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Proteins and peptides bound to long-circulating liposomes

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Liposome formulations with prolonged circulation time have recently been developed as a potential sustained-release drug delivery system. Data shown in this report indicate that such formulations can also be used to prolong the circulation time of proteins and peptides by conjugating them to the surface of liposomes. Increase of the circulation halflife ranged from 2- to 150-fold depending on the protein/lipid ratio of the liposomal formulation, liposome size, and the lipid composition of liposomes. Since the proteins/peptides localize on the liposome surface, instead of being entrapped inside the liposomes, they are directly available for binding to its receptor molecules and express the biological activity. This strategy has been successfully applied to two proteins with known fast clearance rate, i.e. asialofetuin and ricin A-chain. The biological activities of both proteins are preserved when they are formulated in liposomes. Incorporation of a peptide, i.e. a-factor of the yeast *Saccharomyces cerevisiae*, into the liposome membrane also significantly enhanced the circulation time of the peptide.

Introduction

Increasing number of macromolecular drugs, particularly proteins and polypeptides produced by the genetic engineering procedure, are being tested and/or used as parenteral dosage forms. Examples include interleukins, soluble cell surface receptors, enzymes, antibodies and their derivatives. One of the drawbacks of this novel class of drug is their relatively short circulation lifetime when injected systemically. For example, i.v. injected immunotoxins are rapidly cleared from the circulation by the liver, resulting in an inadequate target binding and excessive liver toxicity [1,2]. sCD4, which is a truncated version of the native membrane bound CD4 molecule, also exhibits a short circulation halflife ($t_{1/2}$ = 15 min) [3]. Therapeutic value of these drugs is seriously compromised due to the unfavorable pharmacokinetics.

There are several strategies used to overcome the problem. The most successful one is the covalent conjugation of polymer which forms a steric barrier on the surface of the macromolecular drug. Polyethyleneglycol (PEG) has been widely used for this purpose due to its inert biological activity and the extended linear conformation. Thus, many enzymes, cytokines and others have been conjugated with PEG, and the circulation time of the conjugates is significantly prolonged as compared to that of the native drug (for a review, see Ref. 4). Another strategy is to chemically deglycosylate the protein such that the principal mechanism of clearance, i.e. the lectin-mediated endocytosis, is not operative. Ricin A immunotoxin is rapidly removed from the circulation due to its binding and endocytosis with the liver cells via the mannose and galactose receptors [5,6]. Immunotoxins containing deglycosylated Ricin A, however, exhibit a much longer circulation lifetime and improved therapeutic index [1,6,7]. The third strategy is

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Abbreviations: sCD4, soluble CD4; PEG, polyethyleneglycol; PEG-FE, dioleoyl *N*-(monomethoxy(polyethyleneglycol)succinyl)phosphatidylethanolamine; PBS, phosphate-buffered saline; RES, reticuloendothelial system; PC, egg phosphatidylcholine; NGPE, *N*-glutarylphosphatidylethanolamine; chol, cholesterol; AF, asialofetuin.

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specifically designed for smaller macromolecules (molecular mass < 70 kDa) which are mainly cleared by the kidney glomerular filtration mechanism. For example, recombinant interleukin-2 (molecular mass 19.5 kDa) is rapidly lost from the circulation and appears in the urine, but its conjugates with soluble polymers of larger sizes do not [8]. Thus, increase of the effective molecular size of the drug by conjugating with polymers is at least partially responsible for the prolonged circulation lifetime.

