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LACTOSYLCERAMIDE-INDUCED STIMULATION OF LIPOSOME UPTAKE BY KUPFFER CELLS IN VIVO

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Incorporation of 8 mol percent lactosylceramide into small unilamellar vesicles consisting of cholesterol and sphingomyelin in an equimolar ratio and containing [^{3}H]inulin as a marker resulted in an increase in total liver uptake and a drastic change in intrahepatic distribution of the liposomes after intravenous injection into rats. The control vesicles without glycolipid accumulated predominantly in the hepatocytes, but incorporation of the glycolipid resulted in a larger stimulation of Kupffer-cell uptake (3.2-fold) than of hepatocyte uptake (1.2-fold). Liposome preparations both with and without lactosylceramide in which part of the sphingomyelin was replaced by phosphatidylserine, resulting in a net negative charge of the vesicles, were cleared much more rapidly from the blood and taken up by the liver to higher extents. The negative charge had, however, no influence on the intrahepatic distributions. The fast hepatic uptake of the negatively charged liposomes allowed competition experiments with substrates for the galactose receptors on liver cells. Inhibition of blood clearance and liver uptake of lactosylceramide-containing liposomes by *N*-acetyl-D-galactosamine indicated the involvement of specific recognition sites for the liposomal galactose residues. This inhibitory effect of *N*-acetyl-D-galactosamine was shown to be mainly the result of a decreased liposome uptake by the Kupffer cells, compatible with the reported presence of a galactose specific receptor on this cell type (Kolb-Bachofen et al. (1982) Cell 29, 859–866). The difference between the results on sphingomyelin-based liposomes as described in this paper and those on phosphatidylcholine-based liposomes as published previously (Spanjer and Scherphof (1983) Biochim. Biophys. Acta 734, 40–47) are discussed.

Introduction

In recent years a number of attempts have been reported to target liposomes to hepatocytes in vivo [1–3]. Hepatocytes contain a plasma membrane receptor which facilitates the uptake of particles or molecules containing non-reducing terminal β-galactosyl residues [4]. Incorporation of ligands exposing such galactosyl residues into the liposomal bilayers has been reported to result in an increase in total liver uptake, which was ascribed to specific interaction with this receptor [5,6]. Recently, Kolb-Bachofen et al. reported the presence of a similar receptor on Kupffer cells, which was also shown to recognize specifically galactose or *N*-acetyl-D-galactosamine [7,8]. In previous work [9] we incorporated lactosylceramide into the bilayer of small unilamellar vesicles consisting of cholesterol, dimyristoylphosphatidylcholine and phosphatidylserine and demonstrated that these liposomes were taken up by the liver to a much higher extent than the control liposomes without the glycolipid. Cell separation revealed that the major increase in total liver uptake was due to an increased uptake of lactosylceramide containing liposomes by the hepatocyte fraction. The small increase we found in the uptake of the glycolipid containing liposomes by the Kupffer cells was not

statistically significant. In the present paper liposomes containing sphingomyelin rather than dimyristoylphosphatidylcholine as their main phospholipid constituent were found to have a considerably lower "natural" affinity for Kupffer cells. With such liposomes we found that incorporation of lactosylceramide results in a substantial increase in Kupffer cell uptake rather than in hepatocyte uptake.

Materials

Cholesterol, dimyristoyl-L-α-phosphatidylcholine, L-α-phosphatidylserine, sphingomyelin, *N*-lignoceroyl-dihydrolactocerebroside, collagenase type I, *N*-acetyl-D-galactosamine and *N*-acetyl-D-glucosamine were obtained from Sigma.

Pronase E was from Merck, DNAase type I, grade II was from Boehringer and [^{3}H]inulin (904 mCi/mmol) was obtained from Amersham International, U.K.

Sepharose Cl-2B was purchased from Pharmacia and Diaflow ultrafiltration membranes PM-10 were from Amicon.

