Metabolic Fate of [^{14}C]Triglyceride-Entrapped Lactosylceramide-Bearing Liposomes after Intravenous Injection into Mouse

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We investigated the distribution and fate of liposomes after their intravenous injection into a mouse. Liposomes were composed of dimyristoylphosphatidylcholine, cholesterol and dicetylphosphate (7:2:1, molar ratio) with or without lactosylceramide. They were characterized as small unilamellar vesicles, approximately 100 nm in diameter, using gel-exclusion chromatography on a Sephacryl S-1000, freeze-fracture electron microscope and dynamic light scattering method. Liposomes were very stable in serum as seen by the results of leakage of the entrapped marker and electrophoresis experiments. We demonstrated that liposomes were internalized by way of an endocytotic process *via* coated vesicles detected in the electron microscope. The increase in liver uptake of lactosylceramide-bearing liposomes was mostly accounted for by enhanced uptake in the parenchymal cells, while uptake by non-parenchymal cells was only slightly increased. This observation supported the notion that a galactose-specific receptor was involved in liver uptake of lactosylceramide liposomes. The lactosylceramide-bearing liposomes were preferentially recovered in the liver and were found to first be predominantly localized in the mitochondria-lysosomal fraction. They were then decomposed by lysosomal enzymes, and the hydrolyzed components were reincorporated into membrane phospholipids in the microsomal fraction. At the same time, a rapid and reversible exchange of phosphatidylcholine between microsomes and mitochondria was demonstrated.

Keywords liposome; hepatocytes; targeting; metabolic fate

Introduction

Liposomes have received considerable attention as very useful vehicles for drug delivery systems such as antibiotics, hormones and antitumor agents. Weissman *et al.* suggested that delivery of liposomes to target organs could be attained by appropriate manipulation of the liposomal surface.[1] It is known that a specific receptor for β-galactose is present on the plasma membrane of liver parenchymal cells.[2] Receptors of the cell membrane of hepatocytes specific for terminal galactose residues of these glycoproteins were responsible for rapid endocytosis.[3] In order to target liposomes to hepatocytes, we modified the liposomal surface with asialofetuin sugar moiety or lactosylceramide. In previous *in vitro* experiments, we demonstrated that the *Abrus* lectin did not agglutinate to fluid-phase liposomes under conditions in which solid-phase liposomes were readily agglutinated.[4] Poste and Papahadjopoulos also suggested that the interaction of liposomes with the cell surface is controlled by the membrane fluidity of liposomes.[5] We prepared the liposomes targeting hepatocytes[6]; however, the uptake mechanism and metabolic fate of liposomes is not yet clear. In this communication we examined the subcellular distribution and metabolic fate of lactosylceramide bearing liposomes labeled with [^{14}C]triglyceride after intravenous injection into mice. We also investigated the nature of the interaction by discriminating between adsorption of liposomes at the cell surface and processes leading to movement of liposome contents to the cell interior.

Materials and Methods

Materials Dimyristoylphosphatidylcholine (DMPC), *N*-lignoceroyldihydrolactocerebroside (LacCer), dicetylphosphate (DCP), ferritin, *p*-nitrophenol, glucose 6-phosphate, and *p*-nitrophenylphosphate were products of Sigma Chemicals (St. Louis, MO). Cholesterol (Chol) and calcein were purchased from Nakarai Chemicals (Kyoto, Japan). Glycerol tri-[^{14}C]palmitate ([^{14}C] TG, 50 mCi/mmol), NCS® and ACS-II® were purchased from Amersham (Bucking Hamshire, England). Other chemicals were of the highest grade available.

Preparation of Liposomes Appropriate volumes of the lipid stock solutions in benzene were mixed in the ratio of DMPC : Chol : DCP : [^{14}C] TG (7 : 2 : 1 : 0.5, molar ratio) with or without 10 mol% of LacCer. The solvent was removed under a stream of nitrogen to make a thin film inside a small test tube. The lipid film was dispersed in 1 ml of phosphate buffered saline (NaCl 150 mM, KH_2PO_4 1.5 mM, $Na_2HPO_4 \cdot 7H_2O$ 8.1 mM, pH 7.4), and the dispersion was sonicated for 2 h at 45 °C in a Branson B-12 bath type sonifier at 60 W. To prepare calcein-containing or ferritin-containing liposomes, calcein (32 mM) and ferritin (2 mg) were separately added to the liposomes mentioned above. Excess free calcein and ferritin were respectively separated by chromatography on a 1 × 40 cm Sephadex G-50 column. The liposome dispersion was filtered through a membrane filter (pore size 0.22 μm (Millex GS. Millipore, Ltd., Bedford, MA), and the filtrate was further centrifuged at 100000 × *g* for 30 min. The resultant supernatant was subjected to the following experiments.