While these approaches are effective for different macromolecular drugs, there is not a general method which can be used to prolong the circulation time of most, if not all, of the macromolecular drugs. We describe here a general method which should be applicable to many macromolecules of different classes and sizes. The method is based on a recently described novel class of liposomes which exhibit a reduced affinity to the reticuloendothelial system (RES) in the liver and the spleen [9–11]. These liposomes contain a glycolipid such as the monosialoganglioside GM_1, phosphatidylinositol, or sulfolipids [10,12]. Also, a synthetic phospholipid, *N*-(polyethyleneglycol)phosphatidylethanolamine, has been shown to exhibit the same activity [13–15]. Liposomes containing these lipids exhibit prolonged circulation time, because their affinity of uptake by the RES cells in the liver and the spleen is significantly lower than that of the ordinary liposomes. By covalently conjugating the macromolecular drugs to the surface of these liposomes, we have shown that the circulation lifetime of the drug is significantly prolonged. Furthermore, the liposomal drugs exhibit full biological activities. These improved formulations may be experimentally and clinically useful.

Materials and Methods

Materials

PC and NGPE were purchased from Avanti Polar Lipids, Birmingham, AL. Chol, fetuin, AF, and albumin (bovine) were obtained from Sigma Chemical Co., St. Louis, MO. GM_1 was purchased from Matreya, Inc., Mount Pleasant, PA, and ricin A from Calbiochem, La Jolla, CA. Farnesylated a-factor and unfarnesylated a-factor were a kind gift from Steven Marcus and Chu-biao Xue of the University of Tennessee, Knoxville. Radioiodination of proteins with ^{125}I was done by using Iodogen reagent (Pierce Chemical Co., Milwaukee, WI) to approximately $(1–2) \cdot 10^5$ cpm/μg. ^{111}In-labeled distearyl ester of diethylenetriaminepentaacetic acid (^{111}In-DTPA-SA) was prepared as described [16].

Conjugation of proteins with phospholipid

AF and ricin A were conjugated with NGPE as previously described for the conjugation of antibodies [16]. Briefly, NGPE was activated to the *N*-hydroxysuccinimide ester by using water-soluble carbodiimide in an aqueous buffer (pH 5.0) containing a detergent octyl glucoside as a solubilization agent. After the pH of the solution was adjusted to 7.5, proteins were added at NGPE/protein = 10:1 (w/w). The mixture was incubated overnight at 4 °C to allow a complete reaction. The protein-NGPE conjugates were used for liposome formulation without further purification.

Liposome preparation

Solvent-free lipid mixtures containing ^{111}In-DTPA-SA ($2 \cdot 10^6$ cpm/mg lipid) were vacuum desiccated for at least 1 h before being solubilized by adding octyl glucoside (2 mg/mg lipid). The clear solution was mixed with appropriate amounts of ^{125}I-labeled proteins (AF, ricin A) conjugated with NGPE or with ^{125}I-labeled a-factor. The mixtures were dialysed against PBS for 2 days at 4 °C to remove octyl glucoside. The resulting liposomes were extruded 6–8 times through a stack of two polycarbonate filters of defined pore size to generate liposomes with uniform size distribution. The extruded liposomes were then chromatographed on a Bio-Gel A-1.5-M column to remove the unincorporated proteins or peptides. The protein-to-lipid ratios of the liposome fractions were determined from the ^{125}I and ^{111}In counts and their specific activities. The liposome size was estimated by laser light scattering using a Coulter N4SD instrument.

Biodistribution studies

Free proteins/peptides or liposomal formulations were injected via the tail vein into Balb/c mice, 6–8 weeks old. At different times after injection, mice were anesthetized with metofane, bled via the retroorbital sinus and killed by cervical dislocation. Major organs (lung, heart, liver, spleen and kidney) were collected and counted for both ^{125}I and ^{111}In using a gamma counter. The weight of total blood was assumed to be 7.3% of the body weight [17]. Data are expressed as % injected dose in each organ or blood.

Incorporation of fatty acid into cellular lipids

The effect of AF on the incorporation of oleic acid into cellular lipids was measured as described [18]. Briefly, human skin fibroblasts were grown to subconfluency. After 48 h Dulbecco's Modified Eagle Medium containing albumin or fetuin or AF formulations (100 μg protein/ml) and [^{14}C]oleic acid (250000 dpm/ml) was added and incubated for 24 h. The cells were then washed and removed by trypsinization and sonicated in saline. Aliquots were taken for protein estimation and lipid extraction. Lipid extract was fractionated by thin-layer chromatography [18] and bands corresponding to various lipids were scraped and counted. The results are expressed as dpm/ 100 μg protein.

