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Intrahepatic distribution of small unilamellar liposomes as a function of liposomal lipid composition

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We investigated the intrahepatic distribution of small unilamellar liposomes injected intravenously into rats at a dose of 0.10 mmol of lipid per kg body weight. Sonicated liposomes consisting of cholesterol/sphingomyelin (1:1), (A); cholesterol/egg phosphatidylcholine (1:1), (B); cholesterol/sphingomyelin/phosphatidylserine (5:4:1), (C) or cholesterol/egg-phosphatidylcholine/phosphatidylserine (5:4:1), (D) were labeled by encapsulation of [^{3}H]inulin. The observed differences in rate of blood elimination and hepatic accumulation (A ≪ B ≈ C < D) confirmed earlier observations and reflected the rates of uptake of the four liposome formulations by isolated liver macrophages in monolayer culture. Fractionation of the liver into a parenchymal and a non-parenchymal cell fraction revealed that 80–90% of the slowly clearing type-A liposomes were taken up by the parenchymal cells while of the more rapidly eliminated type-B liposomes even more than 95% was associated with the parenchymal cells. Incorporation of phosphatidylserine into the sphingomyelin-based liposomes caused a significant increase in hepatocyte uptake but a much more substantial increase in non-parenchymal cell uptake, resulting in a major shift of the intrahepatic distribution towards the non-parenchymal cell fraction. For the phosphatidylcholine-based liposomes incorporation of phosphatidylserine did not increase the already high uptake by the parenchymal cells while uptake by the non-parenchymal cells was only moderately elevated; this resulted in only a small shift in distribution towards the non-parenchymal cells. The phosphatidylserine-induced increase in liposome uptake by non-parenchymal liver cells was paralleled by an increase in uptake by the spleen. Fractionation of the non-parenchymal liver cells in a Kupffer cell fraction and an endothelial cell fraction showed that even for the slowly eliminated liposomes of type A endothelial cells do not participate to a measurable extent in the elimination process, thus excluding involvement of fluid-phase pinocytosis in the uptake process.

Introduction

In previous papers [1–3] we described that attempts to target liposomes to the asialoglycoprotein receptor [4] of the parenchymal cells of the liver (the hepatocytes) by means of surface-attached galactose residues may be frustrated by interference on the part of the liver macrophages (Kupffer cells), which also express a cell surface receptor for galactose [5]. Thus, considerable proportions of liposomes carrying surface-galactose were found to be taken up by Kupffer cells instead of hepatocytes. During those studies we observed that, after intravenous injection of low

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doses of liposomes, certain types of control liposomes lacking the galactosyl residues displayed an intrahepatic distribution strongly in favor of the hepatocytes. In the present paper we report on the hepatic uptake and intrahepatic distribution of small unilamellar liposomes of cholesterol/egg phosphatidylcholine or cholesterol/sphingomyelin, either one with or without 10 mol% phosphatidylserine, following intravenous injection of relatively high doses (0.1 mmol lipid per kg body weight) of liposomes. In addition, the rates of blood elimination and the accumulation in the spleen were measured and an attempt was made to correlate these results with the intrahepatic distribution and with observations on liposome uptake by isolated Kupffer cells in maintenance culture.

Materials and Methods

Cholesterol, egg phosphatidylcholine, bovine brain sphingomyelin, L-α-phosphatidylserine and collagenase type I were obtained from Sigma. Pronase-E was from Merck, DNAase type I grade II was from Boehringer and [^{3}H]inulin and cholesteryl [^{14}C]oleate were from Amersham. All other materials were from previously reported sources [1,6].

