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Large unilamellar liposomes with low uptake into the reticuloendothelial system

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Particulate drug carriers, including liposomes, are rapidly removed from blood by cells of the reticuloendothelial system (RES) with resulting adverse effects on this important host defense system. In order to overcome this and other major disadvantages of liposomes, we have altered liposome composition in an effort to achieve prolonged circulation half-lives. Gangliosides and sphingomyelin act synergistically to dramatically diminish the rate and extent of uptake of liposomes by macrophages in vivo. The significantly extended circulation times achieved by these modified large unilamellar liposomes overcome an important barrier to the targeting of particulate drug carriers to specific tissues in vivo.

Liposome; Ganglioside; Sialic acid; Reticuloendothelial system; Sphingomyelin

1. INTRODUCTION

For many years various types of particulate drug carriers have been investigated with the aim of perfecting drug-delivery systems which will localize drugs to their desired in vivo target tissue, thus minimizing their side effects [1]. Liposomes are in some ways ideal drug carriers being biodegradable and of minimal toxicity; however, there are important drawbacks to their use in vivo. We show here how it is possible to overcome one of the major hurdles to the in vivo use of liposomes as drug carriers.

In spite of a lipid composition which closely resembles that of cell membranes, liposomes are recognized as foreign by the cells of the reticuloendothelial system (RES). This results in their rapid removal from circulation following intravenous administration primarily by Kupffer cells of the liver and fixed macrophages of the spleen. Up to 85% of large egg phosphatidylcholine/cholesterol, 2:1 (egg PC:CH) liposomes can be found in liver and spleen 0.5 h post-injection [2,3]. The reason for the efficient uptake of injected liposomes by RES cells is not currently well understood, but is thought to be related to the opsonization of liposomes by plasma proteins [4]. This rapid localization of liposomes in the RES has at least two important consequences. Firstly, drug carriers which localize rapidly in the RES cannot be targeted to non-RES tissues in a specific manner which results, therefore, in a severe limitation of their therapeutic potential. Secondly, multiple liposome injections over a period of several days can result in impaired functioning of the RES [3,5] which is an important component of the host defense system [6].

One of the most notable differences between liposomal membranes and cell membranes is the presence of large amounts of surface carbohydrates on the latter, and in particular the presence of surface sialic acid residues which impart negative charge to the surface membrane. The role of cell surface carbohydrates in cellular

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recognition phenomena is widely appreciated, although poorly understood [7,8]. Enzymatic removal of sialic acid from circulating cells results in their rapid uptake into Kupffer cells of the liver [9–11]. A series of experiments in mice was therefore conducted to examine the effect of liposomal surface sialic acid, in the form of gangliosides, on RES uptake of liposomes in vivo. Earlier experiments have shown that inclusion of ganglioside in egg PC:CH liposomes greatly reduces their susceptibility to lysis and contents leakage induced by plasma components [12]. We also examined the effect of increasing bilayer rigidity, as we have previously found that increasing rigidity decreases interactions of liposomes with high density lipoproteins [13] and increases circulation times [2].

2. EXPERIMENTAL

Gangliosides (Supelco, Bellefonte, PA) were dissolved in chloroform/methanol (2:1) and aliquots were added to appropriate mixtures of egg PC, bovine brain sphingomyelin (SM, Avanti, Birmingham, AL) and cholesterol (Sigma, St. Louis, MO) in chloroform prior to evaporation of organic solvent. Buffer (5 mM Tes, 100 mM NaCl, 0.1 mM EDTA) containing sufficient cpm of ^{125}I-tyraminylinulin [14] was added during liposome preparation to result in 10^5–10^6 cpm/mouse of entrapped label. Large unilamellar liposomes (LUV) were prepared according to Szoka and Papahadjopoulos [15] and extruded through 0.4 μm Nucleophore filters according to Olsen et al. [16]. Liposomes were sized on a Nicomp Instruments laser particle sizer. Liposomes extruded through a 0.4 μm filter averaged 0.17 ± 0.05 μm in diameter and liposomes extruded through a 0.2 μm filter averaged 0.16 ± 0.05 μm diameters. Sizes were very consistent from sample to sample. ICR mice (3 per group) were injected in the tail vein with 0.5 mg phospholipid in 0.2 ml buffer and samples of blood and tissues were obtained at the appropriate times. Some mice were injected with free label. Whole organs, carcass or 100 μl blood were counted for ^{125}I in a Beckman 8000 gamma counter. Blood volume was taken to be 7% of body weight, and tissue counts were corrected for counts present in blood.