Gel Exclusion Chromatography on Sephacryl S-1000 Sizes of sonicated lipid vesicles were determined by column chromatography on a Sephacryl S-1000 column (1.6 × 65 cm) according to the method of Reynolds *et al.*[7] Polystyrene monodisperse latex (Polysciences, Warrington, PA) was used for calibration. Because polystyrene beads tend to aggregate in aqueous media, they were equilibrated in aqueous buffer containing 0.01 M sodium dodecyl sulfate. Columns were presaturated with DMPC to avoid binding of liposomes to lesin. In the experiment, several column volumes of aqueous buffer containing sonicated lipid vesicles were passed through the column until the eluant had the same phospholipid concentration as the buffer.

Particle Size Distribution Mean liposome particle size was determined by the dynamic light scattering (DLS) method (Otsuka Electronics, DLS-700), and expressed in terms of Stokes diameter.

Fluorescence Leakage Assay Efflux from the liposomes was monitored on a fluorescence spectrophotometer (MPF, Hitachi) with attached temperature control and recorder. Excitation was at 490 nm and emission at 520 nm. At the start of each experiment, 10 μl of calcein-containing liposomes (approx. 0.2 μM phospholipid) was pipetted into 2 ml of serum. The change in fluorescence was monitored over time at a constant temperature. The efflux of liposome contents was calculated from the percent leakage noted in the first few minutes of incubation of each sample using a 100% efflux, and the fluorescence reading was obtained when the sample was lyzed with Triton X-100 (final concentration, 0.1%).

Electrophoresis Electrophoresis was performed on 1% agarose gels according to the method of Scherphof *et al.*,[8] albeit slightly modified.

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The liposomes (2 ml) and serum (2 ml) were incubated for 10 min at 37 °C and applied as a 4 μl mixture for gels. The gels were run for 30 min at 70 V in a 0.05 M barbital buffer, pH 8.6. Human lipoprotein was used as a marker and then stained with sudan black B. The gels were cut into 5 mm pieces for radioactivity assay.

Distribution of [^{14}C] Radioactivity after Incubation with Serum *in Vitro* Liposomes (0.2 ml) were incubated with mouse serum (10 ml) at 37 °C for 4 h, then lipids were extracted, and radioactivity of each lipid fraction was determined.

Separation of Phospholipids To incubation mixtures and each subcellular fraction were added 4 ml of chloroform : methanol (1 : 2, v/v), and resultant lipids were extracted by the methods of Bligh and Dyer.[9] The phospholipids were separated by two dimensional thin-layer chromatography (TLC).[10] Lipids were first developed on Silica gel H plates containing 2.5% magnesium acetate with chloroform : methanol : 13.5 N ammonia water (65 : 35 : 6, v/v). The TLC plates were dried *in vacuo* for 30 min at room temperature, and then the samples were redeveloped with chloroform : acetone : methanol : glacial acetic acid : water (3 : 4 : 1 : 1 : 0.5, v/v). Neutral lipids were separated by TLC on Silica gel G plates by developing in petroleum ether : diethyl ether : glacial acetic acid (80 : 30 : 1, v/v). After visualization with I_2 vapor, the areas corresponding to individual phospholipids were scraped off the TLC plate, and radioactivity was measured using a scintillation counter (Beckman LS-9000) with scintillation liquid [toluene : Triton X-100 : water : 2,5-diphenyloxazole : 2,2′-*p*-phenylene-bis(5-phenyloxazole), 800 ml : 200 ml : 50 ml : 3.2 g : 0.24 g]. The radioactive recovery rate from the plate was more than 95%, and counting efficiency was determined with an external standard. All solvents included 0.01% butylated hydroxytoluene. This TLC solvent system was able to effectively separate TG from free fatty acid.

Freeze-Fracture Electron Microscopy Liposomes were first fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer at 37 °C for 20 min. They were resuspended in freshly prepared 30% glycerol in 0.85% NaCl solution, and then frozen in Freon-12 before being transferred to liquid nitrogen (−195 °C). These samples were fractured at −110 °C in a Hitachi HFZ-1 freeze-fracture apparatus. Replicas prepared by platinum–carbon followed by carbon shadowing were cleaned with hypochlorite solution and rinsed in distilled water, and then examined on a JEM-100U electron microscope (JEOL, Japan).