[^{3}H]Inulin-labeled small unilamellar vesicles of compositions given in the legends of figures and tables were prepared by sonication of lipid dispersions in Tris-buffered salt solutions as described previously [1]. The encapsulated volumes of the four liposome types used varied between 0.20 and 0.22 l/mol of lipid. Liposomes were injected into the penile vein of ether-anesthetized male Wistar rats varying in body weight from 170 to 260 g. Liposome elimination from blood was determined by blood sampling from the tail vein. Blood volume was taken as 65 ml per kg body weight. The injected dose was 0.1 mmol of total lipid per kg body weight. For determination of liposome uptake by total liver the organ was perfused in situ via the portal vein with isotonic saline at 37°C to remove blood, and homogenized in a Potter-Elvehjem tube; aliquots of the homogenate were assayed for radioactivity. Splenic uptake was determined similarly without prior perfusion. Intrahepatic distribution of liposomes was determined by measuring radioactivity content of the isolated parenchymal and non-parenchymal liver cell fractions, assuming $450 \cdot 10^7$ hepatocytes and $194 \cdot 10^7$ non-parenchymal cells per kg body weight [7]. Parenchymal cells (hepatocytes) were isolated after perfusion of the liver with collagenase and non-parenchymal cells (Kupffer cells and endothelial cells) after digestion of the liver with pronase as described before [1,6]. Fractionation of the non-parenchymal cell fraction into an endothelial and a Kupffer cell fraction was accomplished by metrizamide gradient centrifugation followed by centrifugal elutriation as described previously [6]. For in vitro uptake studies Kupffer cells were isolated similarly and allowed to attach to 35-mm plastic Petri dishes at a density of $1.5 \cdot 10^6$–$2.0 \cdot 10^6$ cells per dish under the conditions described previously [6]. After at least 24 h the cells were incubated as reported before [6] with liposomes of the compositions given in the legend to Fig. 2.

Results

Vesicles composed of 50 mol% cholesterol and 50 mol% bovine brain sphingomyelin or egg phosphatidylcholine were injected intravenously and elimination of liposome label from blood was followed up to 17 h after administration (Fig. 1). During the initial elimination phase the sphingomyelin-containing liposomes are cleared much more slowly than the phosphatidylcholine-containing liposomes; in the first hour 14 and 67% of the injected dose are eliminated, respectively. During the second phase, up to 17 h after injection, there is closer similarity between the elimination rates of the two liposome types. Incorporation of 10 mol% phosphatidylserine resulted in a considerable increase in blood elimination rate for both types of liposome. For the neutral as well as the negatively charged form of either liposome type we measured uptake by whole liver and spleen and the distribution among the parenchymal and non-parenchymal cell fractions within the liver at two time points, $5\frac{1}{2}$ and 17 h after injection. The results are summarized in Table I. For the sphingomyelin/cholesterol liposomes the slow elimination from the blood is accompanied by a low liver uptake; even after 17 h no more than

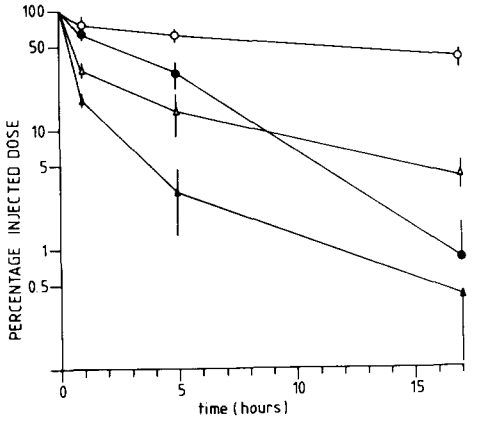

Fig. 1. Elimination of neutral and negatively charged small unilamellar vesicles from blood. Rats were injected with neutral small unilamellar vesicles containing [^{3}H]inulin and composed of cholesterol and sphingomyelin in an equimolar ratio (○), cholesterol, sphingomyelin and phosphatidylserine in a molar ratio of 5:4:1 (●) or cholesterol and phosphatidylcholine in an equimolar ratio (△) or cholesterol, phosphatidylcholine and phosphatidylserine in a molar ratio of 5:4:1 (▲). Liposomes were injected in a dose of 0.10 mmol of total lipid per kg body weight. The amount of radioactivity in blood was plotted against time. Points represent means of 6–8 determinations with standard errors.

