Biochimica et Biophysica Acta, 734 (1983) 40–47
Elsevier

BBA 71827

TARGETING OF LACTOSYLCERAMIDE-CONTAINING LIPOSOMES TO HEPATOCYTES IN VIVO

HALBE H. SPANJER and GERRIT L. SCHERPHOF

Laboratory of Physiological Chemistry, University of Groningen, Bloemsingel 10, 9712 KZ Groningen (The Netherlands)

(Received April 8th, 1983)

Key words: Liposome targeting; Phospholipid; Glycolipid; Galactosyl receptor; Hepatocyte; (Rat liver)

Incorporation of 8 mol% lactosylceramide in small unilamellar vesicles consisting of cholesterol, dimyristoylphosphatidylcholine and phosphatidylserine in a molar ratio of 5:4:1 and containing [^{3}H]inulin as an aqueous-space marker resulted in a 3-fold decreased half-life of the vesicles in blood and a corresponding increase in liver uptake after intracardial injection into rats. The increase in liver uptake was mostly accounted for by an enhanced uptake in the parenchymal cells, while the uptake by the non-parenchymal cells was only slightly increased. The uptake of both the control and the glycolipid-containing vesicles by the non-parenchymal cell fraction could be attributed completely to the Kupffer cells; no radioactivity was found in the endothelial cells. The effect of lactosylceramide on liver uptake and blood disappearance of the liposomes was effectively counteracted by desialylated fetuin, injected shortly before the liposome dose. This observation supports the notion that a galactose-specific receptor is involved in the liver uptake of lactosylceramide liposomes.

Introduction

The liver is one of the few organs in the body in which the parenchymal cells are directly accessible from the blood compartment because of the lack of a continuous basement membrane and because of the presence of a fenestrated endothelial lining [1]. For that reason, the parenchymal cells of the liver, i.e., the hepatocytes, are exceptionally suitable target cells for a drug-delivery system such as liposomes. After intravenous administration, these relatively large particles would, otherwise, have unlimited access only to blood cells, endothelial cells of the vascular system and macrophages in some tissues [2]. Liposomes may vary in size from approx. 0.25 nm to several μm, and we have shown that size to some extent determines their precise intrahepatic disposition after intravenous injection [3]. Large-sized liposomes are preferentially taken up by the liver macrophages, i.e., the Kuppfer cells, while small liposomes also distribute to very appreciable extents to the hepatocytes, presumably because they have easier access to these cells through the endothelial fenestrations measuring an average diameter of 0.1 μm [1]. Nonetheless, a considerable proportion of an intravenous dose of small unilamellar vesicles still finds its way to the Kupffer cells. In an attempt to increase the fraction of vesicles taken up by the hepatocytes, we incorporated a terminal non-reducing β-galactose glycolipid in the liposomal membrane in order to confer an increased affinity for these cells upon the liposomes. Hepatocytes carry on their surface a specific receptor for this sugar residue, which is able to effectuate highly specific endocytosis of glycoproteins exposing such a galactosyl group [4]. Ghosh et al. [5] recently reported results of experiments which were designed to serve the same purpose. They used, however, an ill-defined liposome preparation with low

cholesterol content which may provide insufficient protection against plasma influences [6] and a biodegradable marker of the aqueous space (^{125}I-labeled γ-globulin) which may lead to an underestimation of cellular uptake due to intracellular degradation and release of label from the cells.

Therefore, we applied, in a similar study, preparations of liposomes of fairly homogeneous size, containing a high cholesterol level and a metabolically inert label. At the same time, the vesicles were small enough to experience minimal hindrance in their access to the hepatocytes. We measured blood clearance, liver uptake and intrahepatic distribution in rats of such glycolipid-containing liposomes comparative to control liposomes. While this work was in progress, Szoka and Mayhew reported on the effect of incorporation of lactosylceramide or mannosyldiacylglycerol on blood clearance and organ distribution of small unilamellar liposomes in mice [7].

Materials

Cholesterol, dimyristoyl-L-α-phosphatidylcholine, L-α-phosphatidyl-L-serine, *N*-lignoceroyldihydrolactocerebroside, collagenase type I, fetuin type IV, agarose bound neuraminidase type VI A and dog alkaline phosphatase type X were obtained from Sigma. [^{3}H]Inulin (904 mCi/mmol) was obtained from Amersham International, U.K. Sepharose-Cl-2B was purchased from Pharmacia. Diaflo ultrafiltration membranes PM 10 were obtained from Amicon.

