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Biochimica et Biophysica Acta 1326 (1997) 329–341

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Biodistribution of liposomes containing synthetic galactose-terminated diacylglyceryl-poly(ethyleneglycol)s

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Received 18 November 1996; revised 4 February 1997; accepted 12 February 1997

Abstract

We describe the synthesis of biodegradable poly(ethyleneglycol)-coupled galactolipids in which the galactose moiety is separated from a diacylglyceride lipid anchor by poly(ethylene glycol) chains of 10, 20 or 40 oxyethylene residues ($PEG_{10/20/40}$). These Gal-PEG lipids (Gal-PEG-Lip) were incorporated in the bilayer of liposomes. The surface exposure of the galactose was investigated by aggregation experiments with ricinus communis agglutinin 120. Only the liposomes containing the PEG_{10} galactolipid aggregated with the lectin. Therefore liposomes were prepared containing Gal-PEG_{10}-Lip and a trace amount of [^{3}H]cholesteryl oleyl ether with an average diameter of approximately 100 nm and injected intravenously into rats. The Gal-PEG_{10}-Lip liposomes were cleared from plasma with a T1/2 of 0.3 h. Identically sized and composed control liposomes without the Gal-PEG_{10}-Lip had a T1/2 of approximately 12 h. The rapid plasma elimination of the Gal-PEG_{10}-Lip liposomes could be attributed entirely to increased uptake by the liver amounting to more than 90% of injected dose. Uptake by the spleen was decreased to less than 1% of injected dose. A single injection of N-acetylgalactosamine 1 min prior to Gal-PEG-Lip liposome administration reduced the initial rate of plasma clearance to control levels. The increased liver uptake was almost entirely attributable to increased uptake by the Kupffer cells. Incorporation of PEG-DSPE in the Gal-PEG_{10}-Lip liposomes only partially reversed the effect of the galactolipid with respect to liver and spleen uptake as well as intrahepatic distribution. These experiments demonstrate that liposome surface-exposed galactose residues, even if attached at the distal end of a poly(ethyleneglycol) chain anchored in the liposomal bilayer are effectively recognized by the galactose particle receptor on the Kupffer cells but fail to achieve significant targeting to the asialoglycoprotein receptor on the hepatocytes.

Keywords: N-acetylgalactosamine; Agglutination; Cell-specific drug targeting; Fixed aqueous layer thickness; Galactose receptor; Hepatocyte; Kupffer cell; Lectin; Liver macrophage; Sterically stabilized liposome

Abbreviations: PEG, poly(ethyleneglycol); PEG-DMG, monomethoxy(polyethyleneglycol)-1,2-dimyristoylglycerol; PEG-DSPE, monomethoxypoly(ethyleneglycol)-distearoylphosphatidylethanolamine; ePC, egg yolk phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; CH, cholesterol; RCA, ricinus communis agglutinin; GalNAc, N-acetylgalactosamine; Gal-PEG-Lip, 1-*O*-{3-[ß-D-galactos-1-*O*-yl]carbonylpropionyl}poly(oxyethylene)-ω-yl-2,3-*O*-diacylglycerol

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PII S0005-2736(97)00036-9

1. Introduction

Systemically administered liposomes are predominantly cleared from circulation by liver and spleen [1]. Macrophages, abundantly present in these organs, are mainly responsible for this efficient clearance, although, in the liver, hepatocytes are also able to participate in hepatic uptake [2,3].

Interaction with and uptake of liposomes by liver and spleen macrophages can be substantially reduced, exploiting the properties of poly(ethylene glycol) chains on the liposome surface [4,5]. Such PEG chains are believed to prevent or diminish the adsorption of opsonizing proteins [6,7], which direct the liposomes to macrophages as well as hepatocytes [8]. As a result liposome uptake by these cells is reduced. We measured the thickness of the fixed aqueous layer around such PEG-modified liposomes [9] and demonstrated a good correlation between liposome circulation time and the thickness of the fixed water layer [10].

Attempts to actively target liposomes to hepatocytes, by exploiting the well-known galactose receptor on these cells [11], have produced variable results. Several investigators reported increased liver uptake of liposomes provided with surface-exposed galactose moieties [12–20]. Only in a few instances experimental evidence was offered supporting the conclusion that hepatocytes are involved in this uptake [12,14,18]; others reported only an increase in Kupffer cell uptake [16,19] which was ascribed to the galactose particle receptor on these cells [21].

The way in which the galactose moieties are exposed on the liposome surface may be a determinant in the relative affinities of such liposomes towards the two galactose receptors. Therefore we set out to construct galactose-exposing liposomes in which the sugar is well-separated from the lipid-water interface by a PEG chain of variable length. In this way we anticipated to simultaneously exploit the anti-opsonic action of the PEG chain and the spacer effect, improving the exposure of the galactose moiety and possibly providing an optimal configuration for interaction with the hepatocyte galactose receptor. Thus far only one report appeared in literature describing the simultaneous use of PEG as a macrophage-avoiding agent and a galactosyl ligand, i.c. lactosylceramide [8]. In this case, where PEG and galactose were on different chemical entities, the ligand caused a shift in intrahepatic distribution in favor of the Kupffer cells but not of the hepatocytes.

For the construction of a molecule containing both the ligand sugar and the PEG, galactose-terminated PEG chains of 10, 20 and 40 oxyethylene residues were synthesized and coupled to a long-chain diacyl glyceride for proper anchoring in the liposomal bilayer. The degree of exposure of the galactose residue, anchored in the liposomal bilayer was assayed by lectin-induced agglutination. The PEG chain length resulting in the best galactose exposure was selected for in vivo experiments in which we determined plasma half-lives, liver and spleen uptake as well as intrahepatic distribution.

2. Materials and methods

2.1. Materials

2,3,4,6-Tetra-*O*-benzyl-D-galactose and cholesterol were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylglycerol (DPPG), egg phosphatidylcholine (ePC) and poly(ethylene glycol)-dimyristoylglycerol (PEG-DMG) with an average molecular weight of 2000 (40 oxyethylene residues) and the starting materials for the synthesis of the galactolipids, the carboxypropionyl]poly (oxyethylene)-isopropylideneglycerols, were kindly donated by NOF Corporation (Tokyo, Japan). Poly(ethylene glycol)-distearoylphosphatidylethanolamine (PEG-DSPE), with an average PEG molecular weight of 2000 was purchased from Avanti Polar Lipids (Alabaster, AL, USA). [^{3}H]Cholesteryl oleyl ether was obtained from Amersham (Buckinghamshire, UK). Ricinus communis agglutinin (RCA 120) was obtained from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan). Pronase, collagenase A and DNase (grade II) were obtained from Boehringer Mannheim, (Mannheim, Germany). Nycodenz (analytical grade) was purchased from Nycomed (Oslo, Norway). All other chemicals were commercial products of reagent grade.

