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Effect of cholesterol on the uptake and intracellular degradation of liposomes by liver and spleen; a combined biochemical and γ-ray perturbed angular correlation study

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We investigated the effect of cholesterol on the uptake and intracellular degradation of liposomes by rat liver and spleen macrophages. Multilamellar vesicles (MLV) consisting of distearoylphosphatidylcholine/phosphatidylserine (molar ratio 9:1) or distearoylphosphatidylcholine/cholesterol/phosphatidylserine (molar ratio 4:5:1) were labeled with [^{3}H]cholesteryl hexadecyl ether and/or cholesteryl [^{14}C]oleate. After i.v. injection the cholesterol-containing liposomes were eliminated less rapidly from the bloodstream and taken up to a lesser extent by the liver (macrophages) than the cholesterol-free liposomes. Assessment of the ^{3}H/^{14}C ratios in liver and spleen cells revealed that the cholesterol-containing liposomes are substantially more resistant towards intracellular degradation than the cholesterol-free liposomes. These results could be confirmed by measuring the release of ^{111}In from liposomes after uptake by liver and spleen by means of γ-ray perturbed angular correlation spectroscopy. Experiments with cultured Kupffer cells in monolayer also revealed that incorporation of cholesterol results in a decrease of the uptake and an increase of the intracellular stability of cholesteryl [^{14}C]oleate-labeled liposomes. Finally, incubation of both types of liposomes with lysosomal fractions prepared from rat liver demonstrated a difference in susceptibility to lysosomal degradation: the cholesterol-free vesicles were much more sensitive to lysosomal esterase than the cholesterol-containing liposomes. These results may be relevant to the application of liposomes as a drug carrier system to liver and spleen (macrophages).

Introduction

The application of liposomes as a potential drug delivery system has gained wide interest [1,2]. Liposomes appear to be a specially attractive carrier system for the selective delivery of drugs to macrophages, e.g., in the treatment of intracellular infections such as leishmaniasis [3], candidiasis [4] or listeriosis [5]. Liposomes are also used as carriers of immunomodulators such as muramyl dipeptide to lung and liver macrophages to render these cells cytotoxic to tumor cells [6,7]. Finally, liposomes received widespread attention as a carrier system for anti-tumor drugs such as adriamycin [8].

It has become apparent that both the extent of uptake and the rate of intracellular degradation of liposomes by macrophages are decisive for the rate at which a liposome-encapsulated drug becomes available, either intra- or extracellularly and thus for the therapeutic efficacy. Therefore we have undertaken efforts to manipulate the extent of liposome uptake by macrophages and the rate of intracellular disruption of the vesicles as a function of liposomal lipid composition [9].

In the present study we demonstrate that incorporation of high proportions of cholesterol (up to 50 mol%) into the bilayer of distearoylphosphatidylcholine/phosphatidylserine liposomes not only affects the uptake of liposomes by liver and spleen macrophages, but also results in a decreased rate of intracellular degradation.

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PAC, perturbed angular correlation; PBS, phosphate-buffered saline; DMEM, Dulbecco's modification of Eagles medium; DSPC, distearoylphosphatidylcholine; PS, phosphatidylserine; MLV, multilamellar vesicles.

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Materials and Methods

Materials. Cholesterol and L-α-phosphatidylserine were obtained from Sigma. L-α-Distearoylphosphatidylcholine was from Avanti Polar Lipids. Pronase-E was obtained from Merck; DNase type I, grade II from Boehringer. Cholesteryl [^{14}C]oleate was purchased from Amersham and [^{3}H]cholesteryl hexadecyl ether from New England Nuclear. The trisodium salt of nitrilotriacetic acid was obtained from Aldrich Chemical Co. and carrier-free $^{111}InCl_3$ from Amersham.

Liposomes. Multilamellar vesicles (MLV) consisting of distearoylphosphatidylcholine and phosphatidylserine (molar ratio 9:1) or distearoylphosphatidylcholine, cholesterol and phosphatidylserine (molar ratio 4:5:1) were prepared as follows. Lipids dissolved in chloroform/methanol were dried under reduced nitrogen pressure, redissolved in cyclohexane and lyophilized. Trace amounts of [^{3}H]cholesteryl hexadecyl ether and cholesteryl [^{14}C]oleate were added to the lipid mixtures when required. The lipids were then mixed with 1.5 ml 135 mM NaCl, 10 mM Hepes (pH 7.4) (HN-buffer) and vortexed for ten 30-s periods at 62° C. The vesicles formed were sized by extrusion through a series of polycarbonate membranes (Nuclepore) of 1.0, 0.8, 0.6 and 0.4 μm pore diameter, respectively, at 62° C.