Methods

Preparation of liposomes

Appropriate volumes of lipid stock solutions in chloroform and methanol were mixed to a molecular ratio of cholesterol : dimyristoylphosphatidylcholine : phosphatidylserine(: lactosylceramide) = 5 : 4 : 1(: 0.8). The solvent was removed under a stream of nitrogen. The residue was dissolved in 1–2 ml benzene and lyophilized. The dry lipid was dispersed in 1 ml Tris/NaCl/EDTA (10 mM Tris-HCl/150 mM NaCl/1 mM Na_2EDTA) (pH 7.4) buffer and inulin (1 mM; 100 μCi) and the dispersion was sonicated for 2 h at 20–30°C in a Branson bath sonifier. Larger vesicles and free inulin were separated from the small unilamellar vesicles by gel filtration on Sepharose-Cl-2B with Tris/NaCl/EDTA (pH 7.4) as elution buffer. The small-unilamellar-vesicle-containing fractions were concentrated by ultrafiltration through Amicon PM 10 membranes. Phosphorus in the concentrate was determined according to the method of Böttcher et al. [8] and was used to calculate total lipid content. From phosphorus and radioactivity contents of the concentrate, we calculated an internal volume of the small unilamellar vesicles of 0.36 ± 0.06 μl/μmol ($n = 10$) for control vesicles and 0.34 ± 0.03 μl/μmol ($n = 5$) for lactosylceramide-containing small unilamellar vesicles, corresponding to a mean diameter of 28.8 nm and 27.9 nm, respectively [9].

Animals

Male Wistar-random rats were used varying in weight from 160–260 g. Heart catheters for sampling blood and infusion of the liposomes in conscious animals were built essentially according to the method of Steffens [10] with small modifications. Briefly, a poly(vinyl chloride) catheter was placed in the vena jugularis, passed subcutaneously to the head and fixed to the skull with dental cement. After restraining for at least 3 days the animals were brought under light diethyl ether anesthesia and connected to an injection and sampling tube and received a single dose of 50 U heparin per 100 g body weight. After recovery for at least 0.5 h, the animals were injected intracardially with liposomes at a dose of 2 μmol total lipid per 100 g body weight in a volume not exceeding 0.5 ml. The sampling tube was cleaned by injection of 0.2 ml isotonic saline. Blood samples were taken as follows: at the appropriate time point about 0.1 ml blood was slowly drawn into an Omnifix tuberkuline syringe fitted with a 2G 21 × $1\frac{1}{2}$ inch needle at the end of the sampling tube. Syringe and needle were replaced by empty ones and another 0.1 ml blood was collected. This sample was used for determination of radioactivity. To clean the sampling tube, 0.2 ml saline was reinjected after each blood sample taken. 1 or 3 h after the injection of the liposomes, the animals were brought under diethyl ether anesthesia and the livers were perfused.

Total liver uptake

For the determination of the total liver uptake of the injected liposomes, the liver was perfused in situ via the portal vein for 2 min at 30–40 ml/min with isotonic saline at 37°C to remove blood. Livers were excised and homogenized in a Potter-Elvehjem tube in saline and samples were taken in triplicate for radioactivity measurements.

Hepatocyte uptake

Hepatocytes were isolated according to a combination of the methods of Berry and Friend [11] and Seglen [12] with some slight modifications. The isolation procedure covered a preperfusion in situ with 200 ml preperfusion buffer (142 mM NaCl/6.7 mM KCl/ 10 mM Hepes (pH 7.4)) with 1 mM EGTA followed by a preperfusion with an equal volume of preperfusion buffer without EGTA. The total preperfusion lasted for about 10 min and was followed by a recirculatory perfusion with collagenase buffer (66.7 mM NaCl/6.7 mM KCl/100 mM Hepes/5 mM $CaCl_2$/50 mg collagenase (in 100 ml buffer) (pH 7.6)). At the end of the perfusion, which lasted for maximally 10 min, livers were excised and Glisson's capsule was removed while the liver was kept in 150 ml buffer (118 mM NaCl/4.7 mM KCl/1 mM KH_2PO_4/1 mM $MgSO_4$/2.5 mM $CaCl_2$/5 mM glucose/10 mM Hepes (pH 7.4)). The liver cells were suspended in this buffer and were separated from remaining liver pieces by filtration over nylon gauze with pores of 250 μm. The residue was homogenized and counted for radioactivity.