Animal Experiments Male mice (ddy, weighing 30—35 g) in groups of five were injected *via* the tail vein with 0.2 ml liposome suspensions (0.5 μmol) containing 8×10^5 dpm [^{14}C] TG with or without LacCer and then sacrificed at indicated intervals after injection. Blood volume was taken as 6.5 ml per 100 g body weight.[11] The liver was perfused with saline and homogenized in a 3 vol. cold saline solution to measure radioactivity. When gauging radioactivity, a 0.1 ml aliquot of homogenate, subcellular fraction or cell suspension was directly dissolved in 0.5 ml of the solubilizing agent NCS®, and measured with a scintisol (ACS-II®) in a scintillation counter (Beckman, LS-9000).

Separation of Non-parenchymal and Parenchymal Cells At various time intervals after intravenous injection of liposomes into mice, the liver was perfused *in situ via* the portal vein with Ca^{2+}-free Hanks' solution followed by a minimum essential medium (MEM) containing 0.06% collagenase. The cell suspension was filtered through a double layer of gauze, and the filtrate was subjected to separation of parenchymal and non-parenchymal cell fraction through a Percoll gradient according to the method of Rahman *et al.*[12] The cells were suspended in 40 ml Percoll with a density of 1.059, containing 1×10^6 cells per ml. This Percoll-cell suspension was then centrifuged at $20380 \times g$ for 20 min. After centrifugation, the parenchymal cells were banded at a density of 1.07—1.09 g/ml, while non-parenchymal cells were concentrated at a density of 1.04—1.06 g/ml. The average number of cells obtained from a mouse was $8.7 \pm 0.3 \times 10^7$ cells ($n=3$), representing a recovery rate of about 50—55% of the estimated total liver cells, calculated on the basis of data reported by Seglen.[13] 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 filtrate and the residue. Summation of these recoveries was taken as an estimate of total liver uptake and showed good agreement with the independently determined total liver uptake values.

Thin Section Electron Microscopy Cells were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer. After this cell suspension had been centrifuged, the pellet was fixed with 1% OsO_4 in cacodylate buffer for 1 h and then washed. The samples were dehydrated in a graded ethanol series, and then embedded in epoxy resin (Epon 812).[14] Thin sectioning was performed on a Porter–Blum Mt II ultramicrotome then stained with uranyl acetate and lead citrate and examined on an electron microscope (HS-8 type, Hitachi).

Isolation of Subcellular Fractions The nuclear fraction was obtained by the method of De Duve *et al.*,[15] and the mitochondria and microsomes were isolated according to the procedure of Nozawa and Thompson.[16] The whole liver cells were washed in a potassium phosphate buffer (0.2 M K_2HPO_4/0.2 M KH_2PO_4/2 mM ethylenediaminetetraacetic acid (EDTA)/0.1 M NaCl, pH 7.2) and manually homogenized in a Teflon-glass homogenizer. The resulting yielding slurry was centrifuged at $10000 \times g$ for 1 min. The sediment, which still contained a large number of unbroken cells in addition to nuclei, was rehomogenized in about the same quantity of medium and centrifuged at $6000 \times g$ for 5 min. After repeating this operation once, the nuclear sediment, almost free of intact cells or gross debris, was redispersed by means of the homogenizer, yielding a final volume equivalent to 4 times the weight of tissue processed, for a 1 : 4 nuclear fraction. Mitochondria and microsomes were prepared from the particulate fraction by sequential centrifugation at $19600 \times g$ for 20 min and at $105000 \times g$ for 60 min, respectively. Extraction and separation of phospholipids in each subcellular fraction were performed as described in the section, "Separation of Phospholipids."

Enzyme Assay Glucose 6-phosphatase activity was measured by following the rate of orthophosphate appearance.[17] Incubation mixtures with a final volume of 2.4 ml contained 0.1 M sodium acetate–0.1 M sodium succinate buffer (pH 6) and 0.01 M glucose 6-phosphate. The reaction was initiated by the addition of the subcellular fraction (usually in the range, 0.4 to 0.5 mg of protein). Incubation was done at 37 °C. At 10 min, 0.8 ml of 10% trichloroacetic acid was added. The samples were centrifuged, and the resultant supernatant was analyzed for inorganic phosphate by the method of Ames.[18] One unit of enzyme activity corresponded to the release of 1 μmol of inorganic phosphate in 10 min using a standard assay. Protein concentration was determined by the procedure of Lowry *et al.*[19] with bovine serum albumin as the standard. Acid phosphatase activities were measured at 37 °C and pH 4 by the rate of liberation of *p*-nitrophenol from *p*-nitrophenylphosphate.[20] Assay mixtures contained 100 μmol of sodium acetate, 5 μmol of EDTA, 2.5 μmol of *p*-nitrophenylphosphate, and enzyme in a total volume of 2 ml. After incubation at 37 °C for 10 min, the reactions were stopped by the addition of 1 ml of 1 N NaOH and 2 ml of H_2O, and the absorbance of each solution was measured at 410 nm against an appropriate blank. A standard curve was made using *p*-nitrophenol, and specific enzyme activity was expressed as units of enzyme activity per mg of protein.