2.2. Instrumentation

^{1}H and ^{13}C NMR spectra were measured with a JEOL JNM-GSX 270(270 MHz) or JEOL JNM-

GSX500(500 MHz) spectrometer in $CDCl_3$ using tetramethylsilane (TMS) as an internal standard, in which the following abbreviations were used: s = singlet, d = doublet, dd = double doublet, t = triplet, m = multiplet, br = broad, brs = broad singlet. Chemical shifts (δ) are given in ppm relative to TMS (δ = 0). Column chromatography was performed on silica gel 60 (70–230 mesh) (Merk) and gel filtration on Sephadex LH-20 (Pharmacia) columns. Thin-layer chromatography (TLC) was done on Silica gel 60F254 plates (Merk). The spots were visualized by spraying the plates with a 10% solution of phosphomolybdic acid in ethanol followed by heating on a hot plate.

2.3. Synthesis of Gal-PEG lipids

We synthesized three galactolipids with a poly(ethyleneglycol) chain of various chain lengths separating the galactosyl and the diacylglyceride lipid moieties.

The synthetic procedures are summarized in Fig. 1. The three Gal-PEG lipids are the compounds 7a,b,c, with PEG chains carrying 10, 20 or 40 oxyethylene residues, respectively, and with either a dipalmitoyldiglyceride (a) or a distearoyldiglyceride (b,c) anchor. The synthetic procedure is described in detail for compound 7b as an example of Gal-PEG-lipid synthesis.

2.4. 1-O-{α-[3-Benzyloxycarbonylpropionyl]poly-(oxyethylene)-ω-yl}-2,3-O-isopropylideneglycerol (2)

A solution of 1-*O*-{α-[3-carboxypropionyl]poly-(oxyethylene)-ω-yl}-2,3-*O*-isopropylideneglycerol (1) (5.6 g, 5.1 mmol) in 10 ml N,N-dimethylformamide (DMF) was added to a stirred suspension of potassium bicarbonate (1.0 g, 10 mmol) in 10 ml DMF. To this suspension a solution of benzyl bromide (1.3 g, 7.7 mmol) in 10 ml DMF was added and the mixture was stirred at room temperature for 24 h. Then the solution was poured onto cold water, and the organic layer was extracted with chloroform, washed with water and dried over anhydrous magnesium sulfate. The solvent was removed in vacuo and the residue was purified on a Sephadex LH 20 column, which was eluted with methanol. Compound 2 was obtained as a colorless oil (4.8 g, 79%).

^{1}H-NMR ($CDCl_3$) δ: 1.36, 1.42 (each 3H, s, isopropylidene CH_3), 2.69 (4H, s, $COCH_2CH_2CO$), 3.50 (1H, dd, J = 6.0, 10.5 Hz, glycerol-H), 3.60 (1H, dd, J = 6.0, 10.5 Hz, glycerol-H), 3.61–3.69 (m, oxyethylene-H), 3.67 (2H, m, $OCH_2CH_2OCOCH_2CH_2CO$), 3.73 (1H, dd, J = 6.0, 9.0 Hz, glycerol-H), 4.05 (1H, dd, J = 6.0–6.9 Hz, glycerol-H), 4.23 (2H, m, $OCH_2CH_2OCOCH_2CH_2CO$), 4.28 (1H, m, OCH_2CHCH_2O), 5.17 (2H, brs, CH_2-Ph), 7.36 (5H, m, CH_2-Ph).

^{13}C-NMR ($CDCl_3$) δ: 24.8, 26.2 (q, isopropylidene $CH_3 \times 2$), 28.4, 28.5 (t, $COCH_2CH_2CO$), 63.2, 65.8, 66.1, 68.4, 70.3, 71.7 (t, CH_2O), 69.9 (t, oxyethylene-$CH_2 \times n$), 74.1 (d, CH_2CHCH_2), 108.7 (s, isopropylidene $(CH_3)_2C$), 127.6, 127.7, 127.9 (d, aromatic CH), 135.2 (s, aromatic C), 171.4, 171.6 (s, $COCH_2CH_2CO$).

2.5. 1-O-{α-[3-Benzyloxycarbonylpropionyl]poly-(oxyethylene)-ω-yl}glycerol (3)

Compound 2 (3.36 g, 2.8 mmol) was treated with 40 ml 60% acetic acid aqueous solution (v/v) at room temperature for 2 h. After removal of the solvent, the crude mixture was purified on a silica gel column, eluted with $CHCl_3$/MeOH (20:1 v/v). Compound 3 was obtained as a colorless oil (2.75 g, 86%).

^{1}H-NMR ($CDCl_3$) δ: 2.73 (4H, s, $COCH_2CH_2CO$), 3.45–4.35 (m, oxyethylene-H and glycerol-H), 5.13 (2H, brs, CH_2-Ph), 7.36 (5H, m, CH_2-Ph).

^{13}C-NMR ($CDCl_3$) δ: 29.0, 29.1 (t, $COCH_2CH_2CO$), 63.86, 63.89, 66.50, 69.0, 70.8 (t, CH_2O), 72.9 (d, CH_2CHCH_2), 128.1, 128.2, 128.6 (d, aromatic CH), 135.8 (s, aromatic C), 172.0, 172.2 (s, $COCH_2CH_2CO$).

2.6. 1-O-{α-[3-Benzyloxycarbonylpropionyl]poly-(oxyethylene)-ω-yl}-2,3-O-distearoylglycerol (4)

To a stirred solution of compound 3 (1.7 g, 1.5 mmol), triethylamine (0.4 g, 3.9 mmol) and 4-dimethylaminopyridine (0.09 g, 0.7 mmol) in 50 ml $CHCl_3$, a solution of stearic anhydride (1.8 g, 3.3 mmol) in 50 ml $CHCl_3$ was added dropwise and the mixture was heated at 50–60°C under nitrogen for 2 h. The reaction mixture was diluted with $CHCl_3$ and washed with 1 N HCl, saturated aqueous $NaHCO_3$ and brine. The organic layer was dried over anhydrous magnesium sulfate. The solvent was evaporated