The cell suspension was gently shaken at 37°C for 10–15 min to allow injured cells to disintegrate further. The cell suspension was filtered over a double filter of nylon gauze with pores of 250 μm (above) and 100 μm (below). Hepatocytes in the filtrate were separated from non-parenchymal cells by repeated low-speed centrifugations. Cell number and viability as estimated by Trypan blue exclusion were determined by microscopic examination in a Bürker cell counter. Viabilities were $79.0 \pm 5.2\%$ ($n = 10$) and cell recovery was usually about 40%. Samples from the final hepatocyte suspension were taken in triplicate and counted for radioactivity.

Non-parenchymal cell uptake

Isolation of the non-parenchymal cell fraction and the separation into Kupffer cell and endothelial cell fractions were as described before [13]. Samples of blood, liver homogenates or liver cell suspensions were mixed with an equal volume of 30% H_2O_2 and incubated for 1h at 60°C to decolorize. After addition of plasmasol [14], samples were counted for radioactivity.

Inhibition experiments

Fetuin was desialylated by treatment with agarose-bound neuraminidase; 100 mg fetuin in 20 ml 25 mM acetate buffer (pH 5.0) was incubated for 2 h at 37°C with 2 U agarose-bound neuraminidase. Enzyme was recovered by repeated low-speed centrifugation and the supernatants were dialyzed against distilled water and lyophilized. Successful removal of sialic acid residues was monitored by checking the inhibitory potency of the desialylated fetuin in vivo by measuring its effect on blood clearance of dog alkaline phosphatase [15]. In competition experiments with lactosylceramide-containing (small unilamellar vesicles), asialofetuin was injected at a dose of 10 mg/100 g body weight 1 min prior to injection of the liposomes.

Calculations

Estimates of hepatocyte and non-parenchymal cell uptake were made assuming $450 \cdot 10^6$ hepatocytes and $194 \cdot 10^6$ non-parenchymal cells per 100 g body wt. [16]. Blood volume was taken as 6.5 ml per 100 g body weight. Cell uptake values and blood content values are expressed as percentage of injected dose. A check on total liver uptake of livers from which parenchymal cells or non-parenchymal cells were isolated was made by measuring the recoveries in the first filtrate and the residue. Summation of these recoveries was taken as an estimate for total liver uptake which showed good agreement with the independently determined total liver uptake values.

Results

The half-life of ^{3}H-label in blood of rats injected with a relatively small dose (2 μmol total lipid per 100 g rat) of small unilamellar vesicles consisting of cholesterol, dimyristoylphosphatidylcholine and phosphatidylserine, molar ratio

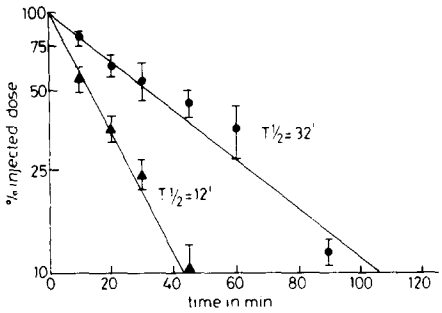

Fig. 1. Elimination of control and lactosylceramide-containing vesicles from blood. Rats were injected intracardially with liposomes (2 μmol of total lipid per 100 g body weight; for composition see Methods). Blood samples were taken and processed as described in Materials and in Methods. Points represent the mean blood content of at least six determinations; bars indicate the standard deviations. Control vesicles, ●———●; lactosylceramide vesicles, ▲———▲.

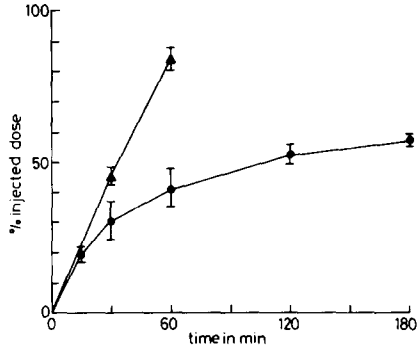

Fig. 2. Liver uptake of control and lactosylceramide-containing vesicles. At times indicated, livers were perfused and processed as described in Materials and in Methods. Points represent the mean liver uptake of at least three animals; bars indicate the standard deviations. Control vesicles, ●———●; lactosylceramide vesicles, ▲———▲.

