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Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives in vivo

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Novel synthetic lipid derivatives of poly(ethylene glycol) (PEG) have been synthesized and tested for their ability to decrease uptake of liposomes into the mononuclear phagocyte system (MPS, reticuloendothelial system) in mice and to prolong circulation half-lives of liposomes. A carbamate derivative of PEG-1900 with distearoylphosphatidylethanolamine (PEG-DSPE) had the greatest ability to decrease MPS uptake of liposomes, at optimum concentrations of 5–7 mol% in liposomes composed of sphingomyelin/egg phosphatidylcholine/cholesterol (SM/PC/Chol, 1:1:1, molar ratio). Results obtained with this compound were equivalent to results previously obtained with 10 mol% monosialoganglioside G_{M1} in liposomes of similar compositions (Allen, T.M. and Chonn, A. (1987) FEBS Lett. 223, 42–46). Non-derivatized methyl PEG or PEG-stearic acid (PEG-SA) were incapable of decreasing MPS uptake of liposomes. PEG-Chol and PEG-dipalmitoylglycerol (PEG-DPG) were intermediate in their effects on MPS uptake. Altering liposome size for liposomes containing PEG-DSPE resulted in only minor changes in blood levels of liposomes. Half-lives of 0.1 μm liposomes of SM/PC/Chol/PEG-DSPE (1:1:1:0.2, molar ratio) in circulation was in excess of 20 h following either i.v. or i.p. injection. Liver plus spleen liposome levels for these liposomes was below 15% of injected label at 48 h following i.v. liposome injection and below 10% following i.p. injection. The major site of liposome uptake was in carcass tissues, with over 50% of label remaining *in vivo* at 48 h post-injections, either i.v. or i.p., in the carcass.

Introduction

One of the major obstacles to the use of liposomes as drug delivery systems for a variety of therapeutic applications has been their rapid removal from circulation by the cells of the mononuclear phagocyte system (MPS, reticuloendothelial system), an important host defense system. Liposome compositions which avoid MPS uptake and achieve prolonged circulation half-lives *in vivo* (Stealth^R liposomes) have recently been described [1–4]. These long-circulating formulations depend on the presence of surface hydrophilicity and the presence of shielded surface negative charges imparted by compounds such as monosialylganglioside, G_{M1} in an optimum concentration of 7–15 mol%, in combination with membrane rigidifying agents such as cholesterol and/or sphingomyelin [3,4]. However, the expense of G_{M1}, and the difficulties in obtaining large quantities of this lipid either by extraction of natural sources or by synthesis, make therapeutic applications of G_{M1}-containing liposomes impractical. These considerations have prompted us to search for inexpensive alternatives to G_{M1} which are equally or more capable of prolonging circulation half-lives of liposomes.

Covalent attachment of certain water-soluble polymers such as poly(ethylene oxide)s to proteins has been shown to decrease their immunogenicity and antigenicity. Poly(ethylene glycol) covalently attached to bovine serum albumin not only decreased the immunogenicity of this protein but also markedly increased blood circulation times of the conjugate [5,6]. Monoethoxypoly(ethylene glycol) has been used to induce tolerance to uricase in mice [7], Abuchowski and colleagues have developed PEG-enzymes such as PEG-adenosine deaminase (PEG-ADA) and PEG-L-asparaginase which have extended circulation half-lives. PEG-ADA has been approved by the U.S. Food and Drug Administration for use in humans [8].

It has been observed by Illum et al. [9] that microspheres coated with hydrophilic materials such as

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poloxamer 338 experienced reduced uptake into the MPS system in rabbits. The prolonged circulation half-lives of liposomes containing G_{M1} has also been ascribed in part to the increased surface hydrophilicity imparted by G_{M1} to the liposomes [3,4].

We have, therefore, synthesized lipid derivatives of poly(ethylene oxide)s and tested them for their ability to prolong the circulation half-lives of liposomes in vivo in mice. The resulting synthetic compounds were very effective in preventing MPS uptake of liposomes and in prolonging their circulation times. A report from our laboratory on the ability of lipid derivatives of poly(ethylene glycol) to dramatically reduce the uptake of liposomes by mouse bone marrow-derived macrophages has been published [10]. Two recent reports on the ability of PEG(5000)-DSPE to increase the circulation half-lives of liposomes have recently appeared in the literature [11,12].

