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Uptake of small liposomes by non-reticuloendothelial tissues *

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The distribution of liposomes within the intravascular space and the extent to which they escape into extravascular space strongly impact on the application of lipid vesicles as a carrier for pharmacologically active agents. The present study investigates how intact small unilamellar vesicles (SUV) may be taken up by different tissues after intravenous injection into mice, using various types of SUV with different entrapped markers, lipid composition, size, doses of liposomal lipids and stability in the blood. Our focus was specifically on sphingomyelin (or distearoyl phosphatidylcholine)/cholesterol (2:1, mol/mol) SUV, which are known to be stable in the blood circulation. Our results indicated that, in addition to the reticuloendothelial tissues, intact SUV were taken up in several other parts of the body, including intestine, skin, carcass and legs. It appears that the accumulation of SUV in the intestine and the skin increases with time post-injection. Furthermore, from the kinetic data, the process of uptake of SUV by the skin and intestine is compatible with a non-saturable pathway, which follows first-order kinetics. This suggests that the cells involved in the uptake of SUV in the intestine and skin are not phagocytic cells, which are normally saturable.

Introduction

Liposomes have demonstrated considerable promise as a vehicle for the delivery of therapeutic agents to tissues of the reticuloendothelial system [1–3]. A key question concerning the applications of liposomes as a drug carrier is their applicability for delivering drugs to tissues other than those of the reticuloendothelial system. Presumably, after entering the blood stream, intact liposomes may either distribute within the intravascular space or escape into extravascular space, depending on the regional permeability of the vascular wall and the size of the liposomes.

Because of the endothelial barrier investing the vascular wall, the probability for large liposomes to escape into extravascular space is low. Previous studies demonstrated that the level of uptake of large liposomes by non-reticuloendothelial tissues was very limited, even under conditions in which the phagocytic activity of the reticuloendothelial tissues were temporarily blocked [4]. In studying the cellular nature of the uptake of liposomes by

* In this paper, the word 'tissue' refers also to organs and other parts of the body.

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Abbreviations: SUV, small unilamellar vesicles; MLV, multilamellar vesicles.

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the liver, where the dimensions of fenestrae of the discontinuous capillaries are quite large, there was virtually no transcapillary passage of large liposomes (larger than 0.2 μm in diameter) [5,6]. Similarly, the narrow intercellular junctions (20–60 Å wide) in the continuous capillaries preclude the transcapillary passage of even the smallest liposomes [7,8].

In contrast, it was shown that small liposomes (less than 0.1 μm in diameter) can penetrate the discontinuous capillaries of the liver and can be taken up by the parenchymal cells [5,7,9,10]. A similar observation of the passage of small neutral liposomes through the capillaries of certain tumors has also been reported [11,12]. This indicates that small liposomes can escape into interstitial tissues and penetrate the diffusion barrier of the mucopolysaccharide-rich interstitium in the spaces of Disse of the discontinuous capillaries, or perhaps the diffusion barriers of the open fenestrae (400–600 Å in diameter) and the protein-rich basa lamina of the fenestrated capillaries [13].

In a given tissue or organ, all the three types of capillary (continuous, fenestrated and discontinuous) are present, although the proportion of each type of capillary may vary [14]. Very little is known regarding the extent to which small liposomes could gain access to non-reticuloendothelial tissues given that they remain in the blood circulation long enough to interact with these tissues. Previously, we have shown that bovine brain sphingomyelin/cholesterol small unilamellar vesciles (SUV) (187 ± 42 Å in diameter) remain intact in the blood circulation with a half-life of 16–24 h [15,16]. The present study investigates the extent of uptake of these small liposomes by non-reticuloendothelial tissues in the BALB/c mouse model.

Materials and Methods

All phospholipids were purchased from Avanti Polar Lipids, Inc. Indium-111 chloride was obtained from Medi + Physics. The AG 1-X8 (chloride form) resin was obtained from Bio-Rad and the gels, Sephadex G-50 and Sepharose 4B and 2B, were purchased from Pharmacia. All other chemicals were obtained commercially and were used as supplied. The purification of $^{111}InCl_3$ and the preparation of AG 1-X8 (phosphate form) were carried out as described previously [15,17].

Preparation of liposomes

Small unilamellar liposomes were prepared by sonicating 20–25 mg dried thin film of sphingomyelin/cholesterol (2 : 1, mol/mol), distearoyl phosphatidylcholine (DSPC)/cholesterol (2 : 1, mol/mol), or dipalmitoyl phosphatidylcholine (DPPC)/cholesterol (2 : 1, mol/mol) in 1 ml of 1 mM nitrilotriacetic acid/0.106 M sodium phosphate isotonic buffer (pH 7.4) solution using a Branson 350 sonicator fitted with a tapered titanium microtip at an input setting of 1.5 for 15 min [15,17]. During sonication, the microtip was immersed 1.4–1.5 cm into the buffered solution of liposomes, which was in a 3 ml conical glass vial. The glass vial was immersed in a glycerol bath at room temperature. At the end of sonication, the temperature of the liposomes solution was about 47 °C. The sonicated liposome preparation was centrifuged at 160000 × *g* for 1 h or 10000 × *g* for 5 min to remove the titanium fragments and aggregate.