5 : 4 : 1, and containing [^{3}H]inulin in the encapsulated volume, was slightly longer than 0.5 h (Fig. 1). Incorporation of 8 mol% lactosylceramide into these vesicles resulted in a very substantial decrease in half-life, to approx. 12 min. Accordingly, the hepatic uptake of label during the first hour after injection increased 2-fold as a result of lactosylceramide incorporation, from an average of 41% of the injected dose for the control vesicles to an average of 84% for the glycolipid vesicles (Fig. 2). Even by the time virtually all control vesicles had been cleared from the blood (3 h), representing a condition comparable to the 60-min situation with the lactosylceramide vesicles, liver uptake amounted to no more than 57%, i.e., well below the 84% reached with the glycolipid vesicles. A considerable fraction of the vesicles apparently accumulates extrahepatically, part of it in the spleen where we recovered 8.2 ± 1.8% of the injected dose in nine animals 3 h after injection. Of the lactosylceramide vesicles, 3.0 ± 0.8% ($n = 6$) was found in the spleen by the time the blood compartment was cleared of liposomes (1 h). For both liposomal preparations, 2–3% of the dose was recovered in the bladder, representing label that had been released from the liposomes. It is remarkable that in the 30 min following the time at which only some 25% of the injected liposome dose is left in circulation, i.e., 0.5 h after injection, liver uptake continues to increase from 45 to 85%. This suggests the existence of a compartment where (some of) the liposomes transiently reside after leaving the circulation and before being taken up by the liver. Possibly, this compartment represents the walls of the vascular bed, including that of the liver, to which liposomes may reversibly adsorb.

In view of the well-established presence of a galactose receptor on the cell surface of hepatocytes [4] one would assume that lactosylceramide effects of the kind described in the foregoing have to be ascribed to a specific interaction between the liganded liposomes and this receptor. Recently, however, the presence of a similar receptor has been reported on the surface of the Kupffer cells [17]. A direct demonstration of the intrahepatic disposition of the injected liposomes would, therefore, be required to show whether or not it is justified to attribute the observed effects to the hepatocyte receptor. Table I shows the results of experiments in which we isolated parenchymal and non-parenchymal cell fractions from rats injected with control or lactosylceramide vesicles. 3 h after injection of control vesicles, i.e., when the blood compartment is completely cleared of liposomes, the hepatocytes and the non-parenchymal cells contain 38% and 23% of the injected dose, respectively. Uptake is slightly in favor of the parenchymal cells. When we compare this to the condition of complete blood elimination for the lactosylceramide-containing vesicles, i.e., 1 h after in-

TABLE I

INTRAHEPATIC CELLULAR DISTRIBUTION OF CONTROL AND LACTOSYLCERAMIDE-CONTAINING VESICLES

1 or 3 h after injection of control or lactosylceramide vesicles (2 μmol lipid per 100 g body weight; for composition see Materials and Methods), whole liver, parenchymal cells or non-parenchymal cells, isolated as described in Materials and in Methods, were analyzed for radioactivity content. Uptake is expressed as percentage of injected dose recovered in total liver and total cell fractions, assuming $450 \cdot 10^6$ parenchymal cells and $194 \cdot 10^6$ non-parenchymal cells per 100 g of rat [16]. LC, lactosylceramide; SUV, small unilamellar vesicles.

Liposomes	Time after injection (h)	Liposome uptake in:		
		Total liver	Parenchymal cells	Non-parenchymal cells
Control-SUV	1	41.2 ± 6.3 ($n = 4$)	20.3 ± 3.0 ($n = 3$)	19.5 ± 3.9 ($n = 3$)
Control-SUV	3	57.0 ± 1.9 ($n = 4$)	38.1 ± 5.1 ($n = 3$)	23.5 ± 4.3 ($n = 3$)
LC-SUV	1	84.7 ± 3.7 ($n = 6$)	48.0 ± 2.7 ($n = 4$)	27.0 ± 5.8 ($n = 3$)

jection, almost 50% of the injected dose is recovered in the parenchymal cells and nearly half that amount (27%) in the non-parenchymal cells, a 1.8-fold difference.