Materials and Methods

Materials

Egg phosphatidylcholine (PC), distearoylphosphatidylcholine (DSPC), bovine brain sphingomyelin (SM), distearoyl-PE (DSPE), dipalmitoylglycerol (DPG) and bovine brain phosphatidylserine (PS) were purchased from Avanti Biochemicals, Birmingham AB. Monosialylganglioside (G_{M1}) was purchased from Makor Chemical, Jerusalem. Cholesterol (Chol) and *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (Tes) was purchased from Sigma Chemical, St. Louis, MO. Poly(ethylene glycol) methyl ethers (PEG), with average molecular masses of 120, 750, 1900 and 5000 daltons, and methyl PEG 1900 were purchased from Aldrich Chemical Co. (Milwaukee, WI). PEG 1750 monostearate (PEG-SA) was purchased from Lonza Inc., Long Beach, CA. Tyraminylinulin was synthesized and ^{125}I-tyraminylinulin (^{125}I-TI) was prepared according to the technique of Sommerman et al. [13]. Pyrogen-free saline for injection (0.9%, USP) was obtained from Travenol Canada Inc., Mississauga, Ont. All containers used in the experiments were sterile and pyrogen-free.

Synthesis of lipid derivatives of poly(ethylene glycol)

Lipid derivatives of poly(ethylene oxide)s, e.g. poly(ethylene glycol), can be made if the lipids contain a primary amine group, e.g. phosphatidylethanolamine (PE), an epoxy group or a diacylglycerol. Three different reaction schemes resulting in three different types of nitrogen-linked poly(ethylene glycol) derivatives of PE have been prepared, namely substituted *N*-alkyl PE, substituted 1,3,5-triazinyl PE and poly(ethylene glycol) carbamate PE. The poly(ethylene glycol) carbamate derivatives were preferred due to the superior yield and ease of preparation. This simple, two-step procedure (Fig. 1) is described in detail below.

Poly(ethylene glycol) methyl ether (I) was dissolved in benzene which had previously been dried over molecular sieves. Carbonyl diimidazole (II) was added to the PEG methyl ether in benzene in a final molar ratio of 1:1.1, and the air displaced with a nitrogen atmosphere. The mixture was enclosed in a vessel, and heated to 75°C for at least 16 h. The mixture was then cooled and allowed to stand at room temperature until the solution of imidazole derivative of the carbamate of PEG methyl ether (III) clarifies. The mixture was then diluted with dry benzene to be stored.

The poly(ethylene glycol) imidazole carbamate (III) was then coupled with PE to form a PEG carbamate-PE as follows. The solution of compound III was dried under vacuum. PE (IV), dissolved in chloroform, was added to compound III in a molar ratio of 1:2. The chloroform was evaporated under vacuum and triethylamine (V) dissolved in 1,1,2,2-tetrachloroethylene or benzene was added to compound III at a molar ratio of 1:1 (V/IV). The flask containing the mixture was closed and heated to 95°C for 6 h. PEG-PE was purified by reverse phase chromatography in ethanol/water (4:1, v/v). The resulting PEG-PE carbamate compound (VI) had a molecular mass of 2654 daltons, if PEG(1900) was used as the starting material, and a yield of approx. 53%.

PEG-cholesterol (PEG-Chol) and PEG-dipalmitoylglycerol (PEG-DPG) were prepared as follows. Because this procedure involves the preparation of ethers of methyl PEG, necessitating the use of potassium hydride, synthetic operations were done under an atmosphere of inert gas. 40 mg (1.0 mmol) of potassium hydride was freed from adherent mineral oil by wash-

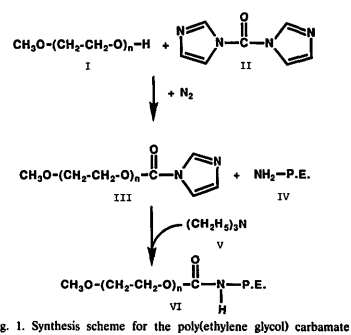

Fig. 1. Synthesis scheme for the poly(ethylene glycol) carbamate derivative of PE.

ing with hexane and drying beneath a gentle stream of inert gas. To the resulting powder was added 3.0 ml of dimethyl formamide (dried over molecular sieves) and 1.0 mmol of the desired alcohol (such as cholesterol or dipalmitoylglycerol). The reaction mixture was warmed to 60°C and agitated until hydrogen evolution ceased (about 2 h). At this time, 2.0 mmol of the appropriate methyl PEG ester or bromide was added and the reaction mixture heated at 100°C for 4 h. The reaction mixture was then acidified with 50 μl of formic acid and the solvent removed by vacuum evaporation to constant weight.

The residue was purified by column chromatography, typically using silica gel adsorbent and chloroform mixed with increasing volumes of 2% concentrated aqueous ammonia in methanol as a developer. Column operation was monitored by thin-layer chromatography (TLC). For some methyl PEG derivatives, it was difficult to distinguish reaction products from starting materials by TLC on silica gel plates. For these situations, analysis was by TLC on Si-C_{18} plates (J.T. Baker Co.) using ethanol/water (4:1, v/v) as a solvent system.