The non-entrapped nitrilotriacetic acid was removed by passing the liposomes through a Sephadex G-50 column (0.8 × 35 cm) equilibrated with and eluted by 0.154 M NaCl/5 mM sodium acetate (pH 5.4). The average size of sphingomyelin/cholesterol (2 : 1, mol/mol) SUV (purified by centrifuging at 160000 × *g* for 1 h) and DPPC/cholesterol (2 : 1, mol/mol) SUV (purified by centrifuging at 10000 × *g* for 5 min) was estimated to be 187 ± 42 Å and 254 ± 48 Å, respectively, from negative-stain electron micrographs of the lipsomes using potassium phosphotungstate as the stain.

Multilamellar liposomes were prepared by bath sonication of 20 mg of dried lipid thin film composed of sphingomyelin/cholesterol (2 : 1, mol/mol) in 1 ml of 1 mM nitrilotriacetic acid/0.106 M sodium phosphate (pH 7.4) or by extrusion through a series of polycarbonate membrane filters with pore sizes ranging from 1.0 to 0.2 μm at 60 °C [17]. Sonication was carried out in a model G112 SPIT bath sonicator (Laboratory Supplies Co.) at 60–80 W in a 55 °C water-bath, using a glass tube (13 × 100 mm), for 10–15 min. The liposome suspension was annealed for 1 h at

65 °C, cooled, and passed over a Sepharose 4B or 2B column equilibrated in 5 mM phosphate-buffered isotonic saline (pH 7.4) [18]. The multilamellar liposomes free from entrapped nitrilotriacetic acid and SUV were collected in the void volume.

Loading procedure

The purified liposomes were loaded with Ga-67 or In-111 using the ionophores 8-hydroxyquinoline or acetylacetone as described previously [18–20]. Briefly, the Sephadex- or Sepharose-purified liposomes were loaded with Ga-67 or In-111 by adding 140–200 μl loading solution to 1 ml liposomes (1–5 mg/ml) dropwise, while the suspension of liposomes was vortexed gently, and incubating at room temperature for 1 h. The 8-hydroxyquinoline-$^{111}In^{3+}$ loading solution was prepared by mixing 70–100 μl indium-111 chloride (0.1–10 pmol) in 3 mM HCl with an equal volume of 360 μM 8-hydroxyquinoline/1.8% NaCl/20 mM sodium acetate (pH 5.5) immediately before the loading process. The same procedure was used to prepare the 8-hydroxyquinoline-$^{67}Ga^{3+}$ loading solution except that 60 μM 8-hydroxyquinoline was used. The loaded liposomes were purified by passage over a small column of AG 1-X8 equilibrated with 0.106 M sodium phosphate buffer (pH 7.4) and were eluted by the same isotonic phosphate buffer. The resin adsorbed 99.9% of the 8-hydroxyquinoline-$^{111}In^{3+}$ and 8-hydroxyquinoline that was not associated with liposomes [15,21].

In some cases, Ga-67 or In-111 was encapsulated in liposomes by sonicating the lipid mixture in 1 ml of the same nitrilotriacetic acid-phosphate isotonic buffer containing a trace of $^{67}Ga^{3+}$ or $^{111}In^{3+}$ and separating the liposome-entrapped Ga-67 or In-111 from the free cations in a Sephadex G-50 column (0.9 × 35 cm). Regardless of the preparation technique, the extent of leakage of the entrapped cations with all liposomes was tested in the presence or absence of serum was determined by chromatography on Sepharose 4B and/or by gamma-ray perturbed angular correlation [19]. Only liposomes of proven stability, as confirmed by the lack of release of In-111 in serum at 37 °C, were used.

Uptake of liposomes by tissues and parts of the body

For the biodistribution studies, various types of liposome were injected via the tail vein of BALB/c mice (mean body weight about 25 g). The lipid concentration was determined by ferrothiocyanate assay [22] or by phosphate analysis [23]. The mice were killed at various times post-injection by cervical dislocation and immediate decapitation.