Cytotoxicity of ricin A

Various amounts of ricin A or liposomal ricin A were added to mouse L929 cells cultured in 24-well plates. The total volume of medium (McCoy's medium plus 10% bovine calf serum) in the well was 2 ml. Approx. $1 \cdot 10^4$ cells were present at the time of addition. Cells were enumerated after 3 days by a Coulter counter, model ZM. Data are expressed as % of control cultures receiving no ricin A.

Results

Size dependence of liposome clearance rate

In order to determine the optimal liposome formulation for a prolonged circulation time, liposomes with (PC/chol/GM_1 = 10:5:1, mol/mol) and without (PC/chol = 2:1, mol/mol) GM_1 were prepared with different mean diameters (d = 100 to 1000 nm). The liposomes were i.v. injected into mice and the amount of liposomes remaining in the blood was measured at 4 h after injection. Fig. 1 shows that the blood content of liposomes is a strong function of the liposome size. Significant amount of the injected liposomes remained in the blood for liposomes of relatively small diameters ($d < 300$ nm), whereas larger liposomes were cleared from the blood more rapidly, mainly by the liver and the spleen. Spleen accumulation was quite pronounced for the larger liposomes containing GM_1, indicating that the enhanced clearance rate of these liposomes was primarily due to the spleen uptake. Also shown in Fig. 1 is the role of GM_1 in the liposome clearance rate. GM_1-containing liposomes were cleared much more slowly than the ones containing no GM_1, especially those liposomes with a mean diameter less than 300 nm. Therefore, the optimal liposome formulation for a prolonged blood residence time appeared to be the ones containing GM_1 and having a mean diameter smaller than 300 nm.