Results and Discussion

Since lysosomal storage diseases are caused by a genetic deficiency of specific acid hydrolases in lysosomes, reversal of such enzyme deficiencies has been attempted by means of direct enzyme replacement. However, a free foreign enzyme injected into the circulation can provoke immunological and other system reactions, and it eventually reaches the reticuloendothelial system. We have postulated that liposomes are suitable candidates for enzyme entrapment because they are made of physiological materials, and their surfaces can be chemically manipulated. In an attempt to use enzyme replacement therapy in an inherited lysosomal storage disease, we attempted to prepare liposomes targeted to hepatocytes. The interactions of liposomes with liver cells were examined especially with regard to their delivery to hepatocytes prepared with glycolipids. The question therefore arises whether or not uptaken liposomes in liver cell can be degraded. The purpose of the present study was to examine the metabolic fate of liposomes after their intravenous injection.

Property of Liposomes It has been shown that plasma is able to degrade liposomes by active transfer of lipid molecules to high density lipoprotein (HDL).[21] When radiolabeled phosphatidylcholine (PC) or Chol is used as a lipid marker of liposomes, there are some difficulties in

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Table I. Latency of Liposomal Calcein in the Presence of Serum

Time (h)	Latent calcein in liposomes (%)
0	100
1	98.3±1.2
2	98.8±1.2
3	98.7±1.5
4	97.8±1.5

Expressed as percentages of the latencies in the respective liposomal preparations (mean±S.D. for three preparations).

Table II. Change in Distribution of [^{14}C] Radioactivity in Serum

Lipid	% of distribution
Phosphatidylcholine	0.08±0.02
Phosphatidylethanolamine	0.08±0.02
Triglyceride	96.66±1.20
Diglyceride	1.50±0.35
Monoglyceride	0.02±0.01
Free fatty acid	0.93±0.02

Values represent mean±S.D. ($n=3$).

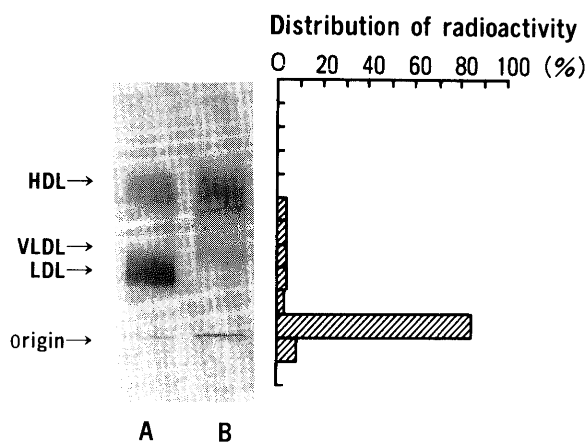

Fig. 1. Electrophoresis of an Incubation Mixture of Labeled Liposomes and Mouse Serum

A, human serum; B, incubation mixture of liposomes and mouse serum. HDL, high density lipoprotein; VLDL, very low density lipoprotein; LDL, low density lipoprotein.

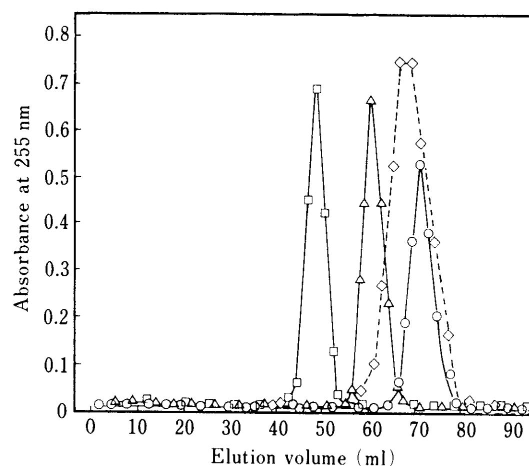

Fig. 2. Elution Profile of Liposomes on Sephacryl S-1000 Column

--◇--, prepared liposomes; ——, polystyrene latex beads (○, 85±5.5; △, 109±2.7; □, 220±5.9 nm).