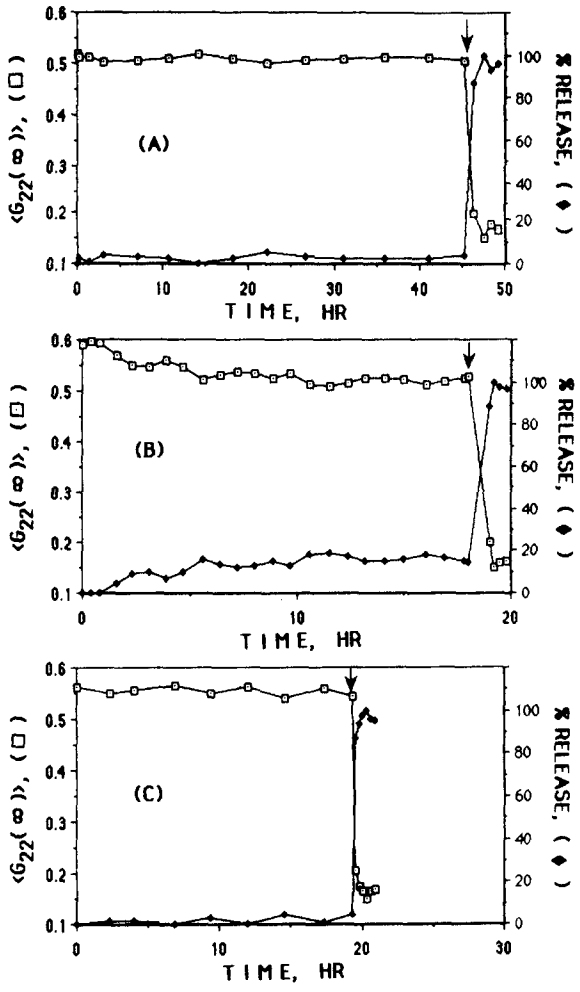

Fig. 1. Stability of liposomes in the presence of serum. (A) Sphingomyelin/cholesterol (2:1, mol/mol) SUV, (B) DSPC/cholesterol (2:1, mol/mol) SUV, and (C) DPPC/cholesterol (2:1, mol/mol) SUV were incubated with 50% rabbit serum at 37 °C, 37 °C and 24 °C, respectively. The time-course of release of the entrapped In-111 was monitored by the techniques of gamma-ray perturbed angular correlation. The arrow indicates the release of all entrapped In-111 by adding Triton X-100 (1% final concentration) to the liposome-serum incubation mixture. The percentages of In-111 released were calculated from the corresponding $\langle G_{22}(\infty)\rangle$ [15] and are shown on the right axis.

In controls, a mixture of free nitrilotriacetic acid-$^{111}In^{3+}$ and/or nitrilotriacetic acid-$^{67}Ga^{3+}$ was injected instead. All tissue distribution data is corrected for the radioactivity due to blood background in each tissue sample, using In-111 labeled erythrocytes [15]. In studying the effect of the injected liposomal dose on the rates of elimination of SUV from the blood and accumulation in tissues, the rates (expressed as the percentage of the total administered liposome dose accumulated in tissues or remained in the blood per 23 h) were plotted as a function of the normalized dose of the injected liposomal lipid (expressed as μg of total lipid per g of mouse body weight or μg/g).

Results

The stability of the various types of liposome used in the present study as estimated by the techniques of gamma-rays perturbed angular correlation is shown in Fig. 1. It appears that in the presence of serum and at 37 °C, both sphingomyelin/cholesterol (2 : 1, mol/mol) and DSPC/cholesterol (2 : 1, mol/mol) liposomes are quite stable, whereas DPPC/cholesterol (2 : 1, mol/mol) liposomes are relatively stable at room temperature. These findings are consistent with reports in the literature [15,24].

The biodistribution of sphingomyelin/cholesterol (2 : 1, mol/mol) MLV and SUV in mice are shown in Tables I and II, respectively. It is apparent that large MLV are taken up primarily by the reticuloendothelial cells in the liver. On the other hand, the SUV accumulate in many parts of the body. Besides the liver, the intestine, skin and carcass show quite a significant uptake of the SUV. Fig. 2 depicts the gradual accumulation of the SUV in the liver, intestine or skin in relation to the corresponding clearance of the liposomes from the blood. It appears that the uptake of the SUV occurs at both high and low dose of liposomal lipid. Fig. 3 shows that, while both the rates (as measured by the activity in tissues at 23 h post-injection) of clearange of SUV from the blood and the accumulation in the liver are affected by the dose of liposomal lipid, the rate of accumulation of SUV in the intestine, skin or carcass is independent of the injected lipid dose of liposomes.

To investigate whether or not the uptake of SUV is unique for sphingomyelin/cholesterol (2 : 1, mol/mol) SUV encapsulating nitrilotriacetic

TABLE I

TIME-COURSE OF BIODISTRIBUTION OF BOVINE BRAIN SPHINGOMYELIN/CHOLESTEROL (2 : 1, MOL/MOL) MLV ENTRAPPING NITRILOTRIACETIC ACID-$^{111}In^{3+}$ IN BALB/c MICE

The data are percentage ± S.D. of total administered dose present in indicated tissue at designated time post-injection.