Preparation of liposomes

Liposomes were composed primarily of SM/PC/Chol, 1:1:1, PC/Chol, 2:1 or DSPC/Chol, 2:1 with various lipid derivatives of PEG or with G_{M1} added in the appropriate molar ratios. Liposomes were made either by the extruded multilamellar vesicle method [14,15] or by the extruded reverse phase evaporation method [15,16], as has been previously described [4]. Liposomes were made in the presence of ^{125}I-TI as an aqueous space marker in a buffer composed of sterile, pyrogen-free 0.9% saline (Travenol Canada) buffered with 10 mM Tes (pH 7.4). Free ^{125}I-TI was removed from liposome-entrapped label by chromatography over Ultrogel AcA34 columns (IBF Biotechnics, France). Liposome concentrations were normally 10 μmol/ml and liposomes were normally extruded 10 times through Nuclepore filters [13] of 0.1 μm pore size, although for some experiments larger or smaller pore size filters were used. The resulting liposomes were sized by dynamic light scattering using a Brookhaven BI90 particle sizer (Brookhaven Instrument Corp., Holtsville, NY) and trapped volumes were determined. MLV liposome preparations which had been extruded through 0.1 μm filters had sizes ranging from 104 to 136 nm in diameter and trapped volumes of approx. 2.2 l/mol phospholipid, as determined from the specific activity of entrapped ^{125}I-TI. MLV liposomes extruded several times through 0.1 μm Nuclepore filters have been shown to be primarily unilamellar [14].

Determination of PEG in liposomes

Various mol% of PEG-DSPE (PEG average molecular weight of 120, 750, 1900 and 5000) were added to liposomes composed of SM/PC/Chol (1:1:1, molar ratio) during liposome formation by the MLV method. Liposomes were then extruded 10 times through 0.1 μm Nuclepore filters and passed over Sepharose 4B columns in buffered saline to separate unincorporated PEG-DSPE micelles from liposome-associated PEG-DSPE. PEG-DSPE in liposomes or in micelles could be readily quantitated by measuring the formation of a blue colour with the BioRad protein assay [17] (Bio-Rad Canada, Mississauga, Ont.). Standard curves were constructed using PEG-DSPE of different average molecular weights of PEG, and results for PEG incorporation into liposomes were calculated from the standard curves.

Animal experiments

Female ICR (outbred) mice in the weight range of 23–27 g were obtained from the Animal Breeding Unit of the University of Alberta and were maintained in standard housing.

Mice (three per group) were injected in the tail vein with 0.5 μmol/mouse of phospholipid in 0.2 ml of sterile, pyrogen-free saline (0.9%, USP) buffered with 10 mM Tes (buffered saline). Individual mice received 10^5 to 10^6 cpm of ^{125}I-tyraminylinulin. At selected times post-injection mice were killed and selected tissues and organs were excised and counted for radiolabel in a Beckman 8000 gamma-counter. Tissues and organs sampled included blood, liver, spleen, lung, heart, kidney, thyroid and carcass, which was the remainder of the animal. Blood-correction factors were applied to all tissues and carcass [2]. Results are sometimes expressed as blood/MPS ratio, which is the ratio of percent injected counts remaining in vivo in blood to percent injected counts remaining in vivo in liver plus spleen.

Results

A comparison of liposomes containing either no additional components, or containing G_{M1} or different lipid derivatives of PEG is given in Table I. Liposomes composed of SM/PC/Chol and 10 mol% of either G_{M1} or PEG(1900)-DSPE had substantially elevated blood levels, particularly at the longer time points compared to liposomes in the absence of these constituents or those prepared with either non-derivatized PEG or PEG-SA. Liposomes containing PEG(1900)-Chol and PEG(1900)-DPG had high blood levels at 2 h post-injection but compared to liposomes containing G_{M1} and PEG(1900)-DSPE the blood levels at 24 h post-injection were much lower. Liposomes containing 10 mol% G_{M1} had blood levels which were not significantly different from those seen for liposomes containing PEG(1900)-DSPE at all time points (Table I). Combinations of G_{M1} with various PEG-lipid derivatives, or

TABLE I

A comparison of the effect of GM_1 with that of various lipid derivatives of PEG-1900 (except PEG-SA, where PEG-1750 was used) on blood levels after i.v. injection in mice

Liposomes were MLV extruded through 0.1 μm Nuclepore filters and averaged 112 to 136 nm in size. Liposomes were labelled with ^{125}I-tyraminylinulin, and mice received 0.5 μmol phospholipid per mouse in 0.2 ml of sterile buffered saline. Results are expressed of % of injected cpm remaining in vivo at various times post-injection. Mean ± S.D.