3. RESULTS AND DISCUSSION

In our initial experiments we explored the effect of the type of ganglioside and ganglioside concentration on large unilamellar liposomes (LUV) composed of egg PC:CH (2:1). LUV have the advantage of entrapping large volumes of drug solution, but their utility is usually limited due to the rapid rate at which they are taken up by the RES. The effect of increasing monosialylganglioside (II^3NeuAc-GgOse$_4$Cer, G_{M1}) concentrations on LUV levels in blood, liver and spleen is shown in fig.1. Liver uptake was dependent on ganglioside concentration, with peak blood levels and minimum liver uptake observed at 5–7 mol% gangliosides. G_{M1} could increase circulating blood levels of liposomes from 3- to 10-fold at optimum ganglioside concentrations for egg PC:CH liposomes. However, uptake into the RES still remained high with more liposomes present in liver and spleen than were present in blood 2 h post-injection. The ratio of % injected liposomes remaining in blood to that in liver plus spleen is termed the blood/RES ratio. No significant levels of liposomes were observed in any other tissues or in carcass (not shown).

In our next experiment we substituted high concentrations of sphingomyelin (SM) for egg PC in LUV and examined the effect of increasing G_{M1} concentration on blood, liver and spleen levels. SM has a bilayer-rigidifying effect due to its ability to form intermolecular hydrogen bonds [13]. The result was a dramatic increase in levels of circulating liposomes and a concomitant decrease in uptake into liver and spleen (fig.1B). Maximum effects were observed in the range 7–15 mol% G_{M1}. However, at 15 mol% G_{M1} the liposomes experienced a significant increase in the rate of contents leakage in blood, and even at 7 mol% G_{M1} the liposomes were rather leaky (not shown). RES levels of SM:PC (4:1) LUV (0.17 μm) at optimum ganglioside concentrations, were more than 20-fold lower than those seen for similar sized egg PC:CH in the absence of gangliosides, and blood/RES ratios at the 2 h sampling time were more than 25-fold greater than those for PC:CH, 2:1 LUV (0.17 μm).

Residence time for intact liposomes in blood reflects both lack of RE uptake and liposome stability to degradation by plasma proteins.