Methods

Preparation of liposomes. Small unilamellar vesicles of compositions given in the legends of the figures and tables were prepared as described previously [9] when phosphatidylserine was incorporated; liposomes without phosphatidylserine were made by probe sonication with a Branson B-12 sonifier at 60 W. In both cases sonication was continued for 3–4 h at room temperature. For all types of liposomes used internal volumes and calculated diameters were determined as described previously [9] and are given in the results.

Turbidity measurements to detect the contribution of vesicles larger than 50 nm to the vesicle population were done according to Barrow and Lentz [10].

Animals. Male Wistar-random rats were used varying in weight from 170–250 g. Animals were equipped with a heart catheter for intravenous injections and blood sampling as described before [9].

Liposomes were injected in a dose of 2 μmol of total lipid per 100 g body weight. Blood sampling, cell separations and determinations of label uptake by total liver or isolated cell fractions were also done as published previously [9].

Inhibition experiments. *N*-Acetyl-D-galactosamine or *N*-acetyl-D-glucosamine were used as potential competitive inhibitors of liposomal uptake. Two min before the injection of the liposomes the animals received a bolus injection of 50 mg *N*-acetylglucosamine or *N*-acetylgalactosamine dissolved in 0.15–0.20 ml (10 mM Tris-HCl/150 mM NaCl/1 mM Na_2 EDTA) (pH 7.4) buffer. Every 10 min an additional dose of 50 mg acetylated sugar was given to maintain an inhibitory concentration of 10 mM acetylated sugar in the blood (cf. Fig. 3 of Ref. 11) for the duration of the experiment.

Results

The effect of incorporation of 8 mol% lactosylceramide on the in vivo behavior of small unilamellar vesicles consisting either of cholesterol and sphingomyelin in an equimolar ratio or cholesterol, sphingomyelin and phosphatidylserine, molar ratio 5 : 4 : 1 was investigated. In order to exclude the possibility that lactosylceramide-containing vesicles have a different blood elimination or intrahepatic distribution as a result of a difference in size [12] we estimated the dimensions of the vesicles by determining the encapsulated volume (Table I). All vesicle preparations had an entrapped volume, based on encapsulation

TABLE I

INCLUDED VOLUME (μl/μmol) AND CALCULATED DIAMETER (nm) OF VESICLE PREPARATIONS USED

From phosphorus and radioactivity contents of the vesicle preparations the included volume was determined. The mean diameter was calculated according to the method described by Wilschut [13]. Chol, cholesterol; SM, sphingomyelin; PS, phosphatidylserine; LC, lactosylceramide.

Vesicle composition		Included volume (μl/μmol)	Diameter (nm)
Chol/SM	(5:5)	0.34	27.6
Chol/SM/LC	(5:4:0.8)	0.29	25.6
Chol/SM/PS	(5:4:1)	0.32	27.0
Chol/SM/PS/LC	(5:1:1:0.8)	0.36	28.8