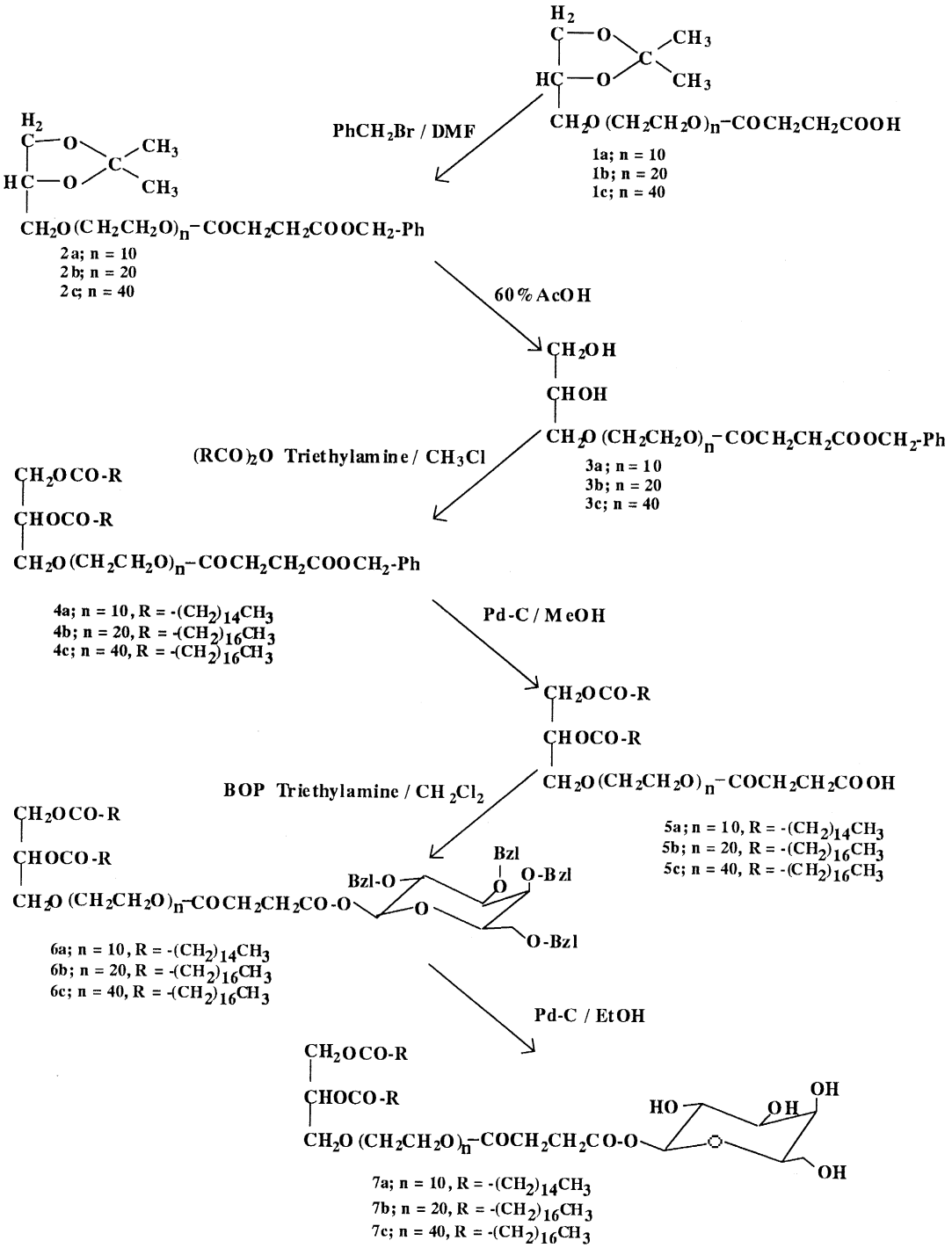

Fig. 1. Synthesis of spacer-armed Gal-PEG lipid.

to dryness and the residue was purified using a column of Sephadex LH 20, which was eluted with methanol. Compound 4 was obtained as a white solid (1.3 g, 51%).

^{1}H-NMR ($CDCl_3$) δ: 0.88 (6H, t, J = 6.3 Hz, $CH_3(CH_2)_{16}$), 1.25 (56 H, brs, $CH_3(CH_2)_{14}CH_2CH_2$), 1.56–1.66 (4H, m, $CH_3(CH_2)_{14}CH_2CH_2$), 2.30 (2H, t, J = 6.5 Hz,

$CH_3(CH_2)_{14}CH_2CH_2$), 2.31 (2H, t, J = 6.5 Hz, $CH_3(CH_2)_{14}CH_2CH_2$), 2.69 (4H, s, $COCH_2CH_2CO$), 3.60–3.69 (m, oxyethylene-H and glycerol-H), 4.15 (1H, dd, J = 11.9, 6.4 Hz, glycerol-H), 4.22–4.24 (2H, m, $(CH_2CH_2O)_{19}CH_2CH_2OCOCH_2CH_2CO$), 4.32 (1H, dd, J = 11.9, 3.7 Hz, glycerol-H), 5.14 (2H, s, CH_2Ph), 5.20–5.22 (1H, m, OCH_2CHCH_2O), 7.35–7.37 (5H, m, CH_2Ph).

2.7. 1-O-{α-[3-Carboxypropionyl]poly(oxyethylene)-ω-yl}-2,3-O-distearoylglycerol (5)

Compound 4 (1.4 g, 0.8 mmol) in 10 ml MeOH was hydrogenolyzed with Pd-C (1.0 g) under H_2 at room temperature overnight. The catalyst was removed by filtration and the filtrate was evaporated to dryness. Compound 5 was obtained as a white solid (1.0 g, 78%).

^{1}H-NMR ($CDCl_3$) δ: 0.88 (6H, t, J = 6.4 Hz, $CH_3(CH_2)_{16}$), 1.27 (56H, brs, $CH_3(CH_2)_{14}CH_2CH_2$), 1.58–1.62 (4H, m, $CH_3(CH_2)_{14}CH_2CH_2$), 2.30 (2H, t, J = 6.5 Hz, $CH_3(CH_2)_{14}CH_2CH_2$), 2.31 (2H, t, J = 6.5 Hz, $(CH_3(CH_2)_{14}CH_2CH_2$), 2.65 (4H, m, $COCH_2CH_2CO$), 3.60–3.73 (m, oxyethylene-H and glycerol-H), 4.15 (1H, dd, J = 11.9, 6.4 Hz, glycerol-H), 4.26–4.27 (2H, m, $(CH_2CH_2O)_{19}CH_2CH_2OC$-$OCH_2CH_2CO$), 4.33 (1H, dd, J = 11.9, 3.7 Hz, glycerol-H), 5.18–5.25 (1H, m, OCH_2CHCH_2O).