interpreting the results of the *in vivo* fate of liposomal lipids, since PC can be exchanged between liposomes and high-density lipoproteins,[22] and also because of a possible exchange of hepatic Chol with liposomal Chol or Chol donation to the cells by liposomes directly or *via* plasma lipoproteins.[23] Also it is known that the addition of Chol causes an increase in vesicle size without any concomitant change in vesicle shape or heterogeneity, suggesting that Chol is capable of regulating or controlling lipid membrane morphology.[24] Therefore, we prepared liposomes with one-fifth of the total phospholipids containing Chol. The property of liposomes was examined using LacCer-bearing liposomes. We demonstrated that our liposomes reduced the leakage of entrapped calcein to nearly negligible levels in serum, and most of this liposomal calcein was still latent 4 h later (Table I). Therefore, we measured the specific radioactivities of TG remaining in the liposomes after incubation with serum (Table II). Uptake of [^{14}C] radioactivity into red blood cells was less than 1% of total radioactivity. No free fatty acid appeared in the serum throughout the duration of the experiments. After incubation of liposomes, prepared from TG of high specific radioactivity in serum, the bulk of the mixture is subjected to electrophoresis to assess the extent of association with high density lipoprotein (HDL). Most of the radioactivity is recovered in the area corresponding to its origin (Fig. 1). It is demonstrated that scarcely any damage occurs to our liposomes resulting from entrapment of calcein in the serum for at least 4 h, and they retain their triglyceride form. Thus, in our experiments, 1-[^{14}C] TG was used as a liposomal lipid marker, since it was not transferred to lipoproteins during blood circulation. Hoekstra *et al.* have shown glycolipid enhanced uptake of liposomal phospholipid by hepatocytes only when containing dimyristoyl PC, but not with egg PC vesicles *in vitro*.[25] In our previous studies, we demonstrated that in the isolated hepatocytes DMPC- and dipalmitoylphosphatidylcholine (DPPC)-liposomes containing LacCer are taken up to a much greater extent than control liposomes without LacCer, while no significant enhancement was observed in the uptake rate of LacCer-targeted egg PC-liposomes compared to control.[26] It has been well known that liposomal size is an important factor in targeting liver cells. In ultra-thin sections of perfused and fixed rat livers, these fenestrae are measured as approximately 0.1 μm in diameter.[27] *In vitro* experiments have shown that isolated Küpffer cells engulf large vesicles to a greater extent than small vesicles by endocytosis.[28] However, it is generally thought that small particles with a size of up to 100 nm in diameter can pass through these fenestrae. Figure 2 shows the elution profiles of prepared liposomes and polystyrene beads as size markers (diameter=85±5.5, 109±2.7, 220±5.9 nm). The prepared liposomes appear homogeneous in size with a diameter of 80—100 nm in elution profiles on Sephacryl S-1000 gel chromatography. This size homogeneity is also supported by freeze-fracture electron microscopy, showing that prepared liposomes are unilamellar and less than 100 nm in diameter (Fig. 3). Furthermore, the particle size distribution of liposomes in the solution has also been measured by the dynamic light scattering (DLS) method, and mean diameter found to be

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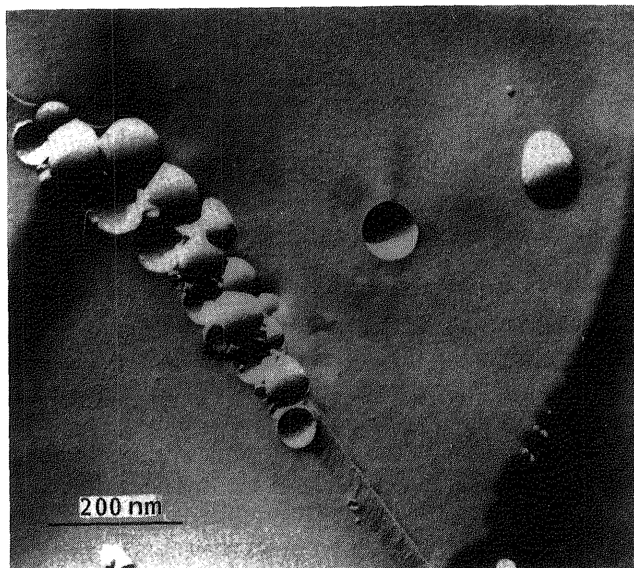

Fig. 3. Freeze-Fracture Electron Microscopic Observation of LacCer-Bearing Liposomes