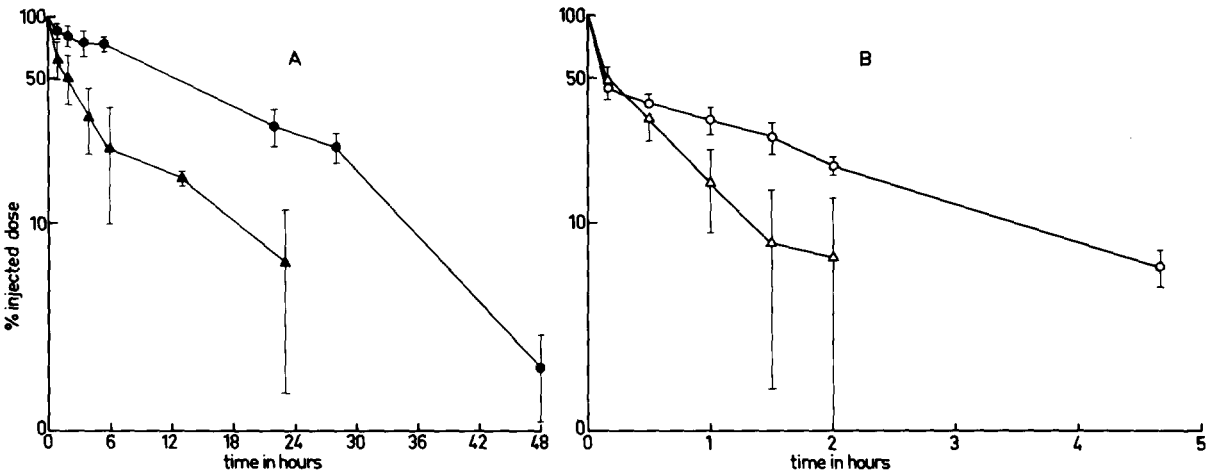

Fig. 1. Elimination of control and lactosylceramide-containing vesicles from blood. Rats were injected with liposomes consisting of cholesterol and sphingomyelin (panel A) or cholesterol, sphingomyelin and phosphatidylserine (panel B) with or without lactosylceramide as described in Methods. Points represent means from 3–6 determinations and bars indicate standard deviation. ●———●, cholesterol/sphingomyelin = 5 : 5; ▲———▲, cholesterol/sphingomyelin/lactosylceramide = 5 : 5 : 0.8; ○———○, cholesterol/sphingomyelin/phosphatidylserine = 5 : 4 : 1; △———△, cholesterol/sphingomyelin/phosphatidylserine/lactosylceramide = 5 : 4 : 1 : 0.8.

of [^{3}H]inulin, of approximately 0.3 μl/μmol lipid, which is characteristic of vesicles with a diameter of 25–30 nm [13]. Vesicles with a diameter of 50 nm would show an entrapped volume of about 1.2 μl/μmol [13]. This suggests that the contribution of vesicles larger than 50 nm is minimal and that there is no significant difference in size between lactosylceramide-containing vesicles and control vesicles. To further corroborate this conclusion we conducted turbidity measurements according to

TABLE II

LIVER UPTAKE AND INTRAHEPATIC DISTRIBUTION OF SMALL UNILAMELLAR VESICLES WITH OR WITHOUT LACTOSYLCERAMIDE

Rats were injected with liposomes of indicated composition and uptake by total liver, parenchymal cells and non-parenchymal cells was determined when less than 10% of the injected dose remained in the circulation. Parenchymal cells were isolated after perfusion of the liver with collagenase and non-parenchymal cells were isolated after perfusion with pronase as described in Methods. Liposome uptake was determined by correcting recovered radioactivity for total cell recovery from literature values ($450 \cdot 10^6$ parenchymal cells and $194 \cdot 10^6$ non-parenchymal cells per 100 g body weight) [21]. Values represent means of 2–6 determinations. Chol, cholesterol; SM, sphingomyelin; LC, lactosylceramide; PS, phosphatidylserine; TL, total liver; PC, parenchymal cells; NPC, non-parenchymal cells.

Liposome composition	Circulation time (h)	Total liver		Uptake (% injected dose)				Uptake (% total liver)		Lipid uptake (nmol/10^6 cells)	
		Mean	Range	PC		NPC					
				Mean	Range	Mean	Range	PC	NPC	PC	NPC
Chol/SM (5:5)	48	32.1	23.8–38.5	26.4	24.9–27.9	8.4	6.6–10.1	82.2	26.2	1.17	0.87
Chol/SM/LC (5:5:0.8)	23	59.7	54.7–64.5	31.8	31.7–31.9	27.4	26.5–28.3	53.3	45.9	1.41	2.82
Chol/SM/PS (5:4:1)	4.5	53.0	48.4–56.9	43.8	32.5–51.5	15.0	14.6–15.3	82.6	26.6	2.11	1.52
Chol/SM/PS/LC (5:4:1:0.8)	2	75.4	67.1–81.3	41.8	41.0–42.5	34.7	33.6–35.7	55.4	46.0	1.86	3.58

Barrow and Lentz [10] and determined the absorbance as a function of $1/\lambda^4$ for all vesicle preparations used. The results (not shown) confirmed the above observations; the contribution of vesicles larger than 50 nm was less than 0.5% for all types of liposome preparations used, irrespective of the presence or absence of the glycolipid in the vesicle bilayer.