Liposome composition (molar ratio)	% of in vivo in blood at time post-injection (number of mice)		
	2 h	24 h	48 h
SM/PC/Chol (1:1:1)	60.0 ± 10.5 (9)	5.1 ± 7.4 (8)	n.d.
SM/PC/Chol/G_{M1} (1:1:1:0.2)	83.1 ± 6.6 (15)	30.8 ± 8.0 (9)	13.2 ± 2.7 (3)
SM/PC/Chol/PEG-DSPE (1:1:1:0.2)	77.5 ± 11.9 (24)	38.2 ± 6.9 (21)	13.1 ± 4.7 (11)
SM/PC/Chol/MePEG (1:1:1:0.2)	59.6 ± 1.9 (3)	15.3 ± 16.6 (3)	n.d.
SM/PC/Chol/PEG-SA (1:1:1:0.2)	54.3 ± 12.2 (6)	10.4 ± 5.6 (3)	n.d.
SM/PC/Chol/PEG-CHOL (1:1:1:0.2)	81.4 ± 8.0 (3)	11.2 ± 4.0 (3)	n.d.
SM/PC/Chol/PEG-DPG (1:1:1:0.2)	70.6 ± 2.9 (3)	17.4 ± 0.6 (3)	n.d.
DSPC/Chol (2:1)	43.2 ± 2.9 (3)	0.3 ± 0.1 (3)	n.d.
DSPC/Chol/G_{M1} (2:1:0.2)	76.6 ± 9.5 (3)	28.1 ± 9.1 (3)	n.d.
DSPC/Chol/PEG-DSPE (2:1:0.2)	80.1 ± 2.0 (3)	30.1 ± 1.2 (3)	n.d.
PC/Chol/PEG-DSPE (2:1:0.2)	76.6 ± 3.0 (3)	33.2 ± 3.6 (3)	14.2 ± 5.0 (3)

combinations of different PEG-lipid derivatives had no advantage over the individual constituents (not shown). When the liposomes were composed of high phase transition phospholipids, e.g. DSPC/Chol, addition of 10 mol% of G_{M1} or PEG(1900)-DSPE again resulted in an elevation of blood levels, to a similar extent as when these lipids were incorporated into SM/PC/Chol liposomes (Table I). Incorporation of PEG(1900)-DSPE also resulted in elevated blood levels of liposomes when the liposomes were composed of low phase transition phospholipids such as PC/Chol (Table I), although these same phospholipids in the presence of G_{M1} did not have as high blood/MPS ratios as did high phase transition phospholipids [1].

It should be pointed out that the initial molar ratios of phospholipid to PEG-DSPE reported in this paper do not represent the real incorporation values of PEG-DSPE into liposomes, as demonstrated by the following experiments. The initial molar ratios of phospholipid to G_{M1} do, however, represent the actual incorporation values [1].

Increasing the mol% of PEG(1900)-DSPE in liposomes composed of SM/PC/Chol (MLV extruded through 0.1 μM Nuclepore filters) between 5 and 20 mol% did not result in any significant change in blood levels or other tissue levels of liposomes (not shown). At higher mol% of PEG(1900)-DSPE in the liposomes there was considerable foaming of the liposome preparations and they became more difficult to handle and extrude, although no increase in contents leakage was observed and no increase in liposome size was observed with increasing PEG(1900)-DSPE content (not shown).

We suspected that the foaming was due to the presence of PEG-DSPE micelles which were not incorporated into the liposomes. Passage over Ultrogel AcA34 columns, a normal procedure to remove free ^{125}I-TI from the liposomes, resulted in elimination of the foaming problem, suggesting that free PEG-DSPE micelles were separated from the liposomes during this procedure. In order to characterize the liposomes further as to their content of PEG-DSPE we performed a series of experiments in which liposome-associated PEG-DSPE was separated from PEG-DSPE micelles by chromatography over Sepharose CL4B columns, and the presence of PEG was quantitated in column fractions by the Bio-Rad protein assay. The results are given in Table II. The level at which PEG(1900)-DSPE incorporation into liposomes becomes saturated is approx. 5 to 7 mol% of the PEG compound, and a similar figure was found for PEG(5000)-DSPE. For lower average molecular weights of PEG-DSPE, higher amounts of the compound were accommodated in liposomes. The blood levels of liposomes at 24 h post-injection (i.v.) were higher for PEG(1900) and PEG(5000) liposomes than for the lower average molecular weights of PEG (Table II), and increasing the amounts of the lower molecular weights of PEG-DSPE to saturating levels did not appear to result in any significant increases of blood levels (not shown).