The capacity of the lactosylceramide to shift liposome uptake from non-parenchymal to parenchymal cells becomes more prominent when we compare two identical time points, e.g., 1 h after injection for both vesicle types. Uptake by the parenchymal cells is increased by a factor of 2.4 (20.3% vs. 48%) as a result of lactosylceramide incorporation and uptake by the non-parenchymal cells by a factor of 1.4 (19.5% vs. 27%). The latter, however, is barely significant).

Our tentative conclusion is that the lactosylceramide-induced increase in blood elimination rate and hepatic accumulation of liposomes is mainly, if not solely, accounted for by increased uptake by the hepatocytes. If the non-parenchymal cells contribute at all, it is to only a minor extent.

In two separate experiments, we further fractionated the non-parenchymal cell fraction into Kupffer cells and endothelial cells. Both for control vesicles and for lactosylceramide vesicles, all radioactivity was associated with the Kupffer cell fraction and none with the endothelial cells (results not shown). This is compatible with what we found for large liposomes [13]. Additional evidence of the involvement of the galactose receptor in the enhanced uptake of lactosylceramide vesicles was obtained from experiments in which a galactose-exposing glycoprotein, asialofetuin, was allowed to compete with the vesicles for the receptor. Fig. 3 shows, in more detail than Fig. 1, the blood elimination curves of control vesicles and glycolipid vesicles during the first hour after injec-

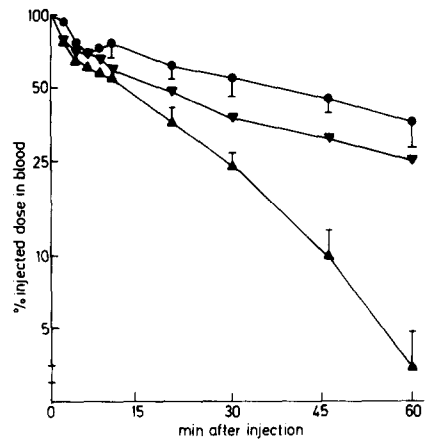

Fig. 3. Elimination of lactosylceramide vesicles from blood: effects of asialofetuin. Rats were injected intracardially with [^{3}H]inulin-containing liposomes (2 μmol total lipid per 100 g body weight). Radioactivities were measured in blood as described for the experiments of Figs. 1 and 2, respectively. Points represent means for at least two determinations and bars, where presented, indicate the standard deviations. ●——●, control vesicles; ▲——▲ lactosylceramide vesicles; ▼——▼, lactosylceramide vesicles injected into animals which had received 10 mg of asialofetuin per 100 g body weight intracardially 1 min prior to administration of the vesicles.

TABLE II

UPTAKE OF LACTOSYLCERAMIDE-CONTAINING VESICLES BY LIVER: INHIBITION BY ASIALOFETUIN

A number of animals used for the experiments described in Fig. 3 were used to measure radioactivity uptake by total liver at 15, 30 and 60 min after injection of the vesicles. Numbers given represent percentage of injected dose ± S.D. For numbers based on two rats only, no standard deviation is given.

Time after injection (min)	Control vesicles	Lactosylceramide vesicles	Lactosylceramide vesicles plus asialofetuin
15	19.4 ± 2.5	23.4	16.7
30	30.6 ± 6.3	45.3 ± 2.9	30.5
60	41.2 ± 6.2	84.7 ± 3.7	42.9

tion. The difference in elimination rates is, again, obvious. In addition, we see that the presence of the asialofetuin shifts the position of the elimination curve of the lactosylceramide vesicles almost back to that of the control vesicles. Apparently, the asialoglycoprotein effectively neutralizes the lactocylceramide-induced increase in elimination rate. The effect of asialofetuin is considered to be specific because the injection of untreated fetuin produced a blood clearance curve essentially similar to that of the control experiment. The effect of the asialoglycoprotein also becomes apparent upon determination of the hepatic radioactivity content Table II. The increment in liver uptake resulting from the glycolipid incorporation is almost completely annihilated when, prior to the liposomes, asialofetuin is injected.

Discussion

Targeting to specific tissues or cells has been a long-cherished goal for the therapeutic use of liposomes [18]. By modifying the liposomal surface with ligands recognizing specific cells, several investigators [7,19,20] have tried to influence tissue distribution of in vivo administered liposomes. As pointed out by Poste et al. [2] the failure of liposomes to cross the continuous endothelial barriers constituting the vascular system is a major obstacle for the widespread use of liposomes as a drug carrier system. Only in liver and spleen are the blood capillaries discontinuous, thus allowing direct access to the liver parenchymal or spleen cells, respectively, from the circulation.