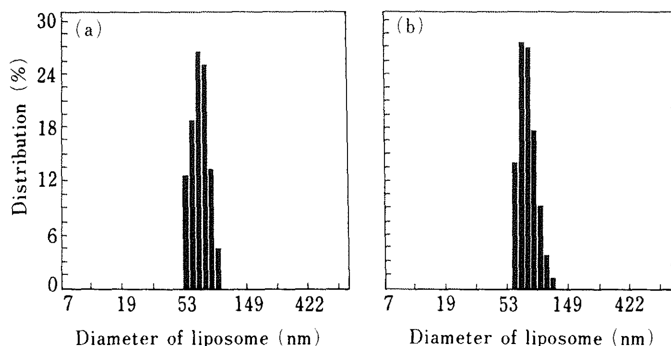

Fig. 4. Size Distribution of Control and LacCer-Bearing Liposomes Determined by the Dynamic Light Scattering Method

(a), control liposomes; (b), LacCer-bearing liposomes.

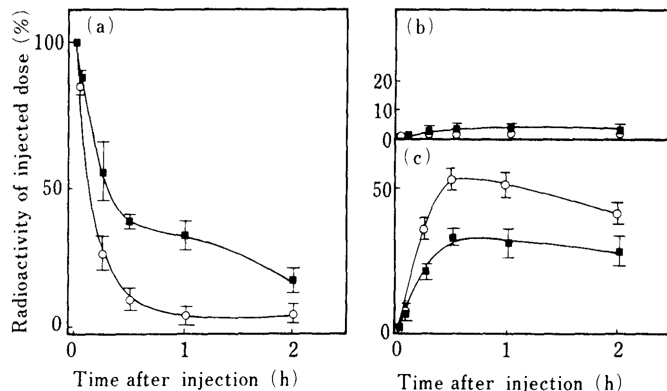

Fig. 5. Blood Clearance and Tissue Uptake of LacCer-Bearing and Control Liposomes

(a), blood; (b), spleen; (c), liver. Each point represents the mean ± S.D. of four different experiments. ■, control liposomes; ○, LacCer-bearing liposomes.

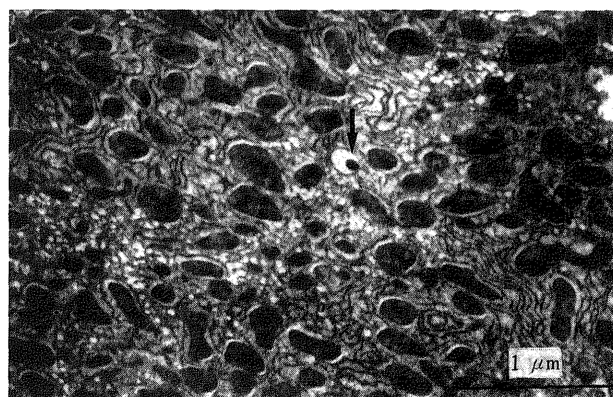

Fig. 6. Thin Sectioning Electron Micrograph of Mouse Hepatocytes after Injection with LacCer-Bearing Liposomes

70.1 ± 10.6 nm, 76.6 ± 10.3 nm for control and LacCer-bearing liposomes, respectively (Fig. 4). The particle size of control liposomes is to a certain extent smaller than LacCer-bearing liposomes. The data produced by the DLS method are in good accordance with the freeze-fracture electron microscopic observation.

Distribution of Liposomes Numerous studies on the reticuloendothelial clearance of foreign particles have shown that the liver possesses remarkable properties for taking up and storing particulate substances. However, this endocytic function has been largely assigned to the Küpffer cells. Because of endothelial linkage to liver sinusoids, parenchymal cells are not readily accessible to particles introduced from the bloodstream. It should be pointed out, however, that the spaces between the endothelial processes have a pore size of about 0.1 μm[27] which is comparable to the size of prepared liposomes. Figure 5 clearly shows that almost all LacCer-bearing liposomes are cleared from the blood within the first 3 h after injection, and the uptake of the LacCer-liposomes by the liver is found to increase rapidly to 52% during the first 1 h. It is thus evident that the liver takes up and stores particulate substances. On the other hand, at 1 h after injection, 34% of the injected dose of control liposomes remain in circulation and 30% are retained in the liver. The spleen and lung possess a low capacity (less than 5%) to take up either control or LacCer-bearing liposomes. Liposomal distribution has been studied by electron microscopy using liposomes with iron-containing, electron dense protein ferritin, and due to this electron density, individual ferritin molecules can be readily detected. Internalisation *via* coated vesicles in other systems has not yet been visually demonstrated. In this process, the coated pits may invaginate to form coated endocytic vesicles. At a time point of 1 h after injection, liposome is observed to be taken up by the hepatocytes (Fig. 6). We identified the hepatocyte cell by excluding other cell types using various staining methods specific for them. The cell was neither stained by oil red O, which visualizes the lipid droplets present in the Stellate cells,[28] peroxidase staining specific for Küpffer cells,[29,30] nor by immunostaining using factor VIII-related antigen antibody specific for endothelial cells.[31] Finkelstein and Weissman have shown a micrograph of phagosome that contains a liposome fusing with a lysosome.[32] They also demonstrated that the liposome is incorporated by endocytosis, which depends on the contents of vacuoles derived from invagination of the plasma membrane. In a previous paper, we have also demonstrated that liposomes composed of egg PC : Chol : DCP (7 : 2 : 1, molar ratio) are taken up by endocytosis based on energy metabolic inhibitor experiments.[33] These vacuoles may subsequently fuse with lysosomes, and become part of the lysosome system.