TABLE II

Percentage of incorporation of PEG-DSPE into liposomes during liposome formation as a function of the size of the PEG headgroup

Liposomes, composed of SM/PC/Chol, 1:1:1, were MLV extruded through 0.1 μm Nuclepore filters and passed over Sepharose 4B columns (1.5×40 cm) in buffered saline. PEG was detected by the Bio-Rad assay. The initial concentration for all molecular weights of PEG-DSPE was 10 mol% with the exception of PEG-120 where the initial concentration was 20 mol%. Blood levels (% of ^{125}I-tyraminylinulin cpm remaining *in vivo*) at 24 h after injection of 0.5 μmol phospholipid per mouse are also reported for the various sizes of PEG added at an initial concentration of 10 mol% PEG-PE. Mean ± S.D., $n = 3$.

Average molecular weight of PEG	% of incorporation (mol%)	blood levels at 24 h (10 mol% PEG-DSPE added)
PEG-120	15.7, 13.7	14.1 ± 1.5
PEG-750	7.8, 8.3	16.6 ± 6.6
PEG-1900	5.7, 5.0, 6.8, 6.5	35.1 ± 3.3
PEG-5000	6.9, 7.3	28.3 ± 1.1

2 h post-injection of SM/PC/Chol, 1:1:1 liposomes (0.1 μm extruded MLV) containing 10 mol% PEG(1900)-DSPE, 7.4 ± 4.0% ($n = 24$) of the ^{125}I-TI counts remaining in vivo were located in liver and spleen (not shown) with 77.5 ± 11.9% ($n = 24$) of the counts remaining in circulation (Table I), i.e. blood/MPS level of 10.5. At 24 h post-injection, 20.0 ± 5.7% ($n = 21$) of the counts were in liver and spleen (not shown) and 38.2 ± 6.9% ($n = 21$) of the counts were in the blood (Table I, blood/MPS ratio of 1.9). By 48 h post-injection the liver and spleen levels had risen to only 30.1 ± 8.6% ($n = 11$) of ^{125}I-TI cpm remaining in vivo (not shown) (blood/MPS ratio of 0.44), which represents 15% of injected cpm, given that the cpm remaining in vivo after 48 h averaged approx. 50% of injected cpm (Table IV, 2nd column), i.e. 50% of the injected ^{125}I-TI had been released from liposomes and removed from the body by filtration through the kidneys [1,4].

The effect of liposome size on blood and MPS levels for liposomes composed of SM/PC/Chol/PEG(1900)-DSPE, 1:1:1:0.2 is shown in Table III. There was no marked size dependence of the pegylated liposomes but the lowest blood levels and the highest MPS levels were found for liposomes extruded through 0.05 μm filters. The increase in liver levels for liposomes extruded through 0.05 μm filters has been reported previously [4], and may be due to passage of small liposomes through liver fenestrations to be taken up by liver parenchymal cells.

Blood/MPS ratios for liposomes composed of SM/PC/Chol/PEG(1900)-DSPE, 1:1:1:0.2 as a function of time following i.v. administration are given in Table IV, and tissue distributions, as a function of time post-injection, for the major tissues of uptake are

TABLE III

Effect of increasing liposome size on blood and MPS levels (% of in vivo cpm) of liposomes composed of SM/PC/Chol/PEG(1900)-DSPE, 1:1:1:0.2 initial molar ratio, labelled with ^{125}I-tyraminylinulin and injected i.v. into mice at a concentration of 0.5 μmol phospholipid per mouse

Mean ± S.D. at 2 h post-injection for two separate experiments of three mice each ($n = 6$). Light scattering results for each liposome preparation are reported separately.

Nuclepore filter size (μm)	% of in vivo in blood	% of in vivo in liver + spleen	Average size (nm) (light scattering)
0.6	85.8 ± 6.8	9.3 ± 5.0	189, 149
0.4	82.2 ± 7.4	10.5 ± 4.8	146, 141
0.2	90.1 ± 4.4	6.2 ± 2.2	138, 127
0.1	82.7 ± 5.3	7.4 ± 1.4	112, 104
0.08	79.2 ± 9.5	10.2 ± 7.1	111, 86
0.05	75.5 ± 6.0	20.3 ± 1.7	92, 87

shown in Fig. 2A. Blood/MPS ratios decreased slowly with increasing time post-injection, and by 24 h post-injection there were still twice as many liposomes in

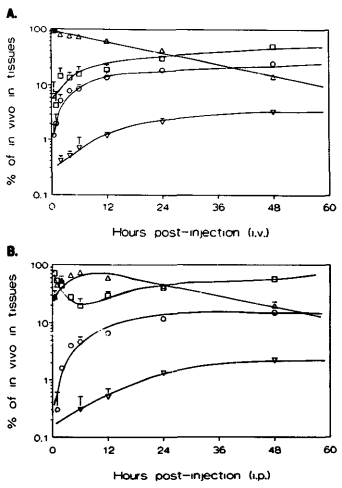

Fig. 2. Tissue distribution of liposomes composed of SM/PC/Chol/PEG(1900)-DSPE, 1:1:1:0.2 initial molar ratio as a function of time after (A) i.v. or (B) i.p. injection in mice. Liposomes were MLV extruded through 0.1 μm Nuclepore filters and contained ^{125}I-tyraminylinulin as an aqueous space marker. Mice received 0.5 μmoles of phospholipid/mouse. Results are expressed as% of injected label remaining *in vivo* at selected times in blood (△), liver (○), spleen (▽) and carcass (□). Mean ± S.D., $n = 3$.

