Biochimica et Biophysica Acta, 1066 (1991) 159–165

ADONIS 0005273691002725

BBAMEM 75283

Large liposomes containing ganglioside GM_1 accumulate effectively in spleen

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(Received 9 November 1990)

Key words: Liposome; Drug delivery system; Ganglioside; Splenic macrophage

Large liposomes, with a composition of egg phosphatidylcholine, cholesterol and ganglioside GM_1, prepared by an extrusion method, were injected intravenously into mice. After 24 h, up to 50% of injected dose was accumulated in spleen compared with about 15% in spleen for liposomes containing no GM_1. The effect of GM_1 on spleen accumulation of liposomes was liposome size dependent. Only relatively large liposomes ($d > 300$ nm) showed high accumulation; smaller liposomes were progressively less accumulated. The spleen accumulation increased with increasing injection dose of the liposomes. It was noted that the enhanced uptake by spleen was accompanied by a decrease in the liver uptake, but the total uptake of liposomes by liver and spleen was not dependent on the diameter of liposome or the presence of the ganglioside GM_1. Autoradiographs of fixed and sectioned spleen using ^{125}I-labeled tyraminylinulin as a content marker for the liposomes, showed that liposomes localized at the reticular meshwork of the red pulp. These results suggest that larger liposomes containing GM_1 are filtered by the spleen during the circulation in blood. The smaller ones with a mean diameter of less than 100 nm are not retained by the filter. The function of GM_1 is to prevent liposomes from a rapid uptake by the liver so that liposomes may circulate through the spleen and be filtered. These results, together with the observation that the liposome-entrapped proteins were degraded by the spleen, suggest the potential use of these liposomes for specific drug delivery to the spleen.

Introduction

Liposomes have demonstrated considerable promise as a carrier for the delivery of drugs *in vivo*. Enhancement of therapeutic efficacy and reduction of toxicity for a variety of drugs have been demonstrated with liposome encapsulated dosage forms (for reviews, see Refs. 1 and 2). Some areas in which liposomes display therapeutic promise as carrier are for anticancer drugs [3,4], antifungal drugs [5], antibacterials [6,7], antivirals [8] and antiparasitics [9]. The application of liposomes in immunomodulation and vaccine development has also been reported (for review, see Ref. 1).

One of the most evident observations in liposome research is that most of liposomes intravenously injected into animals are rapidly removed from the blood circulation by uptake primarily in liver, followed by the spleen, and very slightly in the lung and bone marrow [10]. It has been reported that small liposomes ($d < 200$ nm) can pass through fenestrated endothelium and gain access to liver parenchymal cells [11,12]. Recently, it has also been demonstrated that liposomes ($d \approx 100$ nm) containing ganglioside GM_1 [13,14] exhibit a prolonged circulation time in the blood. More importantly, it has been demonstrated that this type of liposome shows a higher level of uptake by the solid tumors than the ordinary liposomes [14]. Presumably, liposomes with longer circulation halflives stand a better chance to penetrate the leaky vasculature of the solid tumor. However, manipulation of liposomal properties such as the particle diameter, surface charge, and lipid composition causes temporary alterations in the organ disposition, but no qualitative change in the final pattern of liver predominance (on the per organ basis) has been reported.

Abbreviations: aGM_1, gangliotetraosyl ceramide; DTPA-SA, distearylamide of diethylenetriamine-pentaacetic acid; ePC, egg phosphatidylcholine; GM_1, monosialoganglioside; GT_{1b}, trisialoganglioside b; HPI, hydrogenated phosphatidylinositol; PBS, phosphate-buffered saline; RES, reticuloendothelial system; Sulf, sulfatide.

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A detailed study of the localization of lipid-labeled or content-labeled liposomes by Roerdink et al. [11] concluded that liposomes are taken up almost exclusively by Kupffer cells in the liver. Liposomes were phagocytosed and largely degraded in 12–24 h [2]. Most of the drugs are eventually digested since liver is the usual metabolic organ for most chemotherapeutic agents [15].