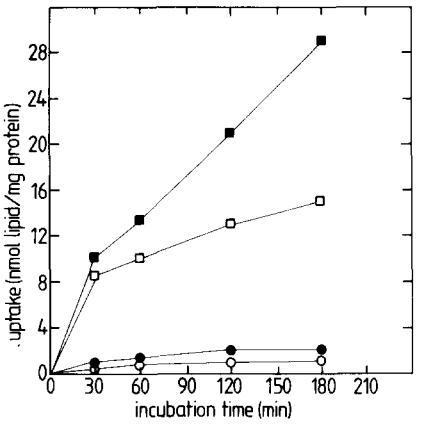

Fig. 2. Effect of lipid composition on the uptake of cholesteryl [^{14}C]oleate labeled SUV by cultured Kupffer cells. SUV of the indicated lipid composition (70 nmol lipid) labeled with cholesteryl [^{14}C-]oleate were incubated with $1.9 \cdot 10^6$ Kupffer cells in maintenance culture for up to 180 min in the absence of serum. At the end of the incubation period the cells were washed six times with cold phosphate-buffered saline (PBS). Uptake of liposomes was calculated from the ^{14}C-label and expressed as nmol of total liposomal lipid per mg cell protein. Cholesterol/sphingomyelin = 1:1 (○); cholesterol/egg phosphatidylcholine = 1:1 (●); cholesterol/sphingomyelin/phosphatidylserine = 5:4:1 (□); cholesterol/egg-phospatidylcholine/phosphatidylserine = 5:4:1 (■).

17% of the injected dose is retained in the liver, while at that time as much as 38% of the injected dose still is in circulation. At both $5\frac{1}{2}$ and 17 h more than eighty percent of the total liver uptake was accounted for by the hepatocytes. The strong preference of the sphingomyelin/cholesterol vesicles for hepatocytes is not merely a reflection of their slow rate of clearance since the phosphatidylcholine/cholesterol vesicles, which are cleared several-fold faster, show an even higher preference for the hepatocytes; more than 95% of the total liver-associated liposomal radioactivity was recovered in this cell type. Incorporation of phosphatidylserine into sphingomyelin-containing liposomes clearly enhanced liver uptake and caused a shift in intrahepatic distribution in favor of the non-parenchymal cells. Incorporation of phosphatidylserine into the phosphatidylcholine-containing vesicles did not further enhance liver uptake to an appreciable extent. The small increase that could be observed, however, appeared to be accounted for entirely by the non-parenchymal cells. For both lipid compositions splenic uptake was significantly increased as a result of phosphatidylserine incorporation.

Table I shows that splenic uptake of liposomes parallels uptake by non-parenchymal liver cells. This suggests that the rate of blood clearance of these liposomes is a reflection of their affinity for tissue macrophages in general. To test this, we incubated the four liposome types used in the in vivo studies with isolated Kupffer cells in maintenance culture (Fig. 2). Both the phosphatidylcholine/cholesterol and the sphingomyelin/cholesterol vesicles were taken up slowly by these cells, the latter type being the slower of the two. Incorporation of phosphatidylserine into either type of vesicle resulted in a 15-fold increased uptake of liposomes. Although these observations are in agreement with the in vivo results on blood clearance (Fig. 1) the correlation with the results on intrahepatic distribution (Table I) is less obvious. Apparently, conferment of negative charge on the liposomes not only affects the affinity for liver and spleen macrophages, but may enhance interaction with cells such as hepatocytes as well. The relative extents of the phosphatidylserine effect on

TABLE I

SPLEEN AND LIVER UPTAKE AND INTRAHEPATIC DISTRIBUTION OF NEUTRAL AND NEGATIVELY CHARGED SMALL UNILAMELLAR VESICLES

[^{3}H]Inulin-labeled liposomes of indicated compositions were injected intravenously and at $5\frac{1}{2}$ and 17 h the amount of radioactivity in blood, spleen and liver were determined as described in Methods. Total uptake by parenchymal (P) and non-parenchymal (NP) cells was determined by assuming the presence of $450 \cdot 10^7$ hepatocytes and $194 \cdot 10^7$ non-parenchymal cells per kg body weight [7]. Values are presented as means with standard errors of 3–6 determinations. In the last two columns the relative contributions of each cell fraction to total liver uptake are given, taking the sum of the two fractions in each experiment at 100%.