blood as were taken up by the MPS (Table IV). The MPS is defined as uptake by liver + spleen, as we have previously found that liposomes of identical size and composition as those used in these experiments were not taken up to any significant extent by mouse bone marrow-derived macrophages [10] or by bone marrow *in vivo* (Fig. 3). The rate of contents leakage from the liposomes is also indicated in Table IV with over 50% of the liposome contents remaining entrapped at 48 h post-injection. The major site of liposome uptake was carcass tissues (50% at 48 h). The blood levels of SM/PC/Chol/PEG(1900)-DSPE liposomes declined in a log-linear fashion after i.v. injection with an apparent half-life of 20 h (Fig. 2A).

Blood/MPS ratios as a function of time following i.p. administration of liposomes are given in Table IV and tissue distributions are given in Fig. 2B. Liposomes were gradually released from the peritoneal cavity into blood over a 6 h time period with very little uptake into liver and spleen. This resulted in some very high blood/MPS ratios at the early time points, drawing attention to one of the drawbacks of relying exclusively on blood/MPS ratios to characterize the behaviour of liposomes. The figures for percentage of injected counts remaining *in vivo* gradually increased over the same time period due to the difficulty of retaining all the liposome-containing fluid in the peritoneal cavity when carcass is sampled, giving an underestimate for total counts remaining *in vivo* at the early time points. As the liposomes were transported from the peritoneal cavity into blood over several hours, this figure began to more accurately reflect the actual situation (Table IV). Also, because carcass counts include residual liposomes in the peritoneal cavity, carcass levels of liposomes were high at early time points and because of liposome spillage, were probably an underestimate of actual values. We would not expect blood, liver and

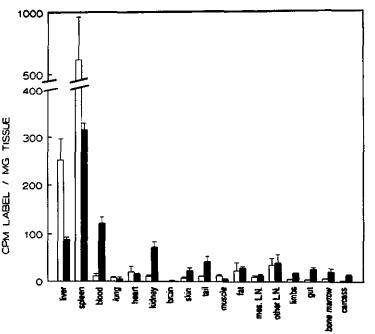

Fig. 3. Tissue distribution, as cpm ^{125}I-TI label per mg tissue, normalized to 10^6 cpm injected, at 24 h post-injection for liposomes (MLV extruded through 0.1 μm Nuclepore filters) composed of PC/Chol, 2:1 (open columns) or SM/PC/Chol/PEG(1900)-DSPE, 1:1:1:0.2 (solid columns). Mice received 0.5 μmol phospholipid per mouse. Mean ± S.D., $n = 3$.

spleen liposome levels to be affected by these considerations.

Blood levels of intact liposomes initially rose, reaching maximum levels at 6 h post-injection (Fig. 2B), levels which were very similar to those seen at the same time point following i.v. injection (Fig. 2B vs. Fig. 2A). After 6 h liposome levels began to fall slowly at a rate similar to that following i.v. administration (Fig. 2B vs. Fig. 2A). Liver and spleen levels of liposome uptake following i.p. injection were lower than those seen following i.v. administration, and carcass levels at the later time points were similar to those seen following i.v. liposome administration. Following subcutaneous injection, similar to the situation after i.p. injection, blood levels rose after a few hours delay and between 6 and 24 h post-injection, blood levels of liposomes between 3 and 6% of injected dose could be maintained (not shown).

A comparison of uptake of PC/Chol and SM/PC/Chol/PEG(1900)-DSPE liposomes (0.1 μm extruded MLV) into several tissues at 24 h post-injection is given in Fig. 3. As has been previously seen with G_{M1}-containing liposomes of similar size [4], compared to PC/Chol liposomes there is decreased uptake of PEG-containing liposomes into liver and spleen, increased blood levels, and moderately increased levels in several other tissues including kidneys, skin, tail, limbs, gut, bone marrow and residual carcass.

The effect of incorporation of PEG(1900)-DSPE into liposomes containing 10 mol% PS is given in Table

TABLE IV

Blood/MPS ratios in mice as a function of time post-injection in the tail vein (i.v.) or intraperitoneally (i.p.) for liposomes composed of SM/PC/Chol/PEG(1900)-DSPE, 1:1:1:0.2 initial molar ratio, labelled with ^{125}I-tyraminylinulin (MLV extruded through 0.1 μm Nuclepore filters), 0.5 μmol per mouse

Mean ± S.D., $n = 3$.