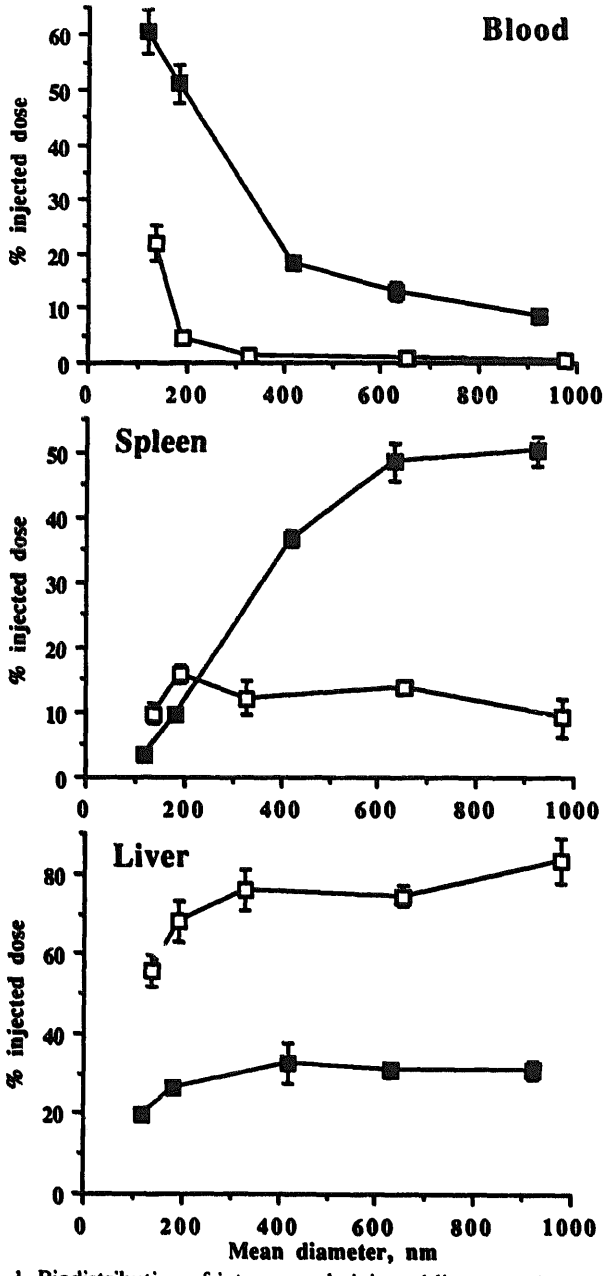

Fig. 1. Biodistribution of intravenously injected liposomes in mouse. Liposomes composed of PC/chol/GM_1 (10:5:1, mol/mol) (■) or PC/chol (2:1, mol/mol) (□) were prepared with different diameters and were i.v. injected into mice (400 μg lipid/mouse). The % of injected dose in blood, spleen and liver 4 h after injection are shown as a function of the liposome diameter. Data are expressed as mean ± S.D. (n = 3).

Clearance of liposomal AF

AF was chosen as a model to study the effect of liposomal formulation on the protein clearance rate, because it is well known that AF clears from the blood very rapidly with a mechanism mediated by the hepatic asialoglycoprotein receptor [19]. We have prepared four different liposomal AF formulations: three with a lipid composition of PC/chol/GM_1, (10:5:1, mol/mol) and one with PC/chol (2:1, mol/mol). Of the three formulations containing GM_1, the protein-to-lipid ratio varied from 1:27 to 1:160 (w/w). As shown in Fig. 2, free AF disappeared from the blood very rapidly ($t_{1/2} < 1$ min); there was no detectable AF in the blood 5 min after injection. Most of the radioactivity was found in the liver (data not shown), presumably in the hepatocytes [19]. All four liposomal formulations showed enhanced residence time in the blood, with the ones containing GM_1 and low AF/lipid ratios (1:160 and 1:74, w/w) being the longest; approximately 50% of these formulations was still in the circulation at 15 min after injection. Liposomal AF containing GM_1 but with a higher AF/lipid ratio (1:27, w/w) cleared more rapidly than the ones with lower ratios. These results show that the clearance rate of the liposomal AF depends on the protein-to-lipid ratio; formulations more enriched with proteins clear faster than the ones less enriched. Furthermore, the liposomal AF without GM_1, although prepared at a low AF/lipid ratio of

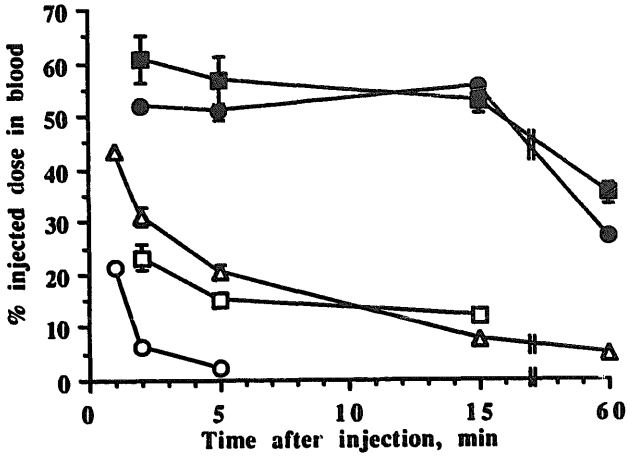

Fig. 2. Blood clearance of AF in mouse. Free AF (○), or AF bound to liposomes composed of PC/chol (2:1, mol/mol) (□) or PC/chol/GM_1 (10:5:1, mol/mol) (■, ●, and △) were injected iv. into mice (3–5 μg AF per mouse) and the amount of AF remaining in the blood was measured at different times. The protein-to-lipid weight ratios were: 1:183 (□), 1:27 (△), 1:74 (●) and 1:160 (■). Mean liposome diameters for all liposome formulations were 200 to 250 nm. Data are expressed as mean ± S.D. (n = 3).