We report here the observations that a high level of accumulation of liposomes in spleen can be achieved by manipulating the vesicle diameter and the inclusion of ganglioside GM_1 in the lipid composition. Time, dose and composition dependent biodistribution of liposomes suggest that the filtration function of spleen is responsible for the high accumulation of the large, GM_1-containing liposomes. Furthermore, the results of the experiments using both lipid and content markers suggest that these liposomes with a relatively high capacity of entrapment may serve as an ideal vehicle to deliver drugs to spleen.

Materials and Methods

Materials. Egg PC and hydrogenated phosphatidylinositol (HPI) were purchased from Avanti Polar Lipids; ganglioside GM_1 and GT_{1b} from Calbiochem; bovine brain sulfatide from Supelco; asialo GM_1 from Matreya; cholesterol and lysozyme were from Sigma. The synthesis of DTPA-SA has been described [16]. The [^{111}In]DTPA-SA was prepared by mixing 1 μl of $^{111}InCl_3 \cdot HCl$ with 500 μl of 1 mM DTPA-SA which was dissolved in hot ethanol (approx. 65°C) with brief sonication. Tyraminylinulin was prepared according to Sommerman et al. [17]. The iodination of tyraminylinulin and lysozyme was performed by using Iodo-Gen reagent from Pierce.

Liposome preparation. Liposomes were prepared by hydrating a thin lipid film containing a trace amount of [^{111}In]DTPA-SA with an isotonic solution of PBS (pH 7.6) or the PBS buffer containing ^{125}I-labeled tyraminylinulin or lysozyme (10 mg/ml), respectively, for overnight at room temperature. The lipid suspension was then extruded through polycarbonate filters of defined pore size to obtain the desired particle diameter. The diameter of the liposomes was measured by dynamic laser light scattering using a Coulter NS4D instrument. Liposome-associated ^{125}I-lysozyme was resistant to trypsin digestion as shown by co-elution of ^{111}In and ^{125}I cpm on a Bio-Gel A15M column after the liposomes had been treated with trypsin (data not shown). Furthermore, ^{125}I-lysozyme stayed liposome-associated even after liposomes had incubated with 90% mouse serum for 2 h at 37°C (data not shown). These results indicate that ^{125}I-lysozyme was stably entrapped in the liposomes.

Biodistribution of liposomes. Six to eight week old Balb/c mice (male; Harlan Sprague Dawley, Indianapolis, IN) were injected intravenously with liposomes. Unless specified, 1 μmol lipids in 150 μl with about 10^6 cpm of radioactivity was injected per mouse. The diameter of liposomes used for most of the experiments was around 600 nm. Mice were bled at the retro orbital sinus and killed by cervical dislocation after 24 h for most experiments. All organs were weighed and their radioactivities were quantitated in a γ-counter. The amount of radioactivity in each organ was expressed as the percentage of total injected dose. The percent recovery was calculated from the radioactivity of the blood, spleen, liver, lung, intestine, heart and kidney. The weight of blood was assumed to be 7.4% of the total body weight [18]. Blood volume and correction factors for the blood content of various tissues were determined in Balb/c mice (same age, sex) by examining the tissue distribution of ^{51}Cr-labeled erythrocytes 30 min after i.v. injection. Labeling of erythrocytes was performed according to a standard procedure. Briefly, red blood cells of the mouse were isolated and washed with PBS five times. The suspended cells ($2 \cdot 10^7$ cells/ml) were incubated for 1 h at 37°C with 50 μCi of $Na_2{}^{51}CrO_4$ (467 mCi/mg Cr, ICN Radiochemicals). The free ^{51}Cr was removed from the cells by centrifugation. The ^{51}Cr-labeled red blood cells were then injected (i.v.) into mice for determination of the blood content of various organs. The correction factors for various organs were relatively small, with liver being the one with greatest correction which was only approx. 8%.

Autoradiographic analysis of liposome localization in spleen. Liposomes containing ^{125}I-tyraminylinulin (10^7 cpm) were injected intravenously and the spleens were collected 4 hours after liposome administration. The spleens were fixed in 5% glutaraldehyde and embedded in paraffin. Sections (5 μm thick) were cut, deparaffinized, coated with emulsion and developed 21 days later, followed by staining with hematoxlin and eosin.