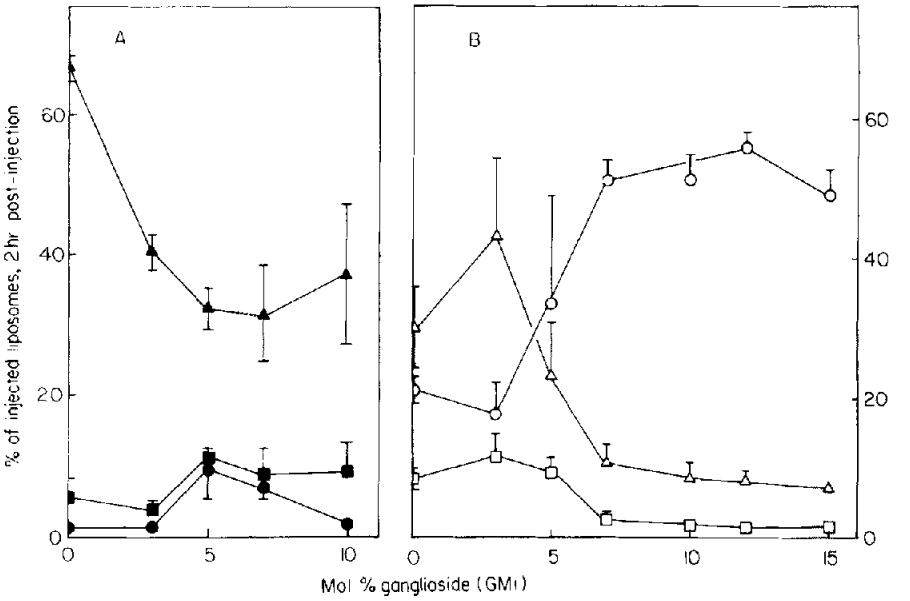

Fig.1. Tissue distribution of liposomes containing entrapped ^{125}I-tyraminylinulin, 2 h post-injection, as a function of ganglioside concentration. (A) LUV (0.17 μm) of egg PC:CH, 2:1 containing ganglioside G_{M1} (Supelco, Bellefonte, PA): liver (▲), spleen (■), blood (●). (B) LUV (0.17 μm) of SM:PC, 4:1 containing G_{M1}: liver (△), spleen (□), blood (○).

Because the SM:PC:G_{M1}, 4:1:0.35 liposomes had lost up to 90% of their entrapped contents by 24 h post-injection, we used the more stable liposome composition SM:PC:G_{M1}, 1:1:0.14 for a subsequent experiment. Less than 20% of injected liposomes, in spite of their large size, were localized in liver and spleen 24 h post-injection and more than 60% of contents were still present in vivo (fig.2). This contrasts with the more than 50% of PC:CH, 2:1, LUV (0.17 μm) present in liver and spleen at the same time point (not shown). Between 6 and 24 h post-injection the intact liposomes moved from circulation into carcass (fig.2A). Free label was rapidly eliminated from the body (fig.2B). Higher whole body levels of free counts 0.5 h post-injection reflect high carcass levels which include free counts in urinary bladder, the site of elimination of this label [16].

The increased circulation times of the modified liposomes can be directly attributed both to the presence of surface sialic acid and to the achievement of optimum bilayer stability (table 1). Addition of G_{M1} at 7 mol% to PC:CH LUV (0.17 μm) caused an increase in the blood/RES ratio by a factor of 13 at 2 h post-injection. However, 7 mol% asialylganglioside (ASG_{M1}, Supelco) increased blood/RES ratios less than 5-fold (table 1). Inclusion of 80 mol% SM in PC liposomes resulted in a 60-fold increase in blood/RES ratio, and addition of 7 mol% G_{M1} caused an additional 6-fold increase (table 1). Inclusion of 7 mol% ASG_{M1} in SM:PC liposomes slightly decreased blood/RES ratio. These results indicate the importance of sialic acid rather than neutral carbohydrate in the observed effect.

The effect of various manipulations of bilayer rigidity 2 h post-injection is also reported in table 1. Addition of cholesterol, which has a bilayer tightening effect in fluid liposomes, to egg PC liposomes increased the blood/RES ratios by 13-fold. Liposomes composed of pure DSPC or SM, which are below or near their phase transition at 37°C, had a low blood/RES ratio, but addition of 20 mol% egg PC or 33 mol% cholesterol to SM liposomes, or 7 mol% G_{M1} to DSPC liposomes (fluidizing effect) resulted in significantly higher blood/RES ratios (table 1) than those seen for either liquid-crystalline or gel state liposomes. Addition of 7 mol% G_{M1} to SM liposomes resulted in liposomes which were very leaky in vivo (table 1). Addition of cholesterol, however, to SM:PC, 4:1 liposomes, with or without G_{M1}, decreased the

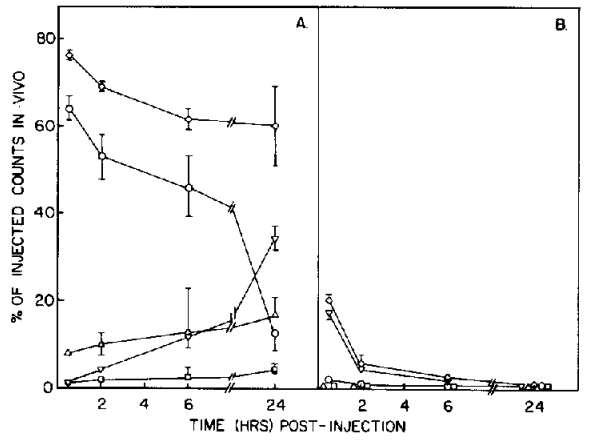

Fig.2. Tissue distribution in mice (n = 3) of liposomes containing ^{125}I-tyraminylinulin of free marker as a function of time post-injection. (A) SM:PC:G_{M1}, 1:1:0.14 (0.2 μm LUV); (B) free ^{125}I-inulin. Total counts in vivo (◇), blood (○), liver (△), spleen (□), carcass, including bladder (▽).