Liposomes used for the PAC measurements were prepared essentially as described above with the following modifications. Liposomal lipids were hydrated in 9.6 ml of 1 mM nitriloacetic acid/Hepes (pH 7.4). After extrusion the MLV were passed over a 10-ml Sephadex G-25 column equilibrated with Hepes buffer to remove untrapped nitrilotriacetic acid. For loading the liposomes with ^{111}In, approx. 7 ml of liposomal suspension was mixed with 1.75 ml Tris-buffer (10 mM tris(hydroxymethyl)aminomethane, 145 mM NaCl (pH 7.6)) containing 1 mCi of $^{111}InCl_3$ and 24 μmol acetylacetone as an ionophore and incubated for 1 h at 50° C. The solution was then passed over 10 ml Sephadex G-25 spin columns as before to remove untrapped ^{111}In and acetylacetone. See for further details Refs. 10 and 11.

Animal experiments. Liposomes (2 μmol of total lipid per 100 g body weight) were injected into the penis vein of ether-anesthetized male Wistar rats, body weight varying from 180 to 220 g. Liposome clearance from the blood was determined by blood sampling from the tail vein. To determine the uptake of the liposomal radioactive labels the liver was perfused via the portal vein with isotonic saline for 3 min at 10° C to remove the blood. Liver and spleen were excised and homogenized in a Potter-Elvehjem tube in water at 4° C and samples were taken in triplicate for measurement of radioactivity.

Uptake by non-parenchymal cells after i.v. injection of liposomes. Non-parenchymal liver cells were isolated at 10° C as described previously [12]. Fractionation of the non-parenchymal cell fraction into an endothelial and a Kupffer cell fraction was accomplished by centrifugal elutriation as described before [13]. Samples of liver cell suspensions and spleen were mixed with water (total volume 0.5 ml) and added to equal volumes of 1% sodium dodecylsulfate. Blood samples were diluted with water and incubated for 20 min with 0.4 ml 30% H_2O_2 to decolorize. Radioactivity was assayed with Plasmasol [14] as a scintillation mixture.

Perturbed angular correlation (PAC) spectroscopy. The two γ-quanta, emitted when ^{111}In decays to ^{111}Cd, show an angular correlation which is determined by the tumbling rate of the ^{111}In nucleus. When chelated liposome-encapsulated ^{111}In is released upon destruction of the liposomes, the ^{111}In ion binds to macromolecules such as proteins and thus undergoes an increase in tumbling rate which is detected by monitoring the time-integrated angular perturbation factor $G_{22}(\infty)$ [10]. Thus, a convenient parameter of in situ liposome integrity is obtained. 0.6 ml of ^{111}In-loaded liposomes (2 μmol lipid/100 g body weight) was injected into the tail vein and at 1, 4 and 24 h after injection the rats were killed. Blood samples were collected by cardiac puncture. The liver was then perfused with 50 ml PBS via the portal vein. Liver and spleen were removed and weighed. Liposome integrity was determined by measurement of the $G_{22}(\infty)$ via the PAC spectrometer [10]. While the sample holder of the PAC equipment could accomodate the entire spleen, only portions of the liver could be counted due to sample size and geometry limitations. No statistical difference was observed between the various liver portions.

In vitro uptake and degradation by cultured Kupffer cells. $3.5 \cdot 10^6$ Kupffer cells were isolated according to Dijkstra et al. [15] and plated out in 1.7 ml DMEM (Flow) containing 10 mM $NaHCO_3$, 20 mM Hepes, 20% fetal calf serum (Gibco), 100 IU/ml penicillin G and 100 μg/ml streptomycin (Gist-Brocades, Delft) on 35-mm petri dishes (Greiner). 24 h after seeding, the medium was replaced by medium containing 10% fetal calf serum. 48 h after seeding, cells were used for experiments. 80 nmol of cholesteryl [^{14}C]oleate-labeled liposomes were incubated with the cells in serum-free medium at 37° C in the presence of 10 mM NH_4Cl to minimize liposomal degradation during uptake of the vesicles by the cells by raising the intralysosomal pH. After 1 h the medium containing the liposomes was removed and the cells were incubated for another 0.5 h in fresh medium without liposomes in the presence of NH_4Cl. After 1.5 h (indicated as zero-time) the medium was replaced by an NH_4Cl-free medium. At times indicated the cells were washed five times with cold PBS and scraped from the culture dishes with a rubber policeman in approx. 1 ml ice-cold water. Aliquots were taken for determination of protein [16] and radioactivity