Time (h)	Blood/MPS ratios (i.v.)	% remaining in vivo (i.v.)	Blood/MPS ratios (i.p.)	% remaining in vivo (i.p.)
0.5	97.2 ± 51.8	98.0 ± 2.9	109.9 ± 59.3	66.4 ± 5.4
1	67.2 ± 48.1	92.4 ± 4.8	404.1 ± 388.8	77.7 ± 1.2
2	15.2 ± 5.1	90.6 ± 4.1	35.6 ± 8.4	87.1 ± 5.3
4	8.9 ± 0.6	88.2 ± 3.3	16.7 ± 0.8	89.1 ± 3.3
6	8.1 ± 2.1	87.3 ± 3.6	16.7 ± 0.8	89.1 ± 3.3
12	4.4 ± 1.3	72.9 ± 3.1	8.5 ± 0.2	81.3 ± 0.1
24	2.0 ± 0.3	67.1 ± 0.6	3.3 ± 0.4	69.7 ± 1.6
48	0.5 ± 0.2	54.8 ± 3.2	1.1 ± 0.1	61.3 ± 0.3

TABLE V

Tissue distribution of liposomes in mice

Liposomes (0.5 μmol per mouse) were composed of SM/PC/Chol/PS, 1:1:1:0.2 (MLV extruded through 0.1 μm Nuclepore filters) containing various mol% of PEG(1900)-DSPE. Tissue distributions were determined for ^{125}I-tyraminylinulin-labelled liposomes at 2 h post-injection. Mean ± S.D., $n = 3$.

Mol% PEG, mol% PS	Liver	Spleen	Carcass	Blood	% *in vivo*
0, 10	46.9 ± 6.7	16.3 ± 2.6	29.0 ± 3.5	6.3 ± 0.7	99.4 ± 3.4
2.5, 10	52.4 ± 2.7	17.5 ± 2.1	22.5 ± 2.1	5.7 ± 0.5	74.1 ± 3.0
5, 10	57.1 ± 0.9	18.2 ± 1.7	19.9 ± 2.1	3.7 ± 0.2	72.4 ± 3.1
10, 10	45.1 ± 6.2	14.2 ± 2.8	21.3 ± 2.3	17.9 ± 8.1	82.6 ± 2.1
10, 0	10.8 ± 1.4	2.9 ± 0.4	9.1 ± 1.0	75.8 ± 3.1	87.9 ± 1.6

V. PEG(1900)-DSPE was not capable of overcoming the strong recognition signal of PS for macrophages, possible via the scavenger receptor for negatively charged phospholipids [18]. We have previously reported that G_{M1} is also not capable of overcoming the recognition signal for PS [19].

Discussion

Liposomes containing 5–7 mol% PEG(1900)-DSPE are equivalent to liposomes containing 10 mol% of G_{M1} in their ability to avoid MPS uptake and circulate for prolonged periods of time. Moreover, synthetic lipid derivatives of PEG can be prepared inexpensively from a rapid, simple two step synthesis, and are easily purified, making liposomes containing these derivatives ideal candidates for therapeutic applications *in vivo*, particularly since PEG has already received approval for some pharmaceutical uses (e.g. Rhinaris^{R}, PEG-ADA).

A lipid derivative of PEG which had two saturated fatty acyl chains (PEG-DSPE) resulted in the longest circulation half-lives. Lipid derivatives lacking surface negative charge (PEG-DPG), or with cholesterol (PEG-Chol) had similar blood levels at earlier time points, but by 24 h were distinctly inferior to PEG-DSPE. It is possible that these lipid derivatives either transferred or exchanged out of liposomes over long periods of time or the bond holding the PEG to the lipid moiety was cleaved over a period of time. A lipid derivative with only one saturated fatty acyl chain (PEG-SA) or un-derivatized methyl PEG were even less effective in preventing MPS uptake, probably due to lack of effective long-lasting association of these compounds with liposomes.

While the longest circulation half-lives in G_{M1}-containing liposomes were obtained with liposomes containing high phase transition phospholipids such as DSPC or SM [1], for liposomes containing PEG(1900)-DSPE, elimination of the high phase transition phospholipid resulted in liposomes with equivalently long circulation half-lives. Therefore, more versatility in the choice of liposomal lipids is possible in the presence of PEG-DSPE.

It was interesting to us that even the bulkier PEG(1900)-DSPE at the liposome surface was not capable of overcoming the recognition signal for macrophage uptake of PS in liposomes. It shows that a strong signal at the liposome surface is still capable of being recognized, even in the presence of PEG-DSPE, and holds out the possibility that antibodies coupled to the surface of liposomes containing PEG-DSPE may be able to be recognized by cell surface antigens.