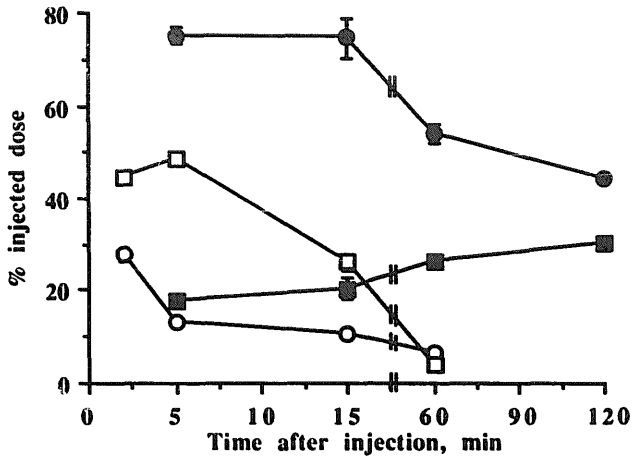

Fig. 3. Blood clearance of ricin A in mouse. Free ricin A (○, □) or ricin A bound to liposomes (●, ■) composed of PC/chol/GM1 (10:5:1, mol/mol) (liposome diameter = 200 nm, protein-to-lipid ratio = 1:78, w/w) were iv. injected (3–5 μg ricin A per mouse) into mice. Amount of ricin A remaining in blood (○, ●) or accumulating in the liver (□, ■) were measured at different times after injection. Data are expressed as mean ± S.D. (n = 3).

1:183, w/w, cleared rather fast from the blood, indicating the importance of GM_1 in prolonging the circulation lifetime of the liposomal formulation.

Biological activity of the liposomal AF

It is important to examine the biological function of the liposomal AF. Fetuin has been shown to facilitate the incorporation of free fatty acids into the cellular lipids, both phospholipids and neutral lipids [18]. We have used a tissue culture system, i.e. the human skin fibroblasts, to test the activities of fetuin, free AF and liposomal AF in promoting the incorporation of [^{14}C]oleic acid into several different classes of lipid (Table I). As can be seen, the activity of free AF was somewhat lower than that of fetuin, particularly for the incorporation into diglycerides and triglycerides (approx. 50% or less). However, the liposomal AF (PC/chol/GM_1 = 10:5:1, mol/mol, AF/lipid = 1:50, w/w) showed approximately the same activity as that of free AF. In the case of incorporation into triglycerides, activity of the liposomal AF was significantly higher (about 2.3-fold) than that of free AF. All fetuin and AF preparations showed activities greater than that of the albumin control. These data clearly indicated that formulating AF in liposomes did not compromise its biological activity; the activity might have even been enhanced.

Clearance of liposomal ricin A

Another protein used for testing the activity of the liposome formulation was ricin A. Ricin A was conjugated to NGPE and incorporated into liposomes composed of PC/chol/GM_1 (10:5:1, mol/mol). The liposomal ricin A showed a significantly higher concentration in the blood as compared to the free ricin A up to 1 h after the injection (Fig. 3). Free ricin A rapidly accumulated in the liver and degraded; whereas the uptake of liposomal ricin A by liver was slow. The amount of liver accumulation of the liposomal ricin A at 2 h after injection was still lower than that remaining

TABLE I

Incorporation of [^{14}C]oleic acid into lipids by human skin fibroblasts in culture: effect of asialofetuin