content; 0.6 ml samples were extracted according to Folch et al. [17] and the chloroform-soluble fraction was chromatographed on 0.25 mm thin-layer plates (Merck, 60F254) with petroleumether (40/60)/diethyl ether/formic acid (60:40:3, v/v) as a solvent. Relevant spots were scraped from the plates, transferred to counting vials and mixed with 0.5 ml water. Radioactivity was determined in Hydrocount (Baker) in a LKB Wallac Liquid scintillation counter.

Degradation of liposomes by lysosomal fractions. Lysosomal fractions were prepared from rat liver by differential centrifugation as described by De Duve et al. [18]. The fractions were sonicated for 15 min in a bath sonifier at 10°C prior to the incubation with liposomes. Incubations of cholesteryl [^{14}C]oleate-labeled liposomes with lysosomal fractions were carried out at 37°C in 0.25 ml medium containing 50 mM sodium acetate (pH 4.8), 25 nmol liposomal lipid and 50 μg of lysosomal protein. The reaction was stopped by adding 4.5 ml chloroform/methanol (1:2, v/v) and lipids were extracted according to Bligh and Dyer [19]. The lipids were separated by thin-layer chromatography and analyzed for radioactivity as described above.

Results

The kinetics of the elimination of cholesterol-containing and cholesterol-free distearoylphosphatidylcholine/phosphatidylserine multilamellar liposomes from the blood is shown in Fig. 1. The cholesterol-containing liposomes are eliminated 3-fold less rapidly ($T_{1/2} \approx 10$ min) than the cholesterol-free liposomes ($T_{1/2} \approx 3$ min). This difference in clearance rate is reflected in the uptake of the liposomes by the liver (Table I). While, 15 min after injection, this organ contains almost 70% of the cholesterol-free liposomes this value is reduced to less than 40% for the cholesterol-containing liposomes. Interestingly, the uptake by the spleen is not reduced by the incorporation of cholesterol into the liposomal bilayer. On the contrary, the uptake of the cholesterol-containing liposomes by the spleen is substantially increased as compared to that of the cholesterol-free liposomes.

To assess the intracellular degradation of the MLV by liver and spleen we double-labeled the liposomes with the degradable marker cholesteryl [^{14}C]oleate and the non-degradable marker [^{3}H]cholesteryl hexadecyl ether. The latter remains tightly associated with the cells after it has been taken up via liposomes. Cholesteryl [^{14}C]oleate, on the other hand, is susceptible to intralysosomal esterase activity resulting in the liberation of the labeled oleate. Most of the [^{14}C]oleate is readily released by the cells [9] and, as a consequence, intracellular degradation of the liposomes will result in an increase of the ^{3}H/^{14}C ratio.

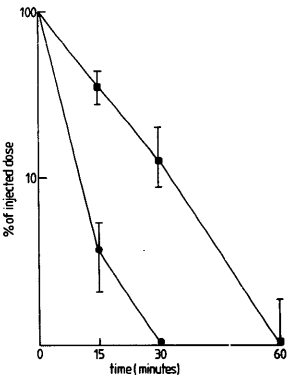

Fig. 1. Effect of cholesterol on the clearance of [^{3}H]cholesteryl hexadecyl ether-labeled liposomes from the blood. Rats were injected with MLV consisting of DSPC/PS (molar ratio 9:1) or DSPC/cholesterol/PS (molar ratio 4:5:1). Liposomes were injected in a dose of 2 μmol of total lipid per 100 g body weight. At times indicated blood samples were collected and assayed for radioactivity (see Materials and Methods). Points represent means ± S.D. of three determinations. ■——■, DSPC/cholesterol/PS; ●——●, DSPC/PS.

Table II shows the results of such an experiment: cholesterol-free and cholesterol-containing liposomes with a ^{3}H/^{14}C ratio of 2.0 were injected. In total liver the isotopic ratio increased approx. 22-fold at 24 h after injection in case of the cholesterol-free liposomes as opposed to a 10-fold increase in case of the cholesterol-containing liposomes. Also in the spleen the rate of increase of the ^{3}H/^{14}C ratio is substantially

TABLE I

Effect of cholesterol on the uptake of intravenously injected liposomes by liver and spleen

MLV of the indicated lipid composition labeled with [^{3}H]cholesteryl hexadecyl ether were injected intravenously into rats. At times indicated the amounts of radioactivity in blood, liver and spleen were determined as described in Materials and Methods. Points represent means ± S.E. of three determinations. Abbreviation: Chol., cholesterol.