Vesicle composition	Time (h)	Radioactivity (% of injected dose)					nmol/10^6 cells		Normalized percentage contribution	
		Blood	Spleen	Total liver	P cells	NP cells	P cells	NP cells	P cells	NP cells
Chol/SM(1:1)	$5\frac{1}{2}$	62.9 ± 2.3	1.7 ± 0.6	6.3 ± 2.3	5.4 ± 1.9	1.2 ± 0.7	1.2	0.6	81.8	18.2
	17	37.8 ± 6.5	2.7 ± 0.5	16.8 ± 5.3	18.0 ± 7.3	2.5 ± 1.1	4.0	1.3	87.8	12.2
Chol/SM/PS(5:4:1)	$5\frac{1}{2}$	29.6 ± 7.5	7.6 ± 1.3	28.4 ± 8.6	20.7 ± 5.1	7.8 ± 2.0	4.6	4.0	72.6	27.4
	17	0.8 ± 0.8	9.4 ± 1.5	46.2 ± 4.6	24.7 ± 0.8	20.3 ± 0.1	5.5	10.5	54.9	45.1
Chol/PC(1:1)	$5\frac{1}{2}$	14.0 ± 5.5	1.1 ± 0.5	45.4 ± 7.0	54.1 ± 5.6	2.3 ± 1.1	12.0	1.2	95.9	4.1
	17	4.2 ± 1.1	1.1 ± 0.5	66.0 ± 7.8	78.1 ± 1.2	2.4 ± 1.3	17.4	1.2	97.0	3.0
Chol/PC/PS(5:4:1)	$5\frac{1}{2}$	3.0 ± 1.7	4.4 ± 1.3	54.5 ± 3.0	57.3 ± 5.7	8.4 ± 3.0	12.7	4.3	87.2	12.8
	17	0.6 ± 0.2	4.5 ± 1.2	64.3 ± 6.9	71.7 ± 4.6	7.1 ± 0.9	15.9	3.7	91.0	9.0

TABLE II

LABEL DISTRIBUTION AMONG NON-PARENCHYMAL LIVER CELLS FOLLOWING INJECTION OF SMALL SPHINGOMYELIN/CHOLESTEROL LIPOSOMES

Rats were injected with small unilamellar sphingomyelin/cholesterol (1 : 1) liposomes labeled with encapsulated [^{3}H]inulin; injected dose, 0.10 mmol total lipid per kg body weight. 45 h after injection the liver was excised and processed for isolation of non-parenchymal cells. Radioactivity was measured in blood, whole liver cell suspension, total non-parenchymal cells, isolated Kupffer cells and endothelial cells. Results are expressed as % of injected dose ± S.D. ($n = 3$). Numbers in parentheses represent uptake in % of injected dose per 10^9 cells.

Blood	Liver	Non-parenchymal cells	Kupffer cells	Endothelial cells
4.8 ± 2.0	45.1 ± 4.4	5.1 ± 2.3 (10.0 ± 2.2)	3.6 ± 1.6 (37.0 ± 13)	0.30 ± 0.11 (1.3 ± 0.5)