Addition	Oleic acid incorporation (dpm/100 μg protein)				
	PL [a]	DG	FFA	TG	CE
Albumin (control)	71027 ± 5267	11283 ± 2052	76791 ± 12148	26751 ± 15726	907 ± 310
Fetuin	114983 ± 12732 [b]	23859 ± 4081 [b]	160867 ± 12532 [b]	66301 ± 19201 [b]	1702 ± 340
AF	91136 ± 12308 [b]	13298 ± 5207	143772 ± 24040 [b]	27403 ± 17921	1113 ± 324
Liposomal AF [d]	96067 ± 6118 [b]	18611 ± 2910	140095 ± 12296 [b]	61620 ± 16116 [b,c]	1208 ± 193

[a] Abbreviations are: AF, asialofetuin; PL, phospholipids; DG, diglycerides; FFA, free fatty acids; TG, triglycerides, and CE, cholesteryl esters.
[b] $P < 0.05$ for difference from controls.
[c] $P < 0.05$ for difference from asialofetuin.
[d] Lipid composition PC/chol/GM_1 = 10:5:1, mol/mol, AF/lipid, 1:50, w/w.

in the blood. These results clearly demonstrated the prolonged circulation time and the reduced liver uptake of the liposomal ricin A as compared to the free toxin.

Cytotoxicity of liposomal ricin A

The biological activity of ricin A, both free and the liposomal formulation, was examined by measuring the growth of mouse L929 cells exposed to different concentrations of the toxin (Fig. 4). Free ricin A was not very toxic to the cells, showing a 50% inhibition of cell growth at approx. 8 μg/mi. This is consistent with the previous reports [20] and reflects the fact that A-chain of ricin can not easily enter the cells without the help of B-chain or a binding ligand such as antibody [20]. Liposomal ricin A also showed a dose-dependent inhibition of cell growth; approx. 50% inhibition was observed at 22 μg toxin/ml. Although the toxic activity of the liposomal ricin A was somewhat lower than the free, unconjugated ricin A, it nevertheless showed that the liposomal formulation of toxin is biologically active.

Clearance of a liposomal peptide

As a model for small macromolecules, the dodecapeptide a-factor of *Saccharomyces cerevisiae* was used to examine the activity of the liposomal formulation to prolong the circulation lifetime. The native a-factor peptide contains a farnesyl chain at its cysteine residue at the C-terminal [21], which renders the peptide insoluble in aqueous solution but is readily incorporated into the liposome membranes. Since free farnesylated peptide formed a precipitate in PBS, it was not possible to inject it into mice to compare with the liposomal a-factor. Instead, we have used an unfarnesylated peptide of identical sequence as a control. Liposomal ^{125}I-labeled a-factor circulated for a much longer period of time ($t_{1/2} \approx 1$ h) than the free unfarnesylated a-factor ($t_{1/2} < 2$ min) (Fig. 5). Most of the free peptides were excreted in urine through the kidney (data not shown); whereas the liposomal a-factor stayed in the blood with a small fraction (12–19%) taken up by the liver. No evidence of kidney excretion of the liposomal a-factor was obtained. Thus, the liposomal formulation has prevented the rapid kidney excretion of a peptide and resulted in a prolonged residence time in the circulation.

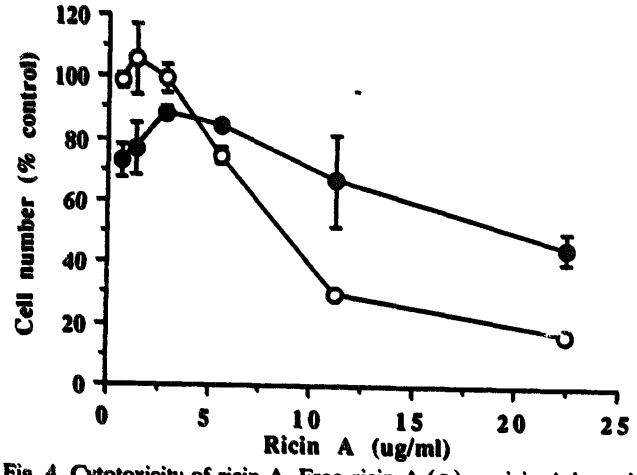

Fig. 4. Cytotoxicity of ricin A. Free ricin A (○) or ricin A bound to liposomes (●) composed of PC/chol/GM_1 (10:5:1, mol/mol), with a mean diameter 200 nm and protein-to-lipid ratio of 1:78, w/w, were added to mouse L929 cells in culture. Cells were enumerated after 3 days. Cell numbers in cultures exposed to different concentrations of ricin A are expressed as % of control culture receiving no ricin A.