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Liposome Uptake in Liver Cell Fractions We examined the characteristics of liposome uptake by liver parenchymal and non-parenchymal cells. The parenchymal cells obtained were not contaminated with non-parenchymal cells or cell fragments as judged by light microscopy. The non-parenchymal cell fraction contained Küpffer and endothelial cells. Uptake rate of the liposomes into the parenchymal and non-parenchymal cells was determined at 30 min after injection into mice. As shown in Table III, approximately the same amount of untargeted liposomes was taken up by the parenchymal and non-parenchymal cells, whereas LacCer-bearing liposomes were taken up by parenchymal cells at a rate two times greater than untargeted liposomes. Moreover, non-parenchymal cells were able to incorporate LacCer-bearing liposomes at a slightly higher rate than untargeted liposomes. However, this result could have been caused by a higher concentration of LacCer-bearing liposomes than of liposomes without LacCer in the liver. Alternatively, the slight enhancement of uptake of LacCer-bearing liposomes by non-parenchymal cells is probably due to the larger size of LacCer-bearing liposomes compared with control liposomes (Fig. 4).

Subcellular Distribution of [^{14}C] Radioactivity Subcellular distribution of liposomal radioactivity in the mouse liver after injection of [^{14}C] TG-containing liposomes was examined (Table IV). In a control experiment, [^{14}C] TG-containing liposomes were added to the buffer prior to homogenization, and non-specific distribution of radioactivity was examined. The control experiment showed that radioactivity was distributed mainly in the postmicrosomal supernatant, and a lower amount was associated with microsomes, mitochondria-lysosomal and nuclear fractions during subcellular fractionation. The same fractionation procedure was carried out on liver cells of liposome-injected mice. At 5 min after injection, 55% of ^{14}C radioactivity was recovered in the mitochondria-lysosomal fraction. There was a gradual decrease of ^{14}C radioactivity in this fraction, whereas radioactivity in the microsomal fraction exhibited a corresponding increase. The observed fractionation patterns were compared against those of acid phosphatase and glucose 6-phosphatase, which served as reference enzymes. In the microsomal fraction, contamination of mitochondrial fraction was less than 5% based on reference enzyme activity. Gregoriadis and Ryman reported entrapment of proteins which were utilized in the mitochondria-lysosomal fraction.[34] Based on this hypothesis, the accumulation of radioactivity in the liver after injection of [^{14}C]-labeled liposomes was supposed to reflect the specific uptake of liposomes into lysosomes. The increase in ^{14}C radioactivity of microsomes at 30 min after injection suggested that the incorporated liposomes containing [^{14}C] TG might be reutilized for synthesis of phospholipids in the endoplasmic reticulum. The rapid sequence of events visualized in the electron micrographs of Fig. 6 precisely paralleled biochemically-derived data on the rapid uptake and degradation of liposomes. Since it was possible that the incorporated [^{14}C] TG could be metabolized to some phospholipid species, the distribution of ^{14}C radioactivity among phospholipids was further investigated. The level of ^{14}C radioactivity in the TG fraction was progressively and markedly reduced within 30 min, with a complementary increase in the phospholipid

Table III. Intrahepatic Cellular Distribution of LacCer-Bearing and Control Liposomes

Liposomes	Time after injection (min)	Radioactivity (%)		
		Total homogenate	Parenchymal cells	Non-parenchymal cells
Control	30	35.7±4.3	16.6±2.6	19.1±2.6
LacCer[a]	30	56.4±7.3	31.9±5.9	24.5±2.1

Data were expressed as percentages of the injected dose. Each value represents the mean±S.D. of three different experiments. *a*) Significantly different from the control group at $p<0.05$.

