-liposomes, followed by wash.

somes interact with erythrocytes or other blood cells electrostatically and escape phagocytosis by macrophages, thus prolonging the circulation time of these cationic liposomes.

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