^{13}C-NMR ($CDCl_3$) δ: 14.1(t, $CH_3(CH_2)_{16}$), 23.0 (t, $CH_3CH_2(CH_2)_{15}$), 24.9, 25.0 ($CH_3(CH_2)_{14}CH_2CH_2CO$), 29.0–29.7 (t, $CH_3CH_2CH_2(CH_2)_{12}CH_2CH_2CO$ and succinyl-C × 2), 31.9 (t, $CH_3CH_2CH_2(CH_2)_{14}$), 34.2, 34.3 (t, $CH_3(CH_2)_{14}CH_2CH_2CO$), 62.7 (t, glycerol-$CH_2$), 69.0 (t, glycerol-$CH_2$), 70.0 (d, glycerol-CH, 70.4–70.7 (t, oxyethylene-CH_2 × n) 172.0, 173.1, 173.5, 173.8 (s, $COCH_2CH_2CO$ and $CH_3(CH_2)_{16}CO$ × 2).

2.8. 1-O-(α-{3-[2,3,4,6-Tetra-O-benzyl-ß-D-galactos-1-O-yl]carbonylpropionyl}poly(oxyethylene)-ω-yl)-2,3-O-distearoylglycerol (6)

Benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP) (0.15 g, 0.34 mmol) was added to a stirred solution of compound 5 (0.3 g, 0.19 mmol), 2,3,4,6-tetra-*O*-benzyl-ß-D-galactose (0.12 g, 0.22 mmol) and triethylamine (0.05 g, 0.5 mmol) in $CHCl_3$ (10 ml) and stirred overnight at 0°C. The reaction mixture was washed once with 0.2 N HCl and brine. The organic layer was dried over magnesium sulfate. After evaporation of the solvent, the residue was purified on a Sephadex LH 20 column, which was eluted with $CHCl_3$:MeOH (1:1 v/v). Compound 6 was obtained as a white solid (0.3 g, 74%).

^{1}H-NMR ($CDCl_3$) δ: 0.88 (6H, t, J = 6.4 Hz, $CH_3(CH_2)_{16}$), 1.26 (56 H, brs, $CH_3(CH_2)_{14}CH_2CH_2$), 1.56–1.66 (4H, m, $CH_3(CH_2)_{14}CH_2CH_2$), 2.30 (2H, t, J = 6.5 Hz, $CH_3(CH_2)_{14}CH_2CH_2$), 2.31 (2H, t, J = 6.5 Hz, $CH_3(CH_2)_{14}CH_2CH_2$), 2.65 (4H, brs, $COCH_2CH_2CO$), 3.56–3.69 (m, oxyethylene-H), 3.96–4.06 (2H, m, H-2 and H-6a), 4.15 (1H, dd, J = 11.5, 6.5 Hz, glycerol-H_2), 4.20–4.22 (1H, m, $(CH_2CH_2O)_{19}CH_2CH_2OCOCH_2CH_2CO$), 4.34 (1H, dd, J = 12.0, 4.0 Hz, glycerol-H_2), 4.39 (1H, d, J = 10.4 Hz, OCH_2-Ph), 4.42 (1H, d, J = 10.4 Hz, OCH_2-Ph), 4.61 (1H, d, J = 11.5 Hz, OCH_2-Ph), 4.71 (2H, s, OCH_2-Ph), 4.75 (1H, d, J = 11.5 Hz, OCH_2-Ph), 4.81 (1H, d, J = 11.0 Hz, OCH_2-Ph), 4.93 (1H, d, J = 11.5 Hz, OCH_2-Ph), 5.21–5.31 (1H, m, CH_2CHCH_2), 5.58 (1H, d, J = 7.5 Hz, H-1), 7.30–7.33 (20H, m, OCH_2-Ph).

^{13}C-NMR ($CDCl_3$) δ: 14.1 (q, $CH_3(CH_2)_{16}CO$), 22.7 (t, $CH_3CH_2(CH_2)_{14}$), 24.9, 25.0 (t, $CH_3(CH_2)_{14}CH_2CH_2CO$), 28.9–29.7 (t, $CH_3CH_2CH_2(CH_2)_{12}CH_2CH_2CO$), 31.9 (t, $CH_3CH_2CH_2(CH_2)_{12}CH_2CH_2CO$), 34.1, 34.2 (t, $CH_3CH_2CH_2(CH_2)_{12}CH_2CH_2CO$) 62.7, 63.9, 67.9, 69.0, 69.6, 70.0, 72.9, 73.1, 73.5, 74.1, 74.7, 75.3, (t, CH_2O), 70.9, (t, oxyethylene-CH_2), 94.6 (d, C-1), 127.6, 127.7, 127.8, 127.9, 128.0, 128.2, 128.3, 128.4 (d, aromatic CH), 137.7, 138.2, 138.3, 138.5 (s, aromatic C), 170.8, 171.9, 173.1, 173.4 (s, $COCH_2CH_2CO$ and $CH_3(CH_2)_{16}CO$ × 2).

2.9. 1-O-(α-{3-[ß-D-Galactos-1-O-yl]carbonylpropionyl}poly(oxyethylene)-ω-yl-2,3-O-distearoylglycerol (7)

Compound 6 (0.27 g, 0.13 mmol) in 10 ml EtOH/AcOH (9:1, v/v) was hydrogenolyzed with 0.27 g Pd-C under H_2 overnight at room temperature. The catalyst was removed by filtration and the filtrate was evaporated to dryness. The residue was purified

on a Sephadex LH20 column, which was eluted with methanol. Compound 7 was obtained as a white solid (0.13 g, 60%).

^{1}H-NMR ($CDCl_3$) δ: 0.88 (6H, t, J = 6.4 Hz, $CH_3(CH_2)_{16}$), 1.26 (56 H, brs, $CH_3(CH_2)_{14}CH_2CH_2$), 1.56–1.66 (4H, m, $CH_3(CH_2)_{14}CH_2CH_2$), 2.28–2.33 (4H, m, $CH_3(CH_2)_{14}CH_2CH_2$), 2.65 (4H, s, $COCH_2CH_2CO$), 3.58–3.75 (m, oxyethylene-H), 3.78–3.79 (1H, m, H-2), 4.15 (1H, dd, J = 11.5, 6.5 Hz, $CH_2OCO(CH_2)_{16}CH_3$), 4.23–4.26 (2H, m, $(CH_2CH_2O)_{19}CH_2CH_2OCOCH_2CH_2CO$), 4.34 (1H, dd, J = 12.0, 4.0 Hz, $CH_2OCO(CH_2)_{16}CH_3$), 5.20–5.22 (1H, m, CH_2CHCH_2), 5.53 (1H, d, J = 7.5 Hz, H-1).