In Fig. 1 the blood elimination curves for all types of liposomes used are shown. Panel A shows the blood elimination for the neutral cholesterol/sphingomyelin vesicles with or without lactosylceramide. The glycolipid caused a very pronounced increase in elimination rate, particularly during the first 5–6 h after injection. With the negatively charged vesicles (panel B), which are cleared much more rapidly, incorporation of lactosylceramide also influenced the elimination rate except during the first 30 min after injection. The differences in elimination rate were reflected in the liver uptake values (Table II). In general, the blood elimination time and liver uptake were related invertedly. No relationship was observed, however, between the rate of liver uptake and the intrahepatic distribution. For the cholesterol/sphingomyelin liposomes only about one third of the injected dose was recovered in total liver by the time the blood compartment contained less than 10% of the injected dose (48 h after injection), whereas for the rapidly clearing negatively charged phosphatidylserine-containing control vesicles more than half of the injected dose was recovered in the liver when, after 4.5 h, less than 10% of the injected dose remained in circulation. The table also shows that in either case about 80% of the liver-associated label was in the parenchymal cells, while an average of 25% was recovered in the non-parenchymal cells. Occasional fractionation of this cell fraction in a Kupffer cell fraction and an endothelial cell fraction by centrifugal elutriation showed that all of the radioactivity in the non-parenchymal cells was associated with the Kupffer cells and none with the endothelial cells as we reported before for other vesicle types [14,15]. For both neutral and negatively charged vesicles incor-

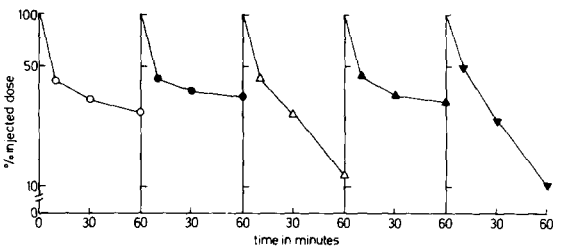

Fig. 2. Specific inhibition by *N*-acetyl-D-galactosamine of the blood elimination of lactosylceramide liposomes. Rats were injected with liposomes composed of cholesterol/sphingomyelin/phosphatidylserine (/lactosylceramide) in a 5:4:1 (:0.8) molar ratio with or without 10 mM *N*-acetyl-D-glucosamine or *N*-acetyl-D-galactosamine present in the blood as described in Methods. Points represent means from 2–6 determinations. ○———○, liposomes without lactosylceramide; ●———●, *N*-acetyl-D-galactosamine and liposomes without lactosylceramide; △———△, lactosylceramide-containing liposomes; ▲———▲, lactosylceramide-containing liposomes and *N*-acetyl-D-galactosamine; ▼———▼, lactosylceramide-containing liposomes and *N*-acetyl-D-glucosamine.

TABLE III

SPECIFIC INHIBITION BY *N*-ACETYL-D-GALACTOSAMINE OF LIVER UPTAKE OF LACTOSYLCERAMIDE LIPOSOMES

Rats were injected with liposomes of the indicated composition with or without 10 mM *N*-acetyl-D-galactosamine or *N*-acetyl-D-glucosamine present in the blood and total liver uptake after one hour was determined as described in Methods. Chol, cholesterol; SM, sphingomyelin; PS, phosphatidylserine; LC, lactosylceramide; GalNac, *N*-acetyl-D-galactosamine; GlcNac, *N*-acetyl-D-glucosamine.