the various cell types seem to be different for the sphingomyelin and the phosphatidylcholine liposomes and are apparently also dose-dependent. We were intrigued by the observation that a major fraction of the slowly clearing sphingomyelin/cholesterol liposomes does not become liver-associated. After 48 h, when less than 5% of the injected dose remains in circulation, no more than 45% of the injected dose is recovered from the liver (Table II). The extremely slow rate of macrophage uptake, both in vivo and in vitro, made us wonder whether uptake of these liposomes might be achieved by fluid-phase pinocytosis rather than by an adsorptive endocytic mechanism. If this were true, the fraction of the dose not accounted for by blood, liver and spleen could perhaps be ascribed to diffuse pinocytotic uptake by endothelial cells possibly including those lining the vascular system. To check this possibility we measured uptake of liposomal label by liver endothelial cells which we were able to obtain from total non-parenchymal liver cells by elutriation centrifugation. Table II shows, in line with our previous observations on differently composed liposomes [2,8] that these cells account for only 0.3% of the injected dose as compared to 3.6% for the Kupffer cells. When we express the uptake as percent of injected dose per 10^9 cells, thus taking into account the excess of endothelial cells over Kupffer cells in the liver, endothelial cell uptake is even further reduced as compared to Kupffer cell uptake (1.3% vs. 37.0%). Even this small amount vanishes completely when we take into account the 4–7% contamination of Kupffer cells in the endothelial cell fraction (not shown). The virtually complete lack of involvement of the endothelial cells in the hepatic component of blood clearance indicates that, also for these liposomes, fluid-phase pinocytosis does not play a role.

Discussion

Macrophages generally have been held responsible for accumulation of intravenously injected liposomes [9]. For liver, it has been shown that also the parenchymal cells, or hepatocytes, may take up substantial amounts of liposomes from the blood circulation, provided that the liposomes are of sufficiently small size to pass through the endothelial fenestrations [10]. In two previous studies [2,3] we found that incorporation of 10 mol% phosphatidylserine into liposomes composed of equimolar amounts of sphingomyelin and cholesterol increased the rate of both blood elimination and liver uptake, without greatly affecting the intrahepatic distribution [2]. In those studies we applied relatively small liposome doses of 20 μmol of total lipid per kg body weight. In the present study, in which we used a 5-fold higher dose, a comparable increase in blood elimination and liver uptake was observed as a result of phosphatidylserine incorporation, but the intrahepatic distribution was clearly changed in favor of the non-parenchymal cells. The extremely low elimination rate of the sphingomyelin/cholesterol vesicles, which is in agreement with previous findings by others [11,12], was virtually identical to the rate we previously found for a 5-fold lower dose [2]; this suggested to us that these neutral liposomes might be cleared from the blood by fluid-phase pinocytosis by the liver cells and, for that matter, by other cells accessible from the circulation such as vascular endothelial cells. However, the minor contribution of the non-parenchymal cells to total hepatic liposome uptake (Table I) is far too low to account for these cells' pinocytotic capacity [13]. In addition, further fractionation of the non-parenchymal cells into a Kupffer cell fraction and an endothelial cell fraction revealed the complete

lack of participation of the latter in liposome uptake, while this cell type, because of its preponderance in the non-parenchymal cells [14], accounts for some 75% of the total non-parenchymal pinocytotic activity in the liver. By extrapolation, these observations would suggest that other vascular endothelial cells are not likely to participate in liposome uptake either.

Incorporation of phosphatidylserine into the liposomes enhances their affinity for the liver cells involved in liposome uptake; differences in uptake capacity between the two cell types may be responsible for the observed phosphatidylserine-induced shift in intrahepatic liposome distribution. Possibly, the high liposome dose saturates the hepatocyte uptake mechanism, thus shifting the uptake in favor of the non-parenchymal cells, whereas the low dose [2] does not saturate the hepatocyte's uptake capacity for these liposomes.