For that reason the hepatocytes constitute one of the few extracapillary cell populations suitable for targeting of liposomes, as the fenestrations in the endothelial cells are of a diameter sufficient to allow passage of liposomes within a limited size range. The mean diameter of the fenestrations has been estimated at 106 nm, with a maximum of 200–300 nm [1]. Although larger pores or gaps with a diameter of more than 250 nm in the endothelial cell lining have been reported [21], their relative frequency is low and it is believed that, in general, particles with a diameter of more than 0.3 μm cannot directly reach the hepatocytes. Multilamellar vesicles in preparations have a size distribution ranging from 0.1 to 3 μm in diameter [22]. Since a considerable fraction of the multilamellar vesicles have a diameter of more than 0.3 μm and are very rapidly cleared from the circulation by the Kupffer cells [3], this type of liposome would not seem the most suitable for targeting towards hepatocytes. Nonetheless, two groups have reported targeting of multilamellar vesicle preparations to hepatocytes in vivo. In those studies, galactose residues were anchored on or in the surface of the liposomes to allow specific uptake via the galactose receptor [19,20]. However, since no cell separations were done by those investigators, the increase in total liver uptake resulting from the galactose-residue incorporation may not necessarily represent an increase in hepatocyte uptake. Kolb-Bachofen et al. [17] have reported on a galactose-specific lectin on Kupffer cells mediating uptake of galactose-residue-bearing particles. Interactions with such a receptor might have in-

fluenced the liver uptake of multilamellar vesicles exposing the galactose residue.

In our experiments we used small unilamellar vesicles, which have optimal access to the hepatocytes, and found, upon isolation of cell fractions, a clear-cut stimulation of hepatocyte uptake as a result of glycolipid incorporation and, perhaps, a slightly enhanced uptake by Kupffer cells. The increased uptake by hepatocytes is in agreement with, at least, the conclusions of Ghosh et al. [5]. These investigators used multilamellar vesicles and may have underestimated the uptake by their non-parenchymal cell fraction, because they used the readily degradable ^{125}I-labeled γ-globulin as a marker, while it is not clear from their paper how long after injection the cells were isolated and assayed for radioactivity. The use of inulin in our experiments as a liposomal marker has the advantage that it is biologically inert and in its free form it is not taken up by the liver or spleen but is quickly excreted via the kidneys. In line with our previous observations on multilamellar vesicles [13] endothelial cells failed to take up small unilamellar vesicles, irrespective of the presence of the galactolipid in the liposomal membrane. With the data available it is impossible to be specific on the way in which the lactosylated liposomes are taken up by the Kupffer cells. Although the level of uptake is barely higher than that of control vesicles, we cannot rule out the possibility that in the case of lactosylated liposomes Kupffer cell uptake proceeds, partly or even entirely, via the specific receptor, while control vesicles are taken up, with about equal affinity, by an aspecific mechanism.

In the experiments reported in this paper we used dimyristoylphosphatidylcholine as the main phospholipid constituent of the liposomes in order to achieve optimal exposition of the galactose residue [23]. The involvement of a galactose-recognizing structure in the uptake of these liposomes is, indeed, strongly suggested by the inhibitory effect of asialofetuin. Yet, in preliminary experiments we found that the lactosylceramide stimulates hepatic liposome uptake equally well when sphingomyelin, with its long hydrocarbon chains, forms the major phospholipid constituent of the vesicles. The relatively short chain-length of the phosphatidylcholine we used may, however, be the reason why we calculate unexpectedly small diameters for our vesicles [24]. Incorporation of cholesterol has been reported to cause substantial increases in vesicle diameter [25].