Liposome composition	Inhibiting sugar	Liver uptake		Number of determinations
		% dose mean	Range	
Chol/SM/PS (5:4:1)	–	41.5	37.2–45.7	2
Chol/SM/PS (5:4:1)	GalNac	42.3	39.1–45.4	2
Chol/SM/PS/LC (5:4:1:0.8)	–	72.4	66.3–76.9	6
Chol/SM/PS/LC (5:4:1:0.8)	GalNac	48.0	37.7–55.6	5
Chol/SM/PS/LC (5:4:1:0.8)	GlcNac	81.6	79.0–84.1	2

TABLE IV

LACK OF UPTAKE OF LACTOSYLCERAMIDE LIPOSOMES BY ENDOTHELIAL CELLS

[^{3}H]Inulin-labeled vesicles (2 μmol lipid per 100 g body weight) were injected intracardially. 1 h after administration non-parenchymal cells were isolated and separated in an endothelial cell fraction (EC) and a Kupffer cell fraction (KC) as described in Ref. 14. Kupffer cells could be identified cytochemically on the basis of endogenous peroxidatic activity, which is absent in endothelial cells. Cells were processed for measurement of radioactivity content as described in Methods.

Cell type isolated	Peroxidase positive cells %		dpm per 10^6 cells		dpm per 10^6 cells corrected for contamination		Uptake (nmol lipid per 10^6 cells)	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range
EC	14.2	13.1–15.3	55.6	11.5–99.6	−64.8	−97.5–(−32.1)	nil	
KC	61.9	61.6–62.1	483.9	452.8–515.0	782.3	734.7–829.8	8.10	7.64–8.56

poration of lactosylceramide caused a substantial increase in the liver uptake; when less than 10% of the injected dose was left in circulation about 60% of the neutral lactosylceramide liposomes and as much as 75% of the negatively charged glycolipid-containing vesicles had accumulated in the liver. For both types of liposomes a substantial increase in non-parenchymal cell uptake is seen as a result of the incorporation of the glycolipid. The amount of liposomes taken up per non-parenchymal cell increased 3.2-fold for the neutral vesicles and 2.3-fold for the negatively charged vesicles, whereas the uptake by the parenchymal cells was stimulated only 1.2- and 0.9-fold for the neutral and negative vesicles, respectively. To determine the involvement of galactose-specific recognition systems in the uptake of lactosylceramide containing liposomes, inhibition experiments with *N*-acetyl-D-galactosamine and *N*-acetyl-D-glucosamine were performed. Because it is practically impossible to maintain inhibiting blood concentrations of the acetylated sugars over a long period of time, which would be necessary in case of the neutral liposomes, the inhibition experiments were performed with the phosphatidylserine-containing liposomes. Inhibition of blood elimination (Fig. 2) and liver uptake (Table III) of the lactosylceramide containing vesicles was achieved specifically with 10 mM *N*-acetyl-D-galactosamine, but not with the same concentration of *N*-acetyl-D-glucosamine. Blood elimination nor liver uptake of the control liposomes were significantly influenced by *N*-acetyl-D-galactosamine. From these results it can be concluded that the lactosylceramide liposomes are taken up, at least partially, via a galactose-specific recognition system. Since the cell separation experiments had revealed that the glycolipid-induced increase in total liver uptake was mainly due to an increase in non-parenchymal cell uptake (Table II), we further examined this cell fraction for the specific involvement of the Kupffer cells (Table IV) and for the inhibiting action of *N*-acetyl-D-

TABLE V

SPECIFIC INHIBITION BY *N*-ACETYL-D-GALACTOSAMINE OF KUPFFER CELL UPTAKE OF LACTOSYLCERAMIDE-CONTAINING LIPOSOMES

Control rats or rats injected with *N*-acetyl-D-galactosamine received liposomes composed of cholesterol, sphingomyelin, phosphatidylserine and lactosylceramide in a molar ratio of 5:4:1:0.8. Kupffer cells were isolated from the nonparenchymal cell fraction by centrifugal elutriation and uptake of liposomes was determined as described in Methods.