2.10. Liposome preparation

Liposomes used in the lectin-induced agglutination experiments were prepared as follows. Solutions of ePC (in chloroform), Gal-PEG-Lip and, when appropriate, PEG-DMG (in chloroform/methanol (2:1 v/v) were mixed in appropriate proportions in a round-bottomed flask, and the solvent was evaporated to dryness. When applicable, 5 mol% PEG-DMG were added to the lipid films. After vacuum desiccation for at least 1 h, 10 ml of a 10 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl was added to the flask and the lipid was suspended by shaking for approximately 1 h at room temperature. The lipid suspension was sonicated for 10 min after nitrogen gas bubbling and extruded once through two stacked polycarbonate membrane filters (Costar, Cambridge MA, USA) with 0.2-μm pore size followed by two extrusions through 0.1-μm pore size filters using a high pressure extruder (Lipex Biomembranes, Vancouver, Canada). Before the lectin-induced agglutination experiments were done, such extruded liposomes were diluted appropriately with 10 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl.

Liposomes for the in vivo experiments were prepared as follows: lipids from stock solutions of DPPC, cholesterol, DPPG, Gal-PEG-Lip and PEG-DSPE in chloroform/methanol (9:1 v/v) were mixed in appropriate proportions with a tracer amount of [^{3}H]cholesteryl oleyl ether and dried under nitrogen gas. The dried lipid film was dissolved in cyclohexane and the lipid solution was lyophilized overnight. The lipids were hydrated at 60°C in HN buffer (10 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (Hepes), 135 mM NaCl, pH 7.4) under mechanical agitation for approximately 1 h. The liposomes thus formed were extruded, as described above, 13 times through 50-nm pore size polycarbonate membrane filters at 60°C.

Phospholipid phosphorus was measured by phosphate assay after perchloric acid destruction [22].

The size of the liposomes was determined by dynamic laser light scattering using a Nicomp model 370 submicron particle analyzer (Nicomp particle sizing systems, Santa Barbara, CA, USA). The mean diameter of the liposomes, obtained from the volume distribution curves produced by the particle analyzer, varied between 75 and 105 nm.

2.11. Lectin-induced agglutination experiments

Lectin-induced agglutination experiments were performed by a modification of the method employed by Yoshioka et al. [23].

Briefly, 2-ml aliquots of the liposome suspension containing galactolipids was put in two cells fixed in a spectrometer. To one of the cells 1 ml 10 mM Tris-HCl buffer containing 150 mM NaCl and various concentrations of RCA 120 was added, and to the other 1 ml of 10 mM Tris-HCl buffer with 150 mM NaCl. The agglutination at 25°C was followed by monitoring the turbidity increase at 450 nm.

2.12. Blood clearance and tissue distribution of liposomes

Male Wag-Rij rats weighing 140–230 g were used in the animal experiments. A dose of 5 μmol (total lipid) of radiolabeled liposomes per 200 g body weight was injected via the penile vein under ether anaesthesia. At the indicated times, blood samples were taken from the tail vein. The samples were centrifuged for 10 min. The amount of liposomes in the serum was determined by measuring radioactivity in a liquid scintillation counter. The total amount of radioactivity in the serum was calculated by applying the equation: serum volume (ml) = (0.0219 × body weight (g)) + 2.66 [28]. 20 h after intravenous injection of liposomes, rats were anaesthesized with nembutal, the spleen was removed and homogenized and

the liver was perfused as described in the following section. Total uptake of liposomes by liver and spleen was determined by measuring radioactivity in the total liver cell suspension obtained during liver cell isolation or in the spleen homogenate, respectively.

2.13. Isolation of liver non-parenchymal and parenchymal cells

Liver non-parenchymal and parenchymal cell suspensions were obtained by liver perfusion with pronase or collagenase, respectively, according to the methods described by Roerdink et al. [2]. The numbers of cells per suspension were determined microscopically and radioactivity of each suspension was determined. From these results the total amounts of radioactivity per cell population, after correction for % recovery, were calculated as described by Roerdink et al. [2].

3. Results

3.1. Lectin-induced agglutination of liposomes containing Gal-PEG lipids

The three Gal-PEG-Lip species were incorporated in phosphatidylcholine liposomes in a molar fraction of 20%. Agglutination experiments with ricinus communis agglutinin, a divalent lectin with high specificity and affinity for ß-D-galactose residues, showed that the galactose moieties in these liposomes were not equally accessible to the lectin. Only the preparations with the Gal-PEG_{10}-Lip were found to aggregate by addition of RCA; preparations with either one of the other two compounds showed no evidence of aggregation at all, as is shown in Fig. 2A. Neither the rate nor the extent of aggregation varied significantly upon lowering the molar fraction of the Gal-PEG-Lip to 10 or even 5% (Fig. 2B).

However, additional incorporation in the bilayer of PEG-DMG, a compound used to confer plasma longevity on liposomes [10], significantly reduced both the rate and extent of RCA-mediated liposome aggregation, but did not abolish it, as is shown in Fig. 3.

In spite of its relatively short lipid chain, the PEG-DMG remains for the larger part associated with the liposomes during prolonged incubation (results not shown). Apparently, a sufficiently large fraction remains liposome-associated to cause the effect on aggregation.

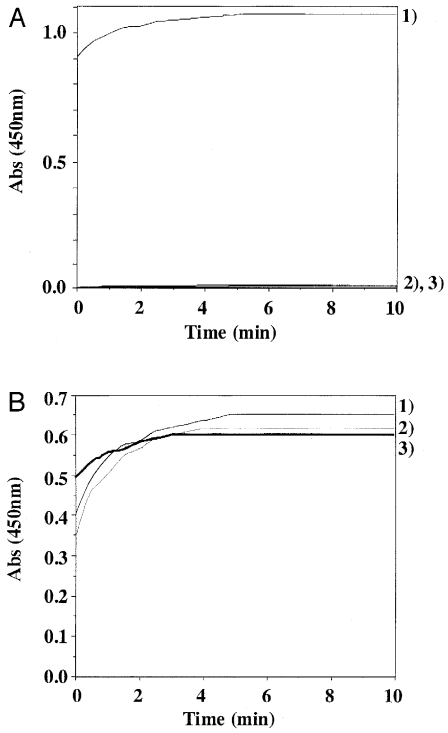

Fig. 2. A: RCA-induced agglutination of Gal-PEG-Lip modified liposomes. Liposomes (1.0 mM lipid, 2 ml) composed of ePC and Gal-PEG-Lip with different chain lengths (8:2 molar ratio) were mixed at 25°C with 10 mM Tris-HCl/150 mM NaCl (pH 7.4), containing 0.5 mg/ml RCA-120, and agglutination was monitored spectrophotometrically at a wavelength of 450 nm. Trace (1) Gal-PEG_{10}-Lip. Trace (2) Gal-PEG_{20}-Lip. Trace (3) Gal-PEG_{40}-Lip. B: Effect of liposomal Gal-PEG-Lip content on RCA-induced agglutination. Liposomes (0.5 mM lipid, 2 ml) composed of ePC/Gal-PEG_{10}-Lip in different molar ratios were mixed at 25°C with 10 mM Tris-HCl/150 mM NaCl (pH 7.4), containing 0.25 mg/ml RCA-120, and agglutination was monitored as an increase in absorption at a wavelength of 450 nm. Trace (1) PC/Gal-PEG_{10}-Lip = 9:1. Trace (2) PC/Gal-PEG_{10}-Lip = 9.5:0.5. Trace (3) PC/Gal-PEG_{10}-Lip = 0:10.