Liposome	Time after injection (min)	% of injected dose		
		blood	liver	spleen
DSPC/PS (9:1)	15	3.8 ± 1.5	68.0 ± 4.5	8.2 ± 1.6
	30	1.1 ± 0.6	74.1 ± 2.5	10.7 ± 1.1
DSPC/Chol./PS (4:5:1)	15	30.0 ± 6.9	39.4 ± 4.3	17.0 ± 2.5
	30	12.0 ± 3.4	53.9 ± 5.2	24.5 ± 1.6

TABLE II

Effect of cholesterol on the intracellular degradation of intravenously injected liposomes by liver and spleen

MLV of the indicated lipid composition, doubly labeled with [^{3}H]cholesteryl hexadecyl ether and cholesterol [^{14}C]oleate were injected intravenously into rats. $^3H/^{14}C$ ratio of the liposome preparation: 2.0. At times indicated Kupffer cells were isolated and samples of cell suspensions obtained from total liver, non-parenchymal cells, Kupffer cells and spleen were processed for determination of radioactivity as described in Materials and Methods. Abbreviations: NPC, non-parenchymal cells; KC, Kupffer cells; Chol., cholesterol.

Liposome	Time after injection (h)	$^3H/^{14}C$ ratio			
		liver	NPC	KC	spleen
DSPC/PS (9:1)	1	3.1	5.1	5.7	7.7
	4	6.9	17.6	23.0	15.4
	24	45.8	144.1	182.2	69.4
DSPC/Chol./PS (4:5:1)	1	2.4	3.2	3.2	2.9
	4	4.3	5.5	6.0	6.3
	24	19.7	67.6	79.1	64.1

higher when cholesterol-free liposomes are injected although the final level at 24 h is roughly the same for both types of liposomes. Table II also shows that for both liposome compositions the increase of the $^3H/^{14}C$ ratio is more pronounced in the spleen than in the liver suggesting that liposomal degradation in the former proceeds at a higher rate. From previous studies [9,21] we know, however, that the values found for liver are an underestimation of the true rate of hydrolysis because part of the liberated oleate is efficiently reutilized by the hepatocytes whereas in the spleen the free oleate is readily released into the circulation.

This is supported by the observations on the non-parenchymal cell fraction and purified Kupffer cells in Table II. In these cells which are responsible for the intrahepatic degradation of intravenously injected MLV [13], the isotopic ratio for cholesterol-free liposomes increases even 70- to 90-fold in 24 h. The isolated cell fractions were obtained by pronase perfusion of the liver at low temperature (10°C) to slow down the intracellular degradation of the liposomes during the isolation procedure [12]. The difference in susceptibility towards intracellular degradation between the two liposome preparations also becomes apparent when the isotopic ratios for the Kupffer cell fractions are compared. The more resistant cholesterol-containing liposomes produce a 40-fold increase in isotopic ratio in contrast to a 90-fold increase for the cholesterol-free liposomes.

The findings described in the preceding paragraphs are fully compatible with the assessment of structural integrity of the liposomes by means of γ-ray perturbed angular correlation spectroscopy techniques (Fig. 2). Measurements of the degree of liposome integrity in liver and spleen at various time points after injection of ^{111}In-labeled MLV also show that the cholesterol-containing liposomes are degraded more slowly than the cholesterol-free liposomes in both organs. This difference was most pronounced at the 1 h time point. Thereafter the rates of ^{111}In release for both lipid compositions converged, reflecting a similar degree of liposomal integrity after 24 h.