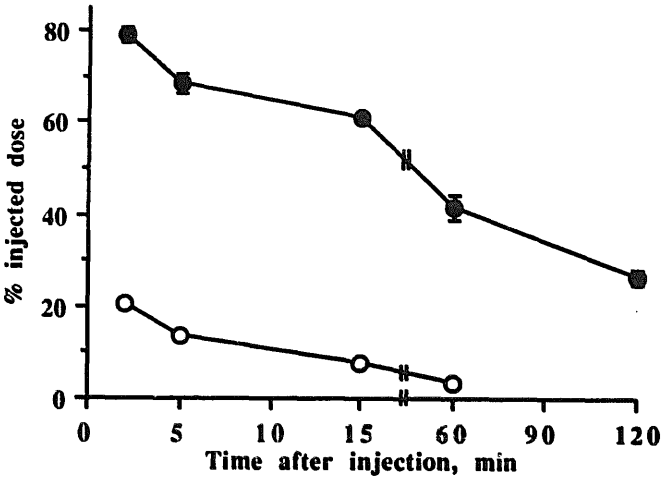

Fig. 5. Blood clearance of a-factor in mouse. Free, unfarnesylated a-factor (○) or a-factor incorporated into liposomes (●) composed of PC/chol/GM_1 (10:5:1, mol/mol) with a diameter of 292 nm and protein-to-lipid ratio of 1:771, w/w, were i.v. injected into mice (3 μg a-factor/mouse). Amount of a-factor remaining in the blood was measured at different times after injection. Data are expressed as mean ± S.D. ($n = 3$).

Discussion

Other investigators [9,10,12] and later also we [11,22] have reported that GM_1, when incorporated into the liposome membrane, significantly reduces the uptake of liposomes by the RES and hence effectively prolongs the residence time in the blood. However, the effect of GM_1 seems to be limited to small liposomes; larger liposomes are rapidly cleared from the blood despite the presence of GM_1 (Fig. 1). This is due to the enhanced spleen uptake of the larger liposomes. Liu et al. [23] and Klibanov et al. [24] have reported similar observations. Liu et al. have further demonstrated that large liposomes are retained in the spleen with a filter mechanism in the sinusoids of the red pulp and that liposomes are taken up by the splenic cells and digested [23]. Thus, the optimal liposome formulation for a reduced blood clearance rate should contain small liposomes ($d < 300$ nm) with GM_1 being included in the lipid composition.

For liposomes containing AF, there is an additional consideration about the size of liposomes. It has been reported that small liposomes ($d < 100$ nm) can readily penetrate the fenestrae of the liver sinusoids and gain access to the parenchymal cells [25,26]. These cells also contain large numbers of the galactose receptor which is primarily responsible for the rapid clearance of free AF from the blood [27]. Since AF covalently conjugated to liposomes can be taken up by the isolated hepatocytes *in vitro* [28,29], we have decided to prepare liposomes with a mean diameter greater than 150 nm. It is known that the mean diameter of the hepatic fenestrae is 50–120 nm [30]. Thus, liposomes of a mean diameter between 150 and 300 nm will not be retained by the spleen sinusoidal filter, nor penetrate the hepatic parenchyma, and should exhibit the maximal chance to stay in the blood. Thus, we have prepared our liposomes with a mean diameter of 200–250 nm.