blood/RES ratio primarily due to increased uptake to liposomes into spleen. Cholesterol can interfere with the hydrogen-bonding ability of SM and cause a destabilization of SM bilayers to serum components [13]. It would appear that an optimum bilayer viscosity, occupying an intermediate value between liquid-crystalline and gel phase lipid, results in optimal circulation times for liposomes. These data clearly demonstrate that surface sialic acid and bilayer viscosity have a synergistic effect in increasing circulating times of liposomes, however sulfatides which, like gangliosides, impart surface hydrophilicity and negative charge to the liposome surface are also capable of increasing blood/RES ratios, although less effectively than G_{M1} (table 1).

Our results have important implications for the use of liposomes as a slow release drug-delivery system within the vasculature, particularly because prolonged circulation times have now been

Table 1

Effect of ganglioside G_{M1}, cholesterol or combination of the two on blood/RES ratios for liposomes of varying compositions, 2 h post-injection (mean ± SD, n = 3)

Composition	Size (μm)	Blood/RES	% remaining in vivo
PC	0.17	0.010 ± 0.005	86.0 ± 5.4
PC:CH, 2:1	0.17	0.13 ± 0.08	78.1 ± 0.04
PC:G_{M1}, 1:0.07	0.17	0.17 ± 0.12	79.4 ± 5.9
PC:CH:G_{M1}, 2:1:0.14	0.16	1.7 ± 0.5	75.6 ± 5.7
PC:CH:ASG_{M1}, 2:1:0.14	0.16	0.62 ± 0.44	64.8 ± 1.6
DSPC	0.17	0.015 ± 0.002	91.2 ± 2.00
DSPC:CH, 2:1	0.17	0.007 ± 0.00	101.2 ± 2.4
DSPC:G_{M1}, 1:0.07	0.17	2.0 ± 0.02	76.7 ± 3.1
DSPC:CH:G_{M1}, 2:1:0.14	0.17	3.2 ± 1.0	64.6 ± 3.5
SM	0.17	0.02 ± 0.01	27.1 ± 3.1
SM:CH, 2:1	0.17	0.7 ± 0.2	71.9 ± 4.4
SM:G_{M1}, 1:0.07	0.17	5.7 ± 1.8	12.4 ± 0.7
SM:CH:G_{M1}, 2:1:0.14	0.17	4.6 ± 0.6	72.1 ± 1.5
SM:PC, 4:1	0.17	0.6 ± 0.2	69.0 ± 3.3
SM:PC:CH, 4:1:3	0.17	0.12 ± 0.06	69.9 ± 2.2
SM:PC:CH:SO_4, 4:1:3:0.35	0.17	0.43 ± 0.21	78.4 ± 1.4
SM:PC:G_{M1}, 4:1:0.35	0.16	3.3 ± 0.3	61.8 ± 2.9
SM:PC:CH:G_{M1}, 4:1:3:0.35	0.16	1.5 ± 0.6	88.5 ± 3.0
SM:PC:ASG_{M1}, 4:1:0.35	0.16	0.9 ± 0.5	80.3 ± 2.5

G_{M1} and ASG_{M1} concentrations are expressed as the molar ratio of total phospholipid. The ratio of % injected counts in blood to % injected counts in liver plus spleen is termed blood/RES ratio

achieved for large unilamellar liposomes which have a large entrapped volume and efficient drug capture characteristics. There are also important applications for the use of these modified liposomes in order to target drugs to tissues other than spleen, liver and lung by means of tissue-specific antibodies covalently coupled to the liposome surface. In addition, the potential of multiple administrations of liposomes to cause impaired functioning of the RES will be considerably reduced through the use of these modified liposomes which appear to avoid substantially uptake by the RES.

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