3.2. Blood clearance

Since for in vivo purposes it was necessary to prepare liposomes with a high stability in plasma, the liposomes used for the animal experiments were composed of dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylglycerol (DPPG) and cholesterol in a 10:1:10 molar ratio rather than of phos-

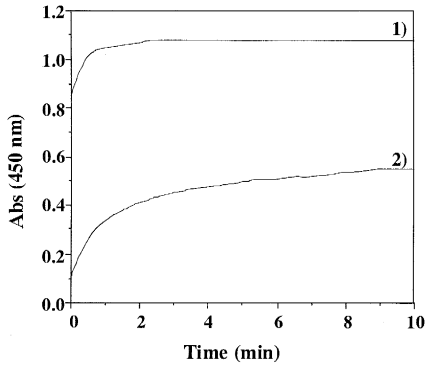

Fig. 3. RCA-induced agglutination of liposomes doubly modified with Gal-PEG$_{10}$-Lip and PEG-DMG. Liposomes (0.8 mM phospholipid, 2 ml) composed of ePC/Gal-PEG$_{10}$-Lip (8:2 molar ratio) with or without 5 mol% PEG-DMG were mixed with 10 mM Tris-HCl/150 mM NaCl, containing 0.5 mg/ml RCA-120 and agglutination was monitored as an increase in light absorption at 450 nm. Trace (1) without PEG-DMG; Trace (2) with PEG-DMG.

phatidylcholine alone. Assuming that this difference in liposome composition is unlikely to significantly influence the accessibility of the galactose residue at the distal end of the PEG chain (see Section 4), we selected the Gal-PEG$_{10}$-Lip for incorporation in the DPPC/DPPG/Chol liposomes, based on the results of the agglutination experiments described in Fig. 2. Fig. 4 presents the plasma elimination data we obtained for such liposomes. Clearly, the incorporation of the Gal-PEG$_{10}$-Lip in the liposomal bilayer caused a dramatic drop in blood circulation time. Independent of the Gal-PEG-Lipid concentration (either 10 or 20 mol%), the liposomes containing this lipid circulate with a T1/2 of less than 20 min. Control liposomes of the same composition but lacking the galactolipid circulate with a T1/2 of approximately 12 h, almost 40 times longer.

Circulation time is clearly prolonged when, in addition to the galactolipid, PEG$_{40}$-DSPE is incorporated in the liposomal bilayer. The T1/2 of liposomes containing 10% Gal-PEG$_{10}$-Lip and 2 mol% PEG$_{40}$-DSPE was increased 3-fold to a value of about 1 h.

A single injection of 100 mg of N-acetylgalactosamine, a well-known inhibitor of the asialoglycoprotein receptor of the hepatocytes as well as of the galactose particle receptor on the Kupffer cells [19,21], resulted in a marked prolongation of the blood circulation time of the Gal-PEG-Lip liposomes, T1/2 being restored to the 12-h value observed for control liposomes. This GalNAc-induced increase in circulation time lasted for at least 2 h. This substantiates our assumption that the Kupffer cell-mediated clearance of these liposomes is mediated by the galactose particle receptor on these cells.

3.3. Organ distribution

The differences in plasma half-lives of the liposomes are reflected in the values for liposome uptake by liver and spleen, as presented in Fig. 5. We analyzed these organs for the amount of liposomal radiolabel they had accumulated in a period of 20 h, a time point when even for the most slowly clearing liposomes the major fraction of the injected dose had been eliminated from the blood.

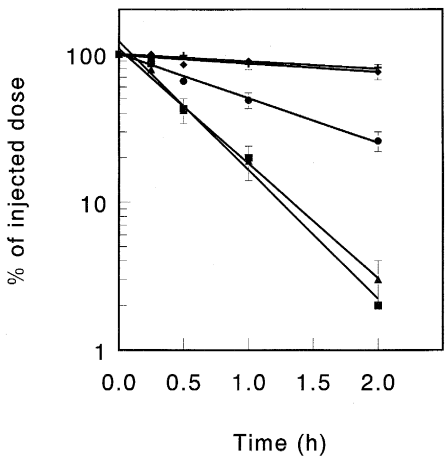

Fig. 4. Plasma elimination of Gal-PEG-Lip liposomes. Liposomes composed of DPPC/DPPG/CH with different amounts of Gal-PEG$_{10}$-Lip and additional PEG$_{40}$-DSPE and labeled with [^{3}H]cholesteryl oleyl ether were injected intravenously into rats at a dose of 25 μmol total lipid per kg. At the times indicated, plasma radioactivity was assayed. Elimination of Gal-PEG$_{10}$-Lip liposomes was also monitored after injection of 100 mg N-acetylgalactosamine (GalNAc) 1 min prior to liposome injection. Results are presented semi-logarithmically as percent of injected dose versus time. Data points are the means of two independent experiments, vertical bars indicating the two separate values. When no bar is given, the two values are within the size of the symbol. (♦) control liposomes without Gal-PEG$_{10}$-Lip (■) liposomes with 10% Gal-PEG$_{10}$-Lip (▲) liposomes with 20% Gal-PEG$_{10}$-Lip (●) liposomes with 10% Gal-PEG$_{10}$-Lip plus 2% PEG$_{40}$-DSPE (+) liposomes with 10% Gal-PEG$_{10}$-Lip, after injection of GalNAc.

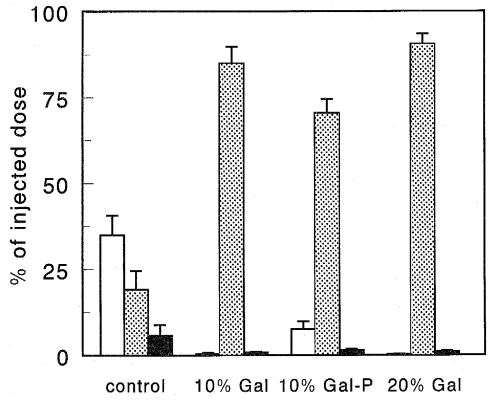

Fig. 5. Organ distribution of Gal-PEG-Lip liposomes. The distribution in liver, spleen and blood of the radiolabeled liposomes described in Fig. 4 was determined 20 h after injection. The total amounts of radioactivity associated with each of these organs is given as percentage of injected dose. The mean of three independent experiments is given with the bars indicating standard deviation. Liposome compositions: control liposomes without galactolipid (control), liposomes with 10 mol% (10% Gal) or 20 mol% (20% Gal) galactolipid and with 10 mol% galactolipid plus 2 mol% PEG-DSPE (10% Gal-P). Open bars, blood; hatched bars, liver; solid bars, spleen.