The in vivo results described in the foregoing on the rates of uptake and intracellular degradation of i.v. injected cholesterol-containing liposomes were confirmed in vitro with cultured Kupffer cells. For four separate macrophage preparations we found that cholesterol-containing liposomes, labeled with cholesteryl [^{14}C]oleate, are taken up to a much lower extent (5.5 ± 1.7 nmol lipid/mg protein) than cholesterol-free liposomes (12.0 ± 0.4 nmol lipid/mg protein) by Kupffer cells in maintenance culture after 1 h incuba-

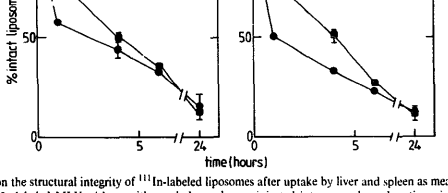

Fig. 2. Effect of cholesterol on the structural integrity of ^{111}In-labeled liposomes after uptake by liver and spleen as measured by perturbed angular correlation spectroscopy. ^{111}In-labeled MLV with or without cholesterol were injected intravenously and at times indicated the time-integrated angular perturbation factor $G_{22}(\infty)$ was measured in liver and spleen as described in Materials and Methods. ■———■, DSPC/cholesterol/PS; ●———●, DSPC/PS.

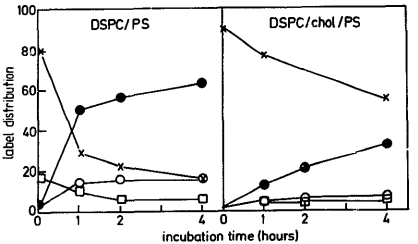

Fig. 3. Effect of cholesterol on the intracellular degradation of cholesteryl [^{14}C]oleate-labeled liposomes by cultured Kupffer cells. Kupffer cells in maintenance culture were incubated with cholesteryl [^{14}C]oleate-labeled MLV of the indicated lipid composition. After 1, 2 and 4 h the cells were washed, the lipids were extracted by thin-layer chromatography and analyzed for radioactivity. See for details Materials and Methods. ×——×, cholesteryl oleate; ●——●, phospholipids; ○——○, oleate; □——□, triacylglycerol.

tion in serum-free medium. NH_4Cl, a lysosomotropic agent which inhibits lysosomal enzyme activity [20], was present in the medium to prevent degradation of the liposomal label during the uptake period.

In Fig. 3 the effect of cholesterol on the intracellular degradation of MLV by cultured Kupffer cells is shown. Cholesteryl [^{14}C]oleate-labeled liposomes (with and without cholesterol) were incubated for 1 h with the cells in the presence of NH_4Cl so as to allow uptake with minimal degradation. After the liposomes were removed from the medium the cells were incubated for another 30 min in the presence of NH_4Cl to allow liposome-containing endosomes to fuse with primary lysosomes. After 1.5 h (indicated as zero-time) the NH_4Cl-containing medium was substituted by an NH_4Cl-free medium and the extent of intracellular degradation was determined at different time points. Again, the cholesterol-containing liposomes are more resistant to lysosomal esterase activity than the cholesterol-free MLV. At the same time efficient incorporation of liberated [^{14}C]oleate into cellular phospholipids is observed, while there is only marginal fatty acid incorporation into triacylglycerols.

Finally, the difference in susceptibility to lysosomal degradation of the two liposome preparations was also demonstrated in a cell-free system by incubating cholesteryl [^{14}C]oleate-labeled liposomes at pH 4.8 with lysosomal fractions isolated from rat liver homogenates (Fig. 4); also under these conditions cholesterol-containing liposomes are less sensitive to lysosomal esterase activity than the cholesterol-free vesicles.

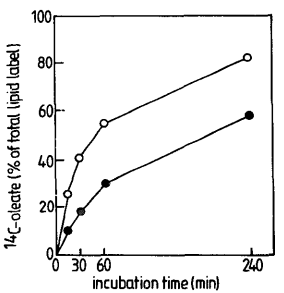

Fig. 4. Effect of cholesterol on the degradation of cholesteryl [^{14}C]oleate-labeled liposomes at pH 4.8 by lysosomal fractions prepared from rat liver. MLV of the indicated lipid composition labeled with cholesteryl [^{14}C]oleate were incubated at pH 4.8 with sonicated rat liver lysosomal fractions. At times indicated the reaction was stopped by adding chloroform/methanol, the lipids were extracted, separated by thin-layer chromatography and analyzed by radioactivity. See for further experimental details Materials and Methods. ○——○, DSPC/PS; ●——●, DSPC/cholesterol/PS.