Our experiments do not allow a definitive conclusion concerning the intracellular location of the liposomes found associated with the various cell fractions. Although the possibility cannot entirely be ruled out that (a fraction of) the liposomes merely remain stuck to the cell surface, there are at least two considerations that argue against such a possibility. Firstly, for the hepatocyte isolation the livers are perfused with EGTA-containing medium, a condition that should readily lead to dissociation of liposomes bound to the receptor at the cell surface [26]. The good correlation which we found between the experimental values for total liver uptake and the values calculated by summation of the uptake by the hepatocytes and that by the non-parenchymal cells would indicate that the bulk of the total liver associated liposomes are localized intracellularly. Secondly, when [*Me*-^{14}C]choline-labeled sphingomyelin is used as the major liposomal phospholipid constituent, a substantial proportion of the ^{14}C-label rapidly accumulates in the phosphatidylcholine fraction of both the parenchymal and the non-parenchymal cells (Roerdink, F.H., et al., unpublished data). This process, involving sphingomyelin degradation and phosphatidylcholine synthesis, can be envisaged to occur only intracellularly.

Acknowledgements

We are indebted to Mr. Jan Wijbenga and Mr. Bert Dontje for excellent biotechnical assistance, to Mrs. Joke Regts, Mrs. Babs van Leeuwen and Mrs. Henriëtte Morselt for some of the non-parenchymal cell isolations and Mrs. Rinske Kuperus for typing the manuscript.

References

1 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
2 Poste, G., Bucana, C., Raz, A., Bugelski, P., Kirsh, R. and Fidler, I.J. (1982) Cancer Res. 42, 1412–1422
3 Scherphof, G., Roerdink, F., Dijkstra, J., Ellens, H., De Zanger, R. and Wisse, E. (1983) Biol. Cell 47, 47–58
4 Ashwell, G. and Morell, A.G. (1974) Adv. Enzymol. 41, 99–128

5 Ghosh, P., Das, P.K. and Bachhawat, B.K. (1982) Arch. Biochem. Biophys. 213, 266–270
6 Kirby, C., Clark, J. and Gregoriadis, G. (1980) FEBS Lett. 111, 324–328
7 Szoka, F.C., Jr. and Mayhew, E. (1983) Biochem. Biophys. Res. Commun. 110, 140–146
8 Böttcher, C.J.F., Van Gent, C.M. and Pries, C. (1961) Anal. Chim. Acta 24, 203–204.
9 Wilschut, J. (1982) in Liposome Methodology in Pharmacology and Biology (Leserman, L.D. and Barbet, J., eds.), pp. 9–24, INSERM, Marseilles
10 Steffens, A.B. (1969) Physiol. Behav. 4, 833–836
11 Berry, M.N. and Friend, D.S. (1969) J. Cell Biol. 43, 506–520
12 Seglen, P.O. (1976) Methods Cell Biol. 13, 29–93
13 Roerdink, F., Dijkstra, J., Hartman, G., Bolscher, B. and Scherphof, G. (1981) Biochim. Biophys. Acta 677, 79–89
14 Wiegman, T., Woldring, M.G. and Pratt, J.J. (1975) Clin. Chim. Acta 59, 347–365
15 Scholtens, H.B., Hardonk, M.J. and Meijer, D.K.F. (1982) Liver 2, 1–13
16 Kooistra, T., Duursma, A.M., Bouma, J.W.M. and Gruber, M. (1979) Biochim. Biophys. Acta 587, 282–298
17 Kolb-Bachofen, V., Schlepper-Schäfer, J., Vogell, W. and Kolb, H. (1982) Cell 29, 859–866
18 Gregoriadis, G. (1977) Nature 265, 407–410
19 Gregoriadis, G. and Neerunjun, E.D. (1975) Biochem. Biophys. Res. Commun. 65, 537–544.
20 Ghosh, P. and Bachhawat, B.K. (1980) Biochim. Biophys. Acta 632, 562–572.
21 De Zanger, R. and Wisse, E. (1982) in Sinusoidal Liver Cells (Knook, D.L. and Wisse, E., eds.), pp. 69–76, Elsevier Biomedical Press, Amsterdam
22 Olson, F., Hunt, C.A., Szoka, F.C., Vail, W.J. and Papahadjopoulos, D. (1979) Biochim. Biophys. Acta 557, 9–23
23 Hoekstra, D., Tomasini, R. and Scherphof, G. (1980) Biochim. Biophys. Acta 603, 336–346
24 Egerdie, S. and Singer, M. (1982) Chem. Phys. Lip. 31, 75–85
25 Forge, A., Knowles, P.F. and Marsh, D. (1978) J. Membrane Biol. 41, 249–263
26 Pricer, W.E. and Ashwell, G. (1971) J. Biol. Chem. 246, 4825–4833