It seems unlikely that the different behaviour of the four liposome types is related to differences in size; the small variation in encapsulated volume between the different preparations allows for a variation in diameter of less than 3%. It may seem remarkable that the cell surface apparently is able to discriminate between a phosphatidylcholine/cholesterol surface and a sphingomyelin/cholesterol surface, which, in a chemical sense, must be very similar. The difference between the two liposomal surfaces rather is likely to be of a physico-chemical nature. Due to the stronger interactions between sphingomyelin and cholesterol as compared to those between phosphatidylcholine and cholesterol [15], the former composition will give rise to a more tightly packed bilayer. It is conceivable that the first step in liposome–cell interaction leading to uptake is penetration of (a) cell-surface protein(s) into the liposomal bilayer and that such penetration is prevented or impeded in case of a very tightly packed bilayer. It is worth noting in this connection, that in the in vitro Kupffer cell system we found that liposome uptake by these cells is protease sensitive; brief treatment of the cells with pronase or trypsin destroyed the cells' capacity to bind and internalize liposomes [16]. Gregoriadis and his associates [11,17] reported a correlation between the rate of liposome clearance from the blood and the rate of plasma-induced solute release. These observations also point to a low susceptibility of slowly-clearing liposomes to penetration by (plasma lipo-) proteins. Also the observations by Patel et al. [18] on the effect of liposomal cholesterol content on blood clearance and liver and spleen uptake of liposomes may find their explanation in a cholesterol-induced impediment of cell-surface protein penetration into the liposomal bilayer.

From our experiments it appears that specific uptake of liposomes by hepatocytes is best achieved with (small) liposomes composed of phosphatidylcholine/cholesterol. Not only do these vesicles display the most extreme distribution in favor of the hepatocytes, they also are cleared at sufficiently high rates to allow accumulation of large amounts of liposomes in these cells. It should be noted that of the sphingomyelin/cholesterol vesicles, which also show a high preference for hepatocytes vs. non-hepatocytes, less than 30% of the amount eliminated from the blood accumulates in the liver after 17 h, as compared to almost 70% of the phosphatidylcholine/cholesterol vesicles.

References

1 Spanjer, H.H. and Scherphof, G.L. (1983) Biochim. Biophys. Acta 734, 40–47
2 Spanjer, H.H., Morselt, H. and Scherphof, G.L. (1984) Biochim. Biophys. Acta 774, 49–55
3 Spanjer, H.H., Van Berkel, T.J.C., Scherphof, G.L. and Kempen, H.J.M. (1985) Biochim. Biophys. Acta 816, 396–402
4 Ashwell, G. and Morell, A.G. (1974) Adv. Enzymol. 41, 99–128
5 Kolb-Bachofen, V., Schlepper-Schäfer, J., Vogell, W. and Kolb, H. (1982) Cell 29, 859–866
6 Dijkstra, J., Van Galen, W.J.M., Hulstaert, C.E., Kalicharan, D., Roerdink, F.H. and Scherphof, G.L. (1984) Exp. Cell Res. 150, 161–176
7 Kooistra, T., Duursma, A.M., Bouma, J.W.M. and Gruber, M. (1979) Biochim. Biophys. Acta 587, 282–298
8 Roerdink, F., Regts, J., Van Leeuwen, B. and Scherphof, G. (1984) Biochim. Biophys. Acta 770, 195–202
9 Poste, G. (1983) Biol. Cell 47, 19–38
10 Wisse, E., De Zanger, R. and Jacobs, R. (1982) in Sinusoidal Liver cells (Knook, D.L. and Wisse, E., eds.), pp. 61–67, Elsevier Biomedical Press, Amsterdam
11 Gregoriadis, G. and Senior, J. (1980) FEBS Lett. 119, 43–46
12 Huang, K., Luk, K-F.S. and Baumier, P.L. (1980) Proc. Natl. Acad. Sci. USA 77, 4030–4034
13 Munniksma, J., Noteborn, M., Brouwer, A., Praaning-van Dalen, D. and Knook, D.L. (1980) Biochem. J. 192, 613–621

14 Knook, D.L. and Sleyster, E.C. (1976) Exp. Cell Res. 99, 444–449
15 Schmidt, C.F., Barenholz, Y. and Thompson, T.E. (1977) Biochemistry 16, 2649–2656
16 Dijkstra, J., Van Galen, M. and Scherphof, G. (1985) Biochim. Biophys. Acta 813, 287–297
17 Senior, J., Gregoriadis, G. and Mitropoulos, K.A. (1983) Biochim. Biophys. Acta 760, 111–118
18 Patel, H.M., Tüzel, N.S. and Ryman, B.E. (1983) Biochim. Biophys. Acta 761, 142–151