Results

Effect of GM_1 and liposome size on the accumulation of liposomes in spleen

The relationship between liposome size and the disposition (24 h after injection) of liposomes *in vivo* was studied with liposomes of defined diameter. Liposomes were extruded through polycarbonate filters with defined pore size and the mean diameter of the liposomes was measured by dynamic light scattering. Fig. 1 shows the distribution of liposomes in spleen and liver as a function of the mean diameter of liposomes. The recovery of total injected marker was about the same (approx. 90%) for all liposome sizes tested. Liposomes

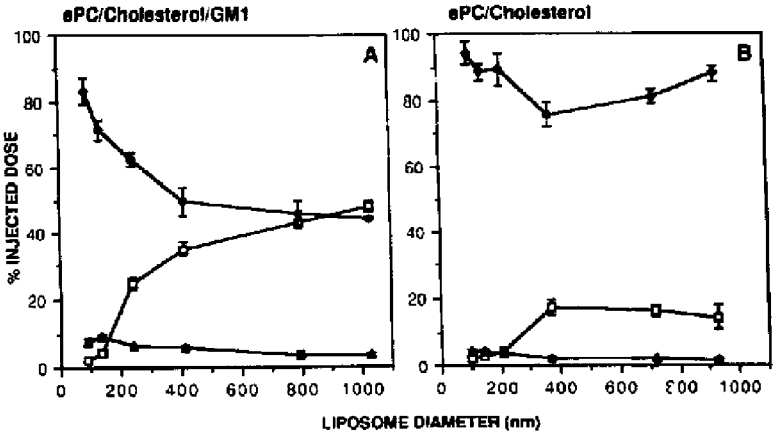

Fig. 1. Effect of the liposome size and ganglioside GM_1 on the distribution of liposomes in RES. ^{111}In-labeled liposomes were injected intravenously at a dose of 1 μmol lipid per mouse and the biodistribution of the liposomes was measured 24 h after administration. Each point represents the mean ± S.D. of three mice. □, Spleen; ●, liver; △, other organs (including lung, heart, blood, intestine and kidney). (A) Liposomes composed of ePC/chol/GM_1 = 10:5:1. (B) Liposomes composed of ePC/chol = 10:5.

containing GM_1 showed a progressive increase of accumulation in the spleen and a decrease in the liver as the diameter of liposomes increased. Approx. 50% of the injected dose was found in the spleen for liposomes with a mean diameter of 1050 nm. However, the effect of the liposome size on the spleen and liver uptake for liposomes without GM_1 was of a much lesser extent; only about 15% of the injected dose was found in the spleen for liposomes with an average diameter of 900 nm. There is no significant correlation between the diameter of the liposomes and the disposition of liposomes in the organs other than liver and spleen (data not shown). The critical diameter of liposomes for the enhanced spleen accumulation was around 300 nm.

Time course of liposome disposition in spleen

The disposition of liposomes containing GM_1 with an average diameter of about 600 nm was determined as a function of time. It is shown in Fig. 2 that the amount of liposomes in blood rapidly decreased. The increase of liposome accumulation in both spleen and liver had a similar kinetics. It took about 4 h to reach the steady state and the distribution of liposomes in spleen and liver did not change thereafter.

Liposome disposition as a function of lipid dose

Increasing amounts of large ($d \approx 600$ nm), GM_1-containing liposomes were injected into mice. The amount of lipid per 100 mg of organ or tissue weight was plotted against the injected lipid dose (Fig. 3). In the dose range of 0.4 to 4 mg (0.5–5 μmol) of lipids injected per mouse, the uptake of liposomes in both spleen and liver was directly proportional to the injected dose. The amount of lipids per 100 mg of spleen was approximately 10-fold higher than that of the liver at a dose of 4 mg per mouse, indicating that the amount of spleen accumulation was much greater than that of the liver. There was not an increase of liposome concentration in blood even in the largest dose injected, indicating that the RES, both the liver and spleen, was not saturated at this dose.