The mechanism with which the liposomal AF exhibited a prolonged circulation lifetime involves several factors. First, the size of the liposomes must play a major role as discussed above. The next factor was the presence of GM_1 in the liposome composition. Although GM_1 and other glycolipids such as sulfatides and the hydrogenated phosphatidylinositol have demonstrated their activities to reduce the liposome affinity to the RES cells [10,12], the precise mechanism of action is not yet elucidated. It probably involves a reduction of interaction of the liposome membrane with some unidentified opsonin proteins in the serum [31]. The presence of AF on the liposome surface apparently affected the activity of GM_1 on the liposome circulation time. Liposomes were cleared faster if they were more enriched with AF (Fig. 2). Thus, the natural clearance mechanism of AF competed with the action of GM_1. A higher concentration of GM_1 may be required to offset this antagonistic effect of AF.

One important observation is that the liposomal AF showed a biological activity equal to, or perhaps even greater than, that of the free AF (Table I). Liposomal formulations of proteins and peptides have been previously prepared by entrapping the proteins/peptides in the interior space of liposomes (see reviews in Ref. 32). The present formulation differs from the previous ones by incorporating the proteins/peptides in the membrane phase of liposomes. Thus, at least a portion of the proteins/peptides are exposed at the surface and are free to bind with their receptor molecules for the expression of biological activity. To this point it is important to note that GM_1 does not interfere with the receptor binding of protein molecules located on the same liposome surface [22] and is particularly suitable for the present purpose. Another glycolipid conjugate, i.e. *N*-(polyethyleneglycol 5000)-phosphatidylethanolamine (PEG5000-PE), although also effective in prolonging the liposome circulation time, prevents liposomes from binding to the receptor molecules due to its strong steric barrier effect [24]. However, we have recently shown that PEG-PE with shorter polymer chains, such as PEG2000-PE, on the liposome surface does not interfere with ligand–receptor interaction due to its weaker steric barrier activity [33]. It is thus of interest to test the activity of such conjugate for the liposome formulations described here.

The circulation time of ricin A was also significantly prolonged by formulating with liposomes containing GM_1 (Fig. 3). This is a significant result because the present method to avoid the rapid clearance by the liver is to chemically remove the oligosaccharides of the molecule [1,6]. The simple approach described here offers an alternative method to reduce the liver uptake of the molecule. The liposomal ricin A would presumably be less liver toxic than the native ricin A. Data in Fig. 4 indicate that the liposomal formulation has slightly decreased the cytotoxicity of the toxin molecule. However, the level of toxicity is still approx. 10^4-fold lower than the level expressed by the immunotoxins containing ricin A [20]. The liposomal ricin A formulation could contain additional specific ligands (such as antibody) on the same liposome surface. The resulting target-specific liposome formulation would be similar to the immunoliposomes containing toxin which have been previously described by this laboratory [34].

Data in Fig. 5 suggest that liposomes prolonged the circulation time of a-factor by increasing the apparent size of the molecule such that it is not rapidly excreted by the renal glomerular filter. This is similar to the existing method of size increase, i.e. conjugation with other macromolecular carriers [8]. Nevertheless, these results have demonstrated that the current liposomal approach is applicable to a relatively small molecular weight drug as well as the large protein molecules.

In summary, we have designed a liposomal formulation which effectively prolongs the circulation lifetimes of two proteins and one peptide. The method is relatively simple and is probably applicable to many other macromolecular drugs. The formulation allows a direct interaction of the drug with its receptor and is potentially targetable by incorporating specific ligands into the same liposome membrane. More than one drug could be simultaneously incorporated into the same formulation. The drug and/or ligand molecules can be incorporated in large numbers as to provide a multivalent binding which generally exhibits an enhanced binding affinity [35]. Such multivalent drugs may be beneficial if the function of drug requires an efficient target binding, such as is the case of sCD4 (see, for example, Ref. 36). Thus, the liposomal formulations described here should be useful for many macromolecular drugs including those produced by the recombinant DNA technology.

Acknowledgments

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