For the control liposomes, still 35% of the injected dose is recovered from the blood at that time, about 19% is in the liver and almost 6% in the spleen. As is usually found for slowly clearing liposomes, as much as 40% of the injected dose is not accounted for by the sum of liver, spleen and blood and presumably represents mostly liposomes that have been able to escape irreversibly from the blood compartment and have become diffusely distributed in the body either in or adsorbed to the vascular endothelial cells or outside the vascular compartment [24–26].

Of the liposomes carrying the Gal-PEG_{10}-Lip, either at a 10% or a 20% concentration, less than 1% was recovered in the blood compartment after 20 h. An overwhelming majority of the eliminated liposomes had accumulated in the liver, whereas the spleen accounted for only 1% of the injected dose or even less.

Liposomes containing, in addition to the galactolipid, PEG_{40}-DSPE took an intermediate position, like in the plasma elimination studies. Twenty h after injection, 70% of the injected dose was recovered in the liver and 1.4% in the spleen. It is clear that with the PEG_{40}-DSPE liposomes the effect of the Gal-PEG_{10}-Lip is still observed, although in this case the extreme bias for the liver has lessened slightly in favor of the spleen.

3.4. Intrahepatic distribution

For obvious reasons, we prepared liposomes of a size allowing access to the hepatocytes [27]. Fig. 6 presents the results of experiments in which we isolated the parenchymal and non-parenchymal cells of rat livers after the animals had been injected with radiolabeled liposomes and determined the radioactivitiy in the isolated cell fractions. The data are presented as % of total hepatic uptake, the sum always amounting to 100%. The data have not been corrected for mutual contamination of the cell fractions. Our hepatocyte preparations contain maximally 2% contamination with Kupffer cells.

It is apparent that of the control liposomes almost 30% is taken up in the hepatocyte fraction as compared to less than 10% of the two Gal-PEG-Lip formulations. These latter values might even be entirely accounted for by the relatively few Kupffer cells contaminating the hepatocyte fraction [28]. It is

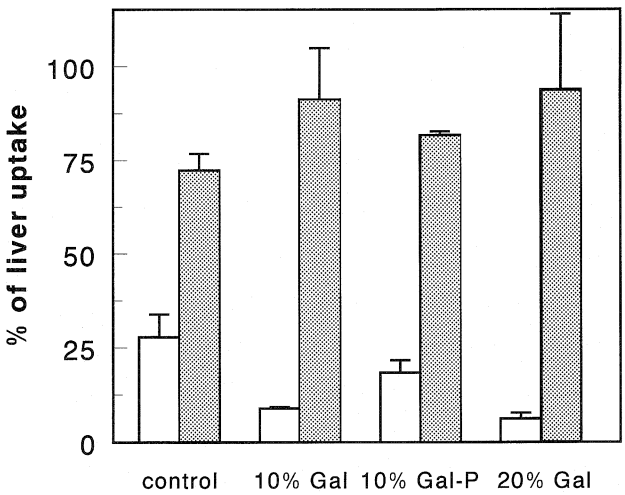

Fig. 6. Intrahepatic distribution of Gal-PEG-Lip liposomes. The livers of the experiments described in Fig. 5 were fractionated into a parenchymal and a non-parenchymal cell fraction, as described in Section 2. The amounts of radioactivity in these cell fractions were converted into the relative uptake values in the two fractions, expressed as percent of total liver uptake. Values given are mean ± S.D. of three independent experiments. Liposome compositions are as in Fig. 4 and Fig. 5. Open bars, parenchymal cell fraction (hepatocytes); hatched bars, non-parenchymal cells.

clear that the rapid hepatic uptake of the Gal-PEG-Lip liposomes can be accounted for almost entirely by the non-parenchymal cells. In a few experiments we further fractionated the non-parenchymal cell fractions into a Kupffer cell and an endothelial cell fraction and found that only the Kupffer cells contributed to non-parenchymal cell uptake of Gal-PEG_{10}-Lip liposomes (not shown).

By including PEG-DSPE in the Gal-PEG-Lip liposomes, we were able to only marginally shift this distribution in favor of the hepatocytes. Probably, the PEG-DSPE sterically interferes with the interaction of the galactose residue with the receptor on the Kupffer cells, in analogy with the reduced agglutination activity in Fig. 2.

4. Discussion

Phosphatidylethanolamine (PE) is the most widely used lipid compound for the construction of lipid-anchored derivatives for proper incorporation in the liposomal bilayer. Its popularity is undoubtedly related to the reactivity of its primary amino group, which allows versatile derivatization, and to the biological safety of the compound as such which is a natural product. From an industrial point of view, however, PE is less desirable for this purpose because of high cost. Therefore, we set out to develop an alternative method for lipophilic derivatization, involving the coupling of the active ingredient (drug or ligand) to a diglyceride residue via a poly(ethylene glycol) spacer. In this paper we specifically describe the synthesis of diglyceride-anchored galactosyl derivatives with medium-length PEG chains as spacer groups. PEG chains of this size have been reported to possess properties favorable to enhance plasma circulation times of particles to which they are attached, presumably by diminishing adsorption of opsonic plasma proteins, which in turn results in diminished delivery to cells of the mononuclear phagocyte system [29–34]. In analogy to the design of immunoliposomes carrying the antibody at the distal end of a bilayer-anchored PEG chain [35,36], we speculated that such a construct would combine the 'macrophage-evading' properties of the PEG chains with the specific targeting properties of the galactose residue.

Since the galactose residue is relatively small and the PEG chains are highly flexible [33], we anticipated that beyond a certain PEG chain length random coil formation might frustrate the formation of galactose clusters required for optimal interaction with the lectin [37,38]. From agglutination experiments with the galactose-specific lectin RCA-120 it appeared that only with the PEG_{10} chains the galactose moieties were properly exposed for adequate interaction with the lectin. The other two PEG spacers completely prevented interaction of the liposomes with the agglutinin.