The effect of different lipid components on the distribution of liposomes in RES

It is clear from the data in Fig. 1 that GM_1 plays an important role in the high level of accumulation of liposomes in spleen. It was interesting to see if other

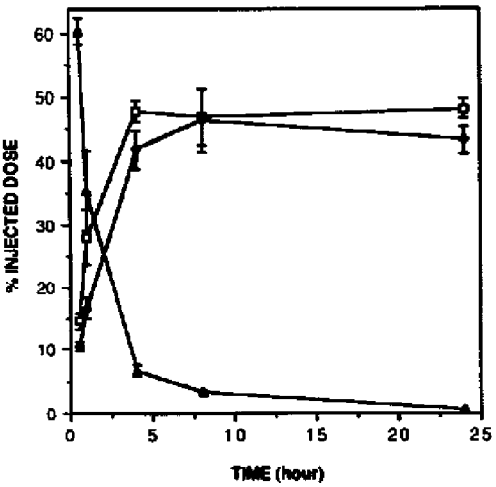

Fig. 2. Time dependent distribution of large, GM_1-containing liposomes in blood and RES. Liposomes (approx. 600 nm in diameter) composed of ePC/chol/GM_1 (10:5:1) were i.v. injected into mice at the dose of 1 μmol lipid in 150 μl per mouse. Mice were killed at different times and the radioactivity in each organ was counted. Each point represents the mean ± S.D. of three mice. □, Spleen; ●, liver; △, blood.

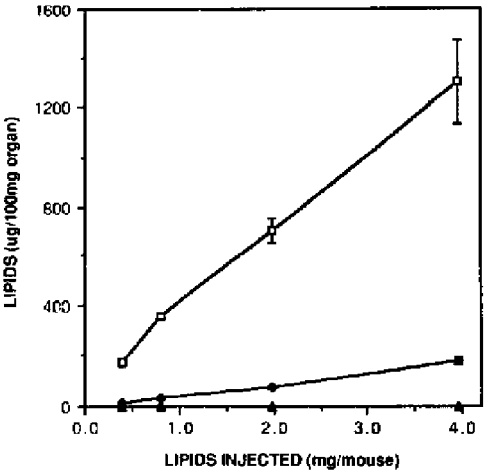

Fig. 3. Dose dependent accumulation of large, GM_1-containing liposomes in spleen and liver 24 h post-injection. Different amounts of liposomes with a diameter of about 600 nm were administered at a volume of 200 μl per mouse. The results were expressed as μg of lipid accumulated per 100 mg of organ weight. The data were the mean ± S.D. of three mice. □, Spleen; ◆, liver; △, blood.

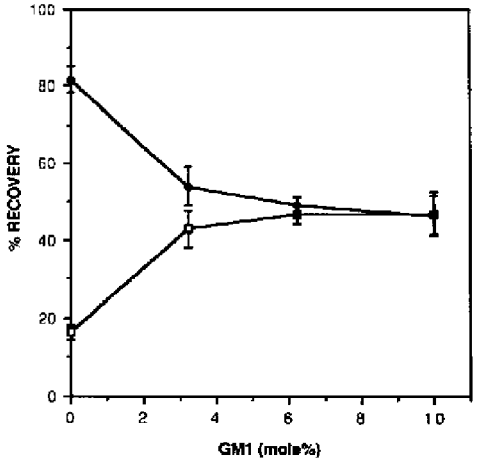

Fig. 5. Dependence on the GM_1 concentration of the distribution of large liposomes (d = 600 nm) in RES. Liposomes composed of ePC/chol (2:1) containing different amounts of GM_1 were administered (i.v.). The distribution was analyzed 24 h after administration. Each point represents the mean ± S.D. of three mice. □, Spleen; ◆, liver.