Liposomes containing PEG-DSPE were effective in reducing MPS uptake at lower PEG-DSPE/phospholipid molar ratios than have been seen previously for G_{M1} in liposomes [1,4]. At 'saturating' levels of PEG-DSPE in liposomes of 5 to 7 mol%, increased circulation half-lives could also be obtained over a wide range of liposome sizes, again demonstrating the versatility to this potential drug delivery system. As with G_{M1}-containing liposomes [4], those containing PEG-DSPE were taken up into a wider variety of carcass tissues than had been seen previously with liposomes of conventional formulations. Using liposomes containing PEG-DSPE, although there was a small increase of uptake of liposomes into bone marrow as compared to PC/Chol liposomes (Fig. 3), we have not seen any significant increase in bone marrow uptake such as that described, using gamma scintigraphy, by Illum et al. [9] for polystyrene microspheres coated with poloxamer 338, another hydrophilic coating which prolongs circulation half-lives of particles. The technique which we used to determine bone marrow uptake was a direct technique involving extrusion of the bone marrow from the femur, which should give very accurate results. In preliminary experiments involving pegylated liposomes labelled with Ga^{67}-desferoxime, gamma scintigraphy in rats did not provide any evidence of increased bone marrow uptake, with a diffuse radioactivity, characteristic of a label confined to the central (circulatory) compartment, present throughout the animals (not shown). In a manuscript submitted for publication (Allen and Hansen), pharmacokinetic studies confirm that the volume of distribution of pegylated liposomes is equivalent to the circulatory volume of the animals. Furthermore, pegylated liposomes, in the presence of serum, had greatly decreased uptake by mouse bone marrow macrophages as compared to non-pegylated liposomes [10], in contrast to the results reported for coated microspheres being taken up by mouse peritoneal macrophages [9]. Clearly, pegylated liposomes are treated differently by bone marrow than poloxamer-coated microspheres, although the reason for the differences is not presently known. The increased bone marrow uptake described in the experiments with

coated microsheres may be related to differences in the nature of the hydrophilic material, differences in opsonization between coated microspheres and pegylated liposomes, differences in the manner in which the hydrophilic materials associates with the microspheres or liposomes, or differences in the length time in which the two remain associated *in vivo*.

It is not presently known whether small liposomes containing either G_{M1} or PEG-DSPE are capable of penetrating through continuous capillary endothelia, or alternatively if they are binding to endothelial tissues throughout the circulatory system, which could also explain the increased carcass uptake. The latter explanation, in the light of some detailed pharmacokinetic experiments in our laboratory, is our preferred explanation for the observed results (Allen, T. and Hansen, C., submitted to BBA).

Although stringent toxicity testing of liposomes containing PEG-DSPE has not been carried out, in experiments designed to test the effects of increasing doses, mice were able to tolerated doses up to the maximum tested dose of 10 μmol/mouse with no signs of adverse reactions and no indications of MPS saturation (Allen, T. and Hansen, C., submitted to BBA).

Intact liposomes containing PEG-DSPE were able to exit the peritoneal cavity effectively, resulting in blood and carcass levels at 6 h post-injection which were not significantly different to levels seen after i.v. injection of liposomes. (The aqueous space label ^{125}I-TI, if released from liposomes, is rapidly cleared from the body [1,13]). Uptake by liver and spleen were lower than that seen following i.v. injection of liposomes. This route of injection results in almost constant blood liposome levels for a period of several hours, a very desirable objective for the use of liposomes as a sustained release system *in vivo*.

The mechanism by which PEG-DSPE prevents MPS uptake of liposomes may be related to its ability to impart a hydrophilic surface to the liposomes. This bulky, hydrophilic surface in turn may prevent opsonization of the surface of the liposomes with plasma proteins involved in the recognition and uptake of liposomes. A similar mechanism has been proposed for the effect of G_{M1} in liposomes [4]. Covalently attached PEG appears to have a significant effect in reducing immunogenicity and antigenicity of proteins and increasing their circulation half-lives [5,6], probably by a similar mechanism.

In summary, the ability of PEG-DSPE to impart MPS-avoiding characteristics to liposomes, the prolonged circulation times of liposomes containing PEG-DSPE and the relative ease of preparing this inexpensive lipid derivative of PEG make it an ideal candidate for therapeutic applications of liposomes. In addition, liposomes containing PEG-DSPE have pharmacokinetics which are favourably disposed towards their use as drug carriers *in vivo*, both as drug slow release systems within the vasculature and as targetable carriers for drug delivery to specific cells or tissues *in vivo*.

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