Monoclonal antibodies as well as some amino acids have been reported to be recognized even when they are attached to the distal end of PEG_{20} or PEG_{40} chains [35,36,39]. Also folate residues attached to liposomes via a PEG_{70} spacer (molecular weight 3350) were shown to be recognized better by the folate receptor than folate coupled to liposomes without a spacer [40]. Apparently, the effect of a PEG spacer between the liposome and the ligand on receptor recognition may vary from ligand to ligand. In the case of galactose recognition, the requirement of cluster formation may be the determinant parameter preventing adequate interaction between ligand and lectin.

The presence of additional PEG_{40} chains, anchored in the liposomal bilayer in the form of PEG-DMG, did influence the agglutination behavior. Both rate and extent of agglutination were substantially decreased. This is taken to reflect the steric hindrance exerted by these relatively long chains, interfering with the establishment of contact between the sugar and the binding site on the agglutinin as well as with the cluster formation required for optimal interaction. In the animal experiments we used liposomes with an average diameter of 75–100 nm, sufficiently small to allow penetration into the space of Disse and thus interaction with the hepatocytes [27]. We also chose for a suitably modified liposomal lipid composition in order to comply with the more rigorous stability requirements applying to in vivo conditions. We consider it unlikely that the flexibility of the PEG chains as such, which is held responsible for the screening effect, would be significantly influenced by the composition of the supporting bilayer. Bilayer viscosity, which clearly is different for the two liposome formulations, may be important, though, since it directly influences lateral mobility of bilayer constituents.

This would, however, not be likely to influence the order in which PEG_{10}, PEG_{20} and PEG_{40} allow recognition of the liposomal galactose by the lectin molecule and would therefore not affect our choice of PEG chains for the in vivo experiments.

Liposomes containing 10 or 20 mol% of the Gal-PEG_{10}-Lip were cleared from circulation almost 40-fold faster than control liposomes without the galactolipid. The eliminated liposomes were almost exclusively recovered in the hepatic macrophages, the Kupffer cells, whereas substantial proportions of the control liposomes were also recovered in spleen and hepatocytes. This very specific and efficient recognition by the Kupffer cells is attributed to interaction with the galactose particle receptor on these cells [21], considering the strongly inhibitory effect of a N-acetylgalactosamine injection. Apparently, the galacto-liposomes do not bind opsonizing proteins in sufficient quantities to successfully compete with the exposed galactose moiety and to allow significant interaction with other cells, such as spleen macrophages. Previously, we reported similar results with a tri-antennary galactosylated cholesterol derivative, which also caused liposomes to be rapidly and almost exclusively taken up by the Kupffer cells [19]. This makes it unlikely that it is the anti-opsonic potency of the PEG chain that is responsible for the lack of uptake by of the Gal-PEG-Lip liposomes by cells other than Kupffer cells. Rather, it is likely the overwhelming affinity of the galactose moieties for the Kupffer cell receptor [21], irrespective of the presence of any opsonizing proteins on the liposomal surface, that causes this highly selective uptake. Peritoneal macrophages, which were also reported to have increased affinity for galactose-exposing liposomes [41], are not accessible from the blood stream and therefore will not interfere with our observations.

Additional incorporation of PEG chains in the bilayers of liposomes containing Gal-PEG-Lip, by means of PEG-DSPE, caused (1) a three-fold increase in blood circulation time, (2) a small but significant decrease in hepatic uptake after 20 h and (3) a small but significant shift in intrahepatic distribution in favor of the hepatocytes, comparable to that of the control liposomes. Probably, the presence of the additional PEG chains impedes the interaction between the galactose and its receptor on the Kupffer cells, in much the same way as it affects the lectin-induced agglutinability of the liposomes. This would allow a larger fraction of the injected liposomes to interact with spleen macrophages, presumably mediated by macrophage-directed opsonins, and with the hepatocytes, either mediated by hepatocyte-directed opsonins [8,28] or by the asialoglycoprotein receptor on these cells, which may have different requirements vis à vis the configuration of the ligand galactose groups. We conclude that the anti-opsonic effect of the PEG-DSPE is predominantly caused by its steric interference with the interaction between galactose and its receptor.

The hepatocytic asialoglycoprotein receptor has been studied in great detail (see Ref. [42] for a comprehensive review). Its affinity for galactose-carrying proteins depends on the number and the spatial arrangement of the terminal galactose moieties: mono-, di-, tri- and tetra-antennary galactose-terminated units bind with increasing affinities [43,44]. Also the spatial arrangement of the galactose residues with respect to each other was shown to be important in receptor affinity [44].

The structural requirements of the macrophage receptor have not been investigated in detail. Van Berkel and co-workers have shown that ligand size has a strong influence. Intravenously administered small lipoprotein particles (HDL) provided with a surface inserted triantennary galactose were taken up by the hepatocyte receptor, while larger particles (LDL) with the same triantennary structure at their surface were predominantly cleared by the liver macrophages [45,46]. This was shown to be caused by specific requirements of the macrophage receptor rather than of the hepatocyte receptor: the affinity of macrophage receptor increased sharply with ligand size, whereas the hepatocyte receptor was virtually insensitive to ligand size [46]. Confirming an earlier report by Lee et al. [47], these authors also demonstrated, however, a strong influence of spacer arm length [48]: When the trigalactosyl moiety was separated from the ligand particle by a distance of 20 Å, both HDL and LDL particles were mainly removed from circulation by the hepatocytic galactose receptor, while the macrophage receptor was not addressed [48]. In our experiments, the use of a long flexible spacer (PEG_{10}) in the galactolipid failed, however, to produce measurable in vivo affinity of the Gal-PEG-Lip liposomes for the asialoglycoprotein receptor;

instead, a high affinity for the galactose receptor on Kupffer cells was observed. The effective length of the PEG_{10} chain is between maximally 35 Å (fully stretched) and minimally 9 Å (fully contracted), comparable to the spacer length applied by Biessen et al. [48]. Rather than the introduction of a long flexible spacer, the branching and number of closely apposing galactose residues in the galactolipids are probably decisive in achieving efficient hepatocyte targeting. We are currently developing methods to synthesize PEG lipids carrying triantennary galactose structures.

We conclude that, as was reported for other galactose-exposing liposomal formulations, galactose residues attached to the liposomal surface via a PEG_{10} spacer fail to target the liposomes to the asialoglycoprotein receptor on hepatocytes but rather deliver them rapidly and highly specifically to the hepatic macrophage population. This phenomenon might be profitably exploited, for example, when highly specific elimination or activation of the liver macrophage population is required, leaving the macrophages of the spleen unaffected, by encapsulating inside the liposomes the macrophage-toxic drug clodronate [49] or an immunomodulator such as muramyldipeptide [50], respectively.

Acknowledgements

The authors thank Bert Dontje for excellent technical assistance. This research was financially supported in part by the Netherlands Organization for Scientific Research (NWO), Grant 902-23-187.

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