lipid components would have the same effect. Among all the lipids tested, GM_1 and GT_{1b} were natural gangliosides containing one and three sialic acid residues, respectively. Asialo GM_1 has no sialic acid. Sulfatide and HPI are both glycolipids with negative charges on the headgroup. It is clear from the data in Fig. 4 that no significant increase of spleen uptake was observed for liposomes containing glycolipids except the ones with GM_1. Thus, GM_1 seems to be unique in having the activity to cause a high level of liposome accumulation in spleen. Thus, it is important to study the GM_1 concentration dependence of the liposome accumulation in spleen. Fig. 5 shows the data of an experiment in which large liposomes containing various amounts of GM_1 were injected and the distribution of liposomes in the RES was measured 24 h later. Statistically significant increase in the percent injected dose in spleen and a concomitant decrease in liver were observed for liposomes containing as little as 3.1% GM_1. There was a nearly equal amount of liposome accumulated in the liver and spleen at the GM_1 concentrations of 6.25% or greater. Thus, it does not require a large amount of GM_1 in liposomes to cause a high level of spleen accumulation of liposomes.

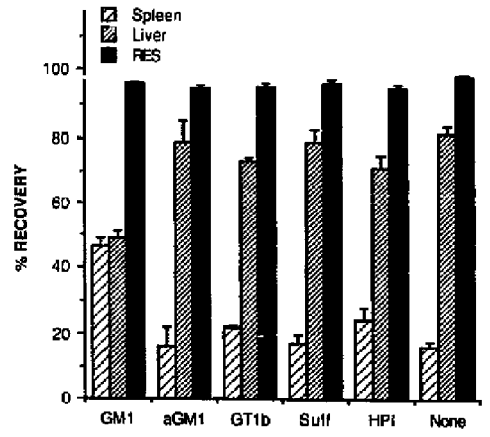

Fig. 4. The effect of different lipid components on the distribution of liposomes (ePC/cholesterol = 10:5). Liposomes with a diameter of 600 nm were administered intravenously at a dose of 1 μmol lipid in 150 μl per mouse. The distribution was analyzed 24 h after administration. The amount of test lipids used was 6.25 mol% of total lipids. The data were presented as mean− + S.D. of three mice.

The biodistribution of water soluble molecules in liposome entrapped and free form

To test the ability of liposomes to carry water-soluble molecules to the spleen, we have used two different entrapped markers, i.e. ^{125}I-labeled lysozyme (M_r 13400) and tyraminylinulin ($M_r \approx 5000$) in addition to the ^{111}In-labeled lipids. The unentrapped molecules were separated from the liposomes by gel filtration using a Bio-Gel A15M column. Unentrapped, free lysozyme and tyraminylinulin were also injected as a comparison. Table I shows the distribution of the radioactivities of both the ^{125}I and ^{111}In markers in the collected organs. It is clear that the molecules entrapped in the liposomes show different distributions than the free ones. The total recovery of the ^{125}I radioactivity in the liposome-entrapped form was higher than that of the free form. Much higher amounts of

TABLE I

Distribution of ^{125}I-labeled, water-soluble molecules as free or liposome-entrapped form in major organs of the mouse at 4 h after injection

Marker	% Dose [a]							
	blood	spleen	liver	lung	intestine	heart	kidney	recovery
^{125}I-tyraminylinulin								
free	0.2 (0.1) [b]	0.1 (0.1)	0.2 (0.1)	0.1 (0.1)	0.3 (0.1)	0.1 (0.1)	0.4 (0.1)	1.4 (0.7)
entrapped	3.5 (2.1)	29.9 (3.9)	31.8 (7.7)	0.7 (0.1)	3.1 (0.6)	0.1 (0.0)	0.3 (0.1)	69.2 (5.2)
^{125}I-lysozyme								
free	2.1 (2.2)	0.0 (0.1)	0.6 (0.4)	0.1 (0.1)	5.4 (3.2)	0.1 (0.0)	1.1 (0.3)	9.1 (6.5)
entrapped	2.3 (2.0)	3.5 (0.7)	4.1 (0.5)	0.2 (0.1)	6.2 (2.3)	0.1 (0.0)	0.7 (0.1)	16.8 (5.5)
^{111}In-lipids	3.0 (0.6)	41.0 (1.1)	41.7 (3.6)	1.1 (0.3)	2.0 (0.3)	0.5 (0.4)	0.6 (0.4)	89.3 (2.5)

[a] 1 μmol lipid in the form of liposome containing 10^6 cpm of content marker and 10^4 cpm lipid marker was administered intravenously per mouse.