Discussion

The experiments described in this paper demonstrate that the cholesterol content of liposomes has a pronounced influence on the rate of uptake and intracellular degradation by liver and spleen (macrophages). Our results show that incorporation of 50 mol% cholesterol in DSPC/PS liposomes results in a decrease of the clearance rate after i.v. injection. Similar results have been published by Patel et al. with reverse-phase evaporation vesicles labeled with [^{14}C]inulin [22]. The effect of the cholesterol is likely to be related to a diminished susceptibility of the lipid interface to perturbation or penetration, either by serum proteins, which may act to opsonize the liposomes [23–25], or by cell

surface proteins serving to establish the initial contact between liposome and cell [26]. In this particular case, where we used DSPC with a transition temperature well above body temperature and brain PS with a transition temperature of approx. 10 °C, the cholesterol probably acted to abolish or diminish the phase boundaries between solid DSPC and fluid PS domains. Such phase boundaries have been suggested to serve as sites where proteins may easily penetrate a lipid bilayer [27]. However, as we observed similar effects of cholesterol with egg phosphatidylcholine/PS liposomes, both with respect to cellular uptake and lysosomal degradation (results not shown), cholesterol may, alternatively, act to tighten the packing of the lower melting phospholipids in a fluid bilayer [28], thus solidifying the liposomal membrane and restraining in this way the penetration of serum or cell surface proteins or lysosomal phospholipases. The potential involvement of opsonizing proteins in the in vivo uptake of liposomes by tissue macrophages has been discussed recently by Moghimi and Patel [25]. They suggested that liver and spleen macrophages may require different opsonizing factors which may aid to explain the differential effect of cholesterol incorporation on liver and spleen uptake, respectively, as observed also by us.

On the other hand, our in vitro experiments with cultured Kupffer cells show that in the absence of serum cholesterol-containing liposomes are also taken up to a lesser extent than the cholesterol-free liposomes. As we pointed out earlier [26] this may be caused by hampered penetration of cell-surface proteins into the cholesterol-containing liposomal bilayer leading to a decreased rate of phagocytosis.

Patel and co-workers could not exclude in their study [22] that the rapid clearance of [^{14}C]inulin encapsulated in cholesterol-free liposomes might be ascribed (at least in part) to leakage of the label from the vesicles into the bloodstream followed by rapid excretion by the kidneys. In our study we used [^{3}H]cholesteryl hexadecyl ether, which remains tightly associated with the liposomes and is therefore a more reliable measure of the in vivo fate of i.v. injected liposomes.

The isotopic ratio we used as a parameter of liposome degradation indicated that, both in liver and spleen, cholesterol-containing liposomes are degraded much more slowly than cholesterol-free liposomes. For the liver, however, the isotopic ratios represent an underestimation of the rate of degradation as became apparent when the ratios in isolated Kupffer cells were determined, which showed substantially higher values. Presumably, the hepatocytes are responsible for efficient reutilization of the [^{14}C]oleate liberated by the Kupffer cells [21].

The observed cholesterol effect on the intracellular stability of the liposomes is supposedly related to an increased resistance to lysosomal enzyme action. These results are compatible with the data publisned by Johnson [29]. She found that release of ^{22}Na from cultured peritoneal macrophages after uptake by means of cholesterol-free liposomes is much more pronounced than in the case of cholesterol-containing liposomes. Penetration of lysosomal lipolytic enzymes into the liposomal bilayer is impeded in the case of the more rigid cholesterol-containing vesicles.

The relative rates of cholesteryl ester hydrolysis of the liposomes are paralleled by the relative rates at which a liposome-encapsulated solute is released, at least during the first hours after uptake, as was demonstrated by the PACS experiments (Fig. 2). At 24 h after injection, however, the ^{111}In release from both types of liposomes has reached the same high level in liver as well as in spleen. These results are compatible with the increase in isotopic ratios in Kupffer cells presented in Table II which also indicate virtually complete cholesteryl ester degradation after 24 h for both types of liposomes.

Finally, the experiments with cultured Kupffer cells and lysosomal fractions from rat liver confirmed the in vivo results: cholesterol-containing liposomes are degraded more slowly than cholesterol-free liposomes. Apparently, the liposomal cholesteryl ester is more accessible to lysosomal esterase activity in case of the cholesterol-free liposomes.

In conclusion, incorporation of 50 mol% cholesterol in distearoylphosphatidylcholine/phosphatidylserine liposomes results in a decreased uptake of the vesicles by liver but not by spleen macrophages and an increased intracellular stability. These results may be relevant to the application of liposomes as drug carrier system to the liver (macrophages). Manipulating the liposomal lipid composition influences the intracellular degradation of liposomal lipids and thereby the therapeutic availability of liposome-encapsulated drugs [21,30].

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