Body weight (g)	*N*-Acetyl-D-galactosamine administered	Total liver uptake (% of injected dose)	Kupffer cell uptake (nmol/10^6 cells)
200	−	66.3	10.1
250	−	76.9	9.7
240	+	46.9	6.3
200	+	37.7	3.2

galactosamine (Table V). Thus, one hour after administration of lactosylceramide liposomes, with or without prior injection of *N*-acetyl-D-galactosamine, the non-parenchymal cells were isolated and separated into a Kupffer-cell fraction and an endothelial cell fraction by centrifugal elutriation. Table IV shows that only the Kupffer cells are involved in the non-parenchymal cell uptake of the lactosylceramide containing liposomes; this is compatible with results reported before for liposomes of different compositions which also revealed lack of involvement of the endothelial cell fraction in the hepatic uptake of liposomes [14,16]. Table V, finally, demonstrates that the inhibitory effect of the *N*-acetyl-D-galactosamine on blood clearance and liver uptake as presented in Fig. 2 and Table III, can, at least to a considerable extent, explicitly be ascribed to inhibition of Kupffer cell uptake.

Discussion

In previous work we reported targeting of lactosylceramide-containing liposomes to hepatocytes in vivo [9]. In those experiments we used liposomes composed of cholesterol/dimyristoylphosphatidylcholine/phosphatidylserine in a 5 : 4 : 1 molar ratio and showed that incorporation of lactosylceramide resulted in an increase in total liver uptake mainly as a result of an increase in hepatocyte uptake. The experiments described in the present paper lead us to conclude, on the other hand, that increased blood elimination and liver uptake due to incorporation of lactosylceramide can also be caused by a stimulation of Kupffer cell uptake. Apparently, the bulk lipid composition of the liposomes determines whether lactosylceramide incorporation leads to stimulation of hepatocyte uptake, Kupffer cell uptake or both. With the dimyristoylphosphatidylcholine liposomes which by themselves have already a relatively high tendency to accumulate in the Kupffer cells, stimulation of Kupffer cell uptake due to lactosylceramide incorporation is modest when compared to sphingomyelin liposomes, which by themselves, tend to distribute intrahepatically more in favor of the hepatocytes.

From the results of the encapsulated volume- and turbidity measurements differences in size [12] can be excluded as an explanation for the observed differences in intrahepatic distribution.

When the characteristics of two types of control vesicles, e.g., cholesterol/dimyristoylphosphatidylcholine/phosphatidylserine = 5 : 4 : 1 and cholesterol/sphingomyelin/phosphatidylserine = 5 : 4 : 1 are compared it can be seen that the sphingomyelin containing liposomes have an almost 2-fold longer blood circulation time than the ones with an equal amount of dimyristoylphosphatidylcholine.

It is tempting to speculate on the existence of a relationship between the rate of blood elimination and the intrahepatic distribution of liposomes. Liposomes with a low affinity for the Kupffer cells would thus be cleared relatively slowly and would have a greater chance of being taken up by the hepatocytes. From in vitro work with Kupffer cells (Roerdink, F.H. et al., unpublished data) we have evidence that phosphatidylcholine-containing liposomes have a substantially higher affinity for these cells than sphingomyelin-containing liposomes. In addition, introduction of negative charge in the liposomal bilayer further increases this affinity, which is fully compatible with our and other investigators' observations on the in vivo behavior of such liposomes [17,18]. Whether lactosylceramide incorporation into the liposomal bilayer will lead to stimulation of uptake by hepatocytes or Kupffer cells or both will therefore be determined by a number of factors, including those that determine the intrahepatic distribution of the control liposomes without glycolipid. In addition, similar variables may influence the affinity and capacity of the galactose receptors on Kupffer cells and hepatocytes, respectively. For Kupffer cells we have clearly demonstrated that liposome uptake by these cells takes place by way of an endocytic mechanism [19]. It is conceivable that the less fluid membranes of sphingomyelin-containing liposomes provide a more suitable matrix for the glycolipid to act as a successful ligand, leading to endocytosis of the liposomes. Munn and Parce [20] recently demonstrated in an elegant study that IgG-coated solid-membrane liposomes are internalized much more efficiently by neutrophils than fluid-membrane liposomes. This difference was ascribed to restricted lateral mobility of the ligand in the solid-type vesicles leading to a better 'grip'

of the receptor-covered cell membrane on the liposome. Whether similar effects play a role in the ultimate in vivo distribution of liganded liposomes in the liver remains to be investigated.

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