[b] Data represent the mean (S.D.) of three mice.

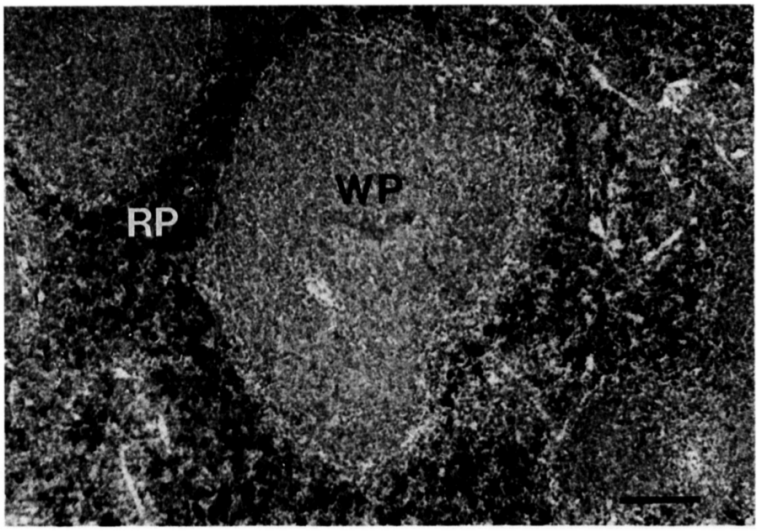

Fig. 6. Autoradiograph of a histologic section of mouse spleen. Liposomes of ePC/cholesterol/GM_1 (10:5:1) with a diameter of about 600 nm and containing ^{125}I-tyraminylinulin were i.v. injected (10 μmol lipids and 10^7 cpm in 400 μl). The mouse was killed 4 h later and the spleen was excised and fixed in 5% glutaraldehyde. Sections of spleen were coated with emulsion, exposed for 21 days, developed and visualized under a light microscope. Magnification is 465.6 x. Bar is 25 μm. WP, white pulp; RP, red pulp.

^{125}I-tyraminylinulin (30–32%) was found in the spleen and liver than ^{125}I-lysozyme (3–4%) when they were administered in the liposomal form. However, both markers showed lower amounts in these RES organs than the ^{111}In-labeled lipid (approx. 41%).

The distribution of liposomes within the spleen

In order to explore the mechanisms of high uptake of large, GM_1-containing liposomes by spleen, we have examined the distribution of liposomes within the organ. Fig. 6 shows an autoradiograph of a section of spleen from a mouse injected with liposomes containing ^{125}I-tyraminylinulin as a radioactive marker. It is clear that the silver grains were only found in the reticular meshwork of the red pulp. Furthermore, the distribution of the silver grains in the red pulp was uneven; clusters of grains were seen throughout the red pulp and the marginal zone.

Discussion

It has been well documented that intravenously injected liposomes of various size, charge content and membrane fluidity are rapidly and predominantly taken up by the Kupffer cells in the liver (for review, see Ref. 1). Spleen uptake is usually minor and increases only after the liver uptake is saturated [19]. The observations described here deviate from the liver dominance rule and document the fact that the spleen uptake of liposomes can be approx. equal to that of the liver (approx. 10-times greater if calculated on the per organ weight basis). Data in Fig. 1 also indicate that the

enhanced spleen uptake is at the expense of the liver uptake; i.e. there is a redistribution of liposomes within the two RES organs.