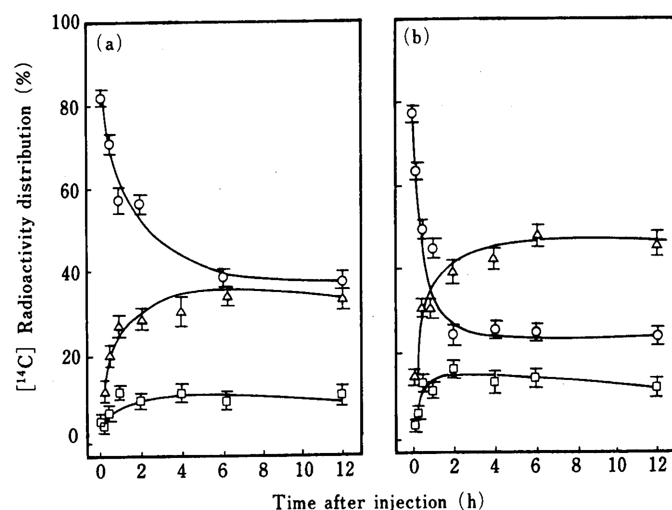

Fig. 7. Change in Distribution of [^{14}C] Radioactivity in Liver Subcellular Fraction

(a), Mt-Lys; (b), Ms. Each value represents the mean±S.D. of three different experiments. ○, TG; △, PC; □, PE. Mt-Lys, mitochondria-lysosomal fraction; Ms, microsomal fraction.

Table IV. Subcellular Distribution of [^{14}C] Radioactivity in Mouse Liver

Fraction	Enzyme activity		Distribution of radioactivity (%)					
	Acid phosphatase	Glucose 6-phosphatase	5 min	10 min	15 min	20 min	30 min	*In vitro* control
Nuclei	N.D.	N.D.	8.2±1.0	7.5±0.9	8.3±0.5	9.5±0.5	10.5±0.6	3.5±0.5
Mitochondria-lysosomes	2010.4±40.9	15.2± 2.1	55.4±5.8	46.3±4.5	40.1±2.6	36.0±2.7	32.5±3.2	4.5±0.8
Microsomes	50.1± 6.2	238.3±15.9	25.0±1.8	34.2±1.5	42.5±2.9	46.3±3.1	47.0±4.2	15.2±1.5
Post microsomal supernatant	N.D.	N.D.	11.4±0.6	12.0±0.9	9.1±0.7	8.2±0.6	10.0±1.2	76.8±3.1

As a control, the liver from a mouse without liposome administration was homogenized in 0.25 M sucrose containing [^{14}C] TG-labeled LacCer-bearing liposomes. Each value represents the mean±S.D. of four different experiments. N.D., not detected.

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fraction, especially PC (Fig. 7); however, only a small increase in radioactivity of PE was observed. Other phospholipid species (phosphatidylinositol and phosphatidylserine) retained less than 5% of the total ^{14}C radioactivity. ^{14}C Radioactivity of PC and PE was found not only in microsomes but also in mitochondria-lysosomal fractions. Thus, it was conceivable that the liposomal TG taken up by endocytosis may first have been delivered to lysosomes and degraded to free fatty acids by lysosomal lipases. Since no accumulation of [^{14}C]-free fatty acids in any intracellular fraction was observed (data not shown), it was suggested that the acids were rapidly reutilized for the synthesis of phospholipids in the endoplasmic reticulum. Two pathways of phospholipid synthesis are known: One is the deacylation–reacylation pathway,[35] and the other is *de novo* synthesis.[36] Although it is not clear whether one or both pathways were operating in these cells, the decomposed free fatty acids were reutilized rapidly for phospholipid synthesis. [^{14}C]-Labeled phospholipids were thought to be further transferred to other organelles, such as mitochondria and lysosomes by specific exchange proteins.[37]

In conclusion, liposomes coated with LacCer appear to have a higher affinity to parenchymal cells than liposomes without LacCer. No hydrolysis of labeled TG is observed in serum, but uptake and hydrolysis occur in the liver. Biodegradable liposomal lipids are reutilized more for synthesis of PC and less in PE. Phospholipid degradation might also occur following incorporation of the liposomal lipid into the lysosomal fraction as a result of endocytosis. At the same time, a rapid and reversible exchange of PC between microsomes and mitochondria is demonstrated. Since liposomes could be directly delivered to the lysosomes, one could envisage an approach in the utilization of liposomes in lysosomal enzyme-replacement therapy.

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