This novel observation can be understood on the basis of two independent facts: (1) the presence of GM_1 in liposomes significantly reduces the Kupffer cell uptake of liposomes and results in a prolonged circulation time of liposomes [13,14,20,21], (2) the spleen contains a filtration mechanism which removes unwanted red blood cells and particles from the blood [22]. Data in Fig. 1 indicate that only those liposomes containing GM_1 and having a mean diameter greater than 300 nm are accumulated in the spleen. Thus, GM_1 functions to reduce the liver uptake of liposomes which showed a prolonged residence time in blood. The volume of blood flow through spleen is usually a small fraction of that of the liver. Ordinary liposomes without GM_1 would have little chance to circulate through the spleen, hence they are predominantly taken up by the liver. Liposomes containing GM_1, because of the prolonged residence in blood, encounter spleen much more readily than the liposomes without GM_1. If the GM_1-containing liposomes are large enough, they would be retained by the spleen filter, resulting in an elevated level of liposome accumulation in the organ.

Spleen is a spongy organ which contains the white pulp and the red pulp. The red pulp contains a meshwork of reticular cells which form the sinuses of the spleen. It serves as a filter to purify the humors derived from the liver or from the blood [22]. Damaged or aged red blood cells are retained by the filter and are ingested by the splenic macrophages residing in the red pulp. Large liposomes must also be retained by the reticular filter in the same manner. Autoradiographic study of the spleen revealed that the retained liposomes were found exclusively in the red pulp (Fig. 6). They seem to cluster in regions resembling the sinusoid structure. These results strongly support that the filtration mechanism of the spleen is responsible for the high level of spleen accumulation of large liposome containing GM_1.

GM_1 seems to be the only tested glycolipid which endows the liposomes with a high level of spleen accumulation (Fig. 4). Sulfatide and HPI are effective in prolonging the circulation time of liposomes composed of distearoyl PC and cholesterol [14]. In our own studies, GT_{1b} also causes a reduction in the liver uptake of liposomes composed of unsaturated phosphatidylethanolamine (unpublished data). These glycolipids are, however, inefficient in reducing the liver uptake of liposomes composed of PC and cholesterol. This result strongly indicates that the activity of a glycolipid to prolong the liposome circulation time depends on the matrix lipid composition of liposomes, perhaps also on the size and other physical parameters of liposomes. Further studies will undoubtedly reveal more details of the chemical and physical mechanisms which control the uptake of liposomes by the Kupffer cells in liver. While these mechanisms remain unclear at the present time, data from this lab [23] indicate that polyethyleneglycol conjugated to phosphatidylethanolamine can effectively enhance the spleen uptake of the large liposomes containing the conjugate. It has been shown that the conjugate effectively reduces the liver uptake of small liposomes composed of ePC and cholesterol, resulting in a prolonged residence time in the blood. Therefore, GM_1 is not unique in causing an elevated level of accumulation of liposomes in spleen.

Data in Table I indicate that liposomes accumulated in the spleen are subjected to the metabolic process of the organ. The ^{111}In-labeled lipid marker, which is non-metabolizable and non-excretable [24], accumulated efficiently in the spleen. The accumulation of the ^{125}I-labeled entrapped marker, however, depended on the molecular nature of the marker itself. Tyraminylinulin is known as a non-metabolizable marker and it was accumulated at a relatively high level. Lysozyme, on the other hand, can be readily degraded by proteinase and was not accumulated at any significant level in the spleen. The fact that a protein marker entrapped in liposomes is rapidly degraded and removed from the spleen indicates that liposomes retained by the filter must also be taken up by the splenic macrophages, just like the damaged and aged red cells. This observation has an important implication because it suggests that the GM_1-containing liposomes are still ingestable by the spleen macrophages. In other words, GM_1 may only reduce the liposome's affinity to the hepatic (the Kupffer cells), but not the splenic, macrophages. While this hypothesis will have to be further tested, the observation that liposomes can deliver proteins to the splenic macrophages may be significant in terms of the vaccine development. Splenic macrophages are active in antigen presentation. A high dose of antigen delivered to these cells may facilitate an effective antigen processing and presentation to other immune cells. This possibility is certainly worthy of further experimentation.

Acknowledgment

This work was supported by NIH grants AI25834 and CA24553. We thank Carolyn Drake for manuscript preparation and James Wesley for preparing the spleen sections and autoradiographs.

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