Tissue	0.017 h ($n = 2$) [a]	0.25 h		1.00 h			5 h	12 h	24 h
		($n = 3$) [a]	($n = 2$) [b]	($n = 4$) [a]	($n = 2$) [b]	($n = 2$) [c]	($n = 2$) [b]	($n = 2$) [c]	($n = 2$) [c]
Blood	42.7±4.8	9.0±4.5	16.7±1.7	4.7±2.1	11.3±0.2	46.5±5.8	0.3±0.1	1.1±0.2	0.5±0.0
Liver	42.9±4.9	80.5±5.0	72.5±2.4	84.8±2.7	72.5±2.4	32.0±9.9	94.1±1.5	70.0±0.2	62.1±0.9
Spleen	0.7±0.2	4.4±0.6	3.6±0.2	5.6±0.8	3.0±0.7	1.5±0.4	1.8±1.3	3.1±0.3	2.0±0.3
Intestine	0.6±0.2	0.3±0.1	0.5±0.1	0.4±0.1	0.5±0.2	2.0±1.0	0.4±1.0	1.2±0.1	1.6±1.1
Skin	3.1±1.6	0.6±0.2	1.5±0.5	0.8±0.2	1.2±0.0	4.5±0.2	0.3±0.2	2.5±0.1	1.4±0.5
Carcass	5.1±0.2	1.8±0.5	2.3±0.2	1.3±0.3	1.2±0.4	12.4±2.8	0.6±0.1	4.6±0.3	3.4±1.1
Legs	0.9±0.2	0.5±0.2	1.0±0.1	0.5±0.0	0.6±0.1	1.2±0.2	0.2±0.1	0.9±0.2	0.5±0.2
Tail	1.8±1.4	2.0±1.0	1.0±0.0	1.3±0.7	2.4±0.5	2.0±0.4	2.0±0.7	2.1±1.0	1.8±0.4

[a] The liposomes were prepared by membrane extrusion. The liposomal dose was 4 μg total lipid/g mouse body weight. The liposomes were loaded with In-111 by 8-hydroxyquinoline-$^{111}In^{3+}$.

[b] The liposomes were prepared by 10 min bath sonication and isolated from the void volume of a Sepharose 2B column. The liposomal dose was 1 μg total lipid/g mouse body weight. The liposomes were loaded with In-111 by acetylacetone-$^{111}In^{3+}$.

[c] The liposomes were prepared by 10 min bath sonication and isolated from the void volume of a Sepharose 4B column. The liposomal dose was 0.1–0.2 μg total lipd/g mouse body weight. The liposomes were loaded with In-111 by 8-hydroxyquinoline-$^{111}In^{3+}$. The activity in the legs in this set of data was measured from dissected muscle.

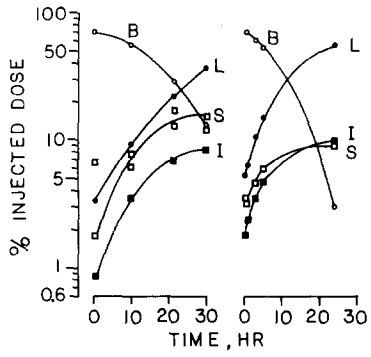

Fig. 2. The blood clearance and tissue uptake of sphingomyelin/cholesterol (2:1, mol/mol) SUV at low and high doses. Low doses correspond to 6 μg lipid/g body weight injected intravenously via the tail vein into BALB/c mice (right frame). Each point is an average of six measurements, with the standard deviation less than the size of the symbols. The abbreviations B, L, I and S next to the curves stand for blood, liver, intestine and skin, respectively. High doses correspond to an average of 93 μg lipid/g body wt. injected intravenously via the tail vein into BALB/c (left frame) mice. Except for the skin, each point is an average of two measurements with the standard deviation less than the size of the symbols. Indium-111 was loaded into liposomes by acetylacetone [19].

acid-$^{111}In^{3+}$, the biodistribution of SUV of different lipid compositions and entrapped markers were studied. Table III shows that the activity in the skin and intestine persists, when a different radioactive marker, $^{67}Ga^{3+}$, was encapsulated in the sphingomyelin/cholesterol (2:1, mol/mol) SUV. Table IV shows a similar pattern of the time-course of uptake of DSPC/cholesterol (2:1, mol/mol) SUV encapsulating nitrilotriacetic acid-$^{67}Ga^{3+}$. Table V shows the biodistribution of DPPC/cholesterol (2:1, mol/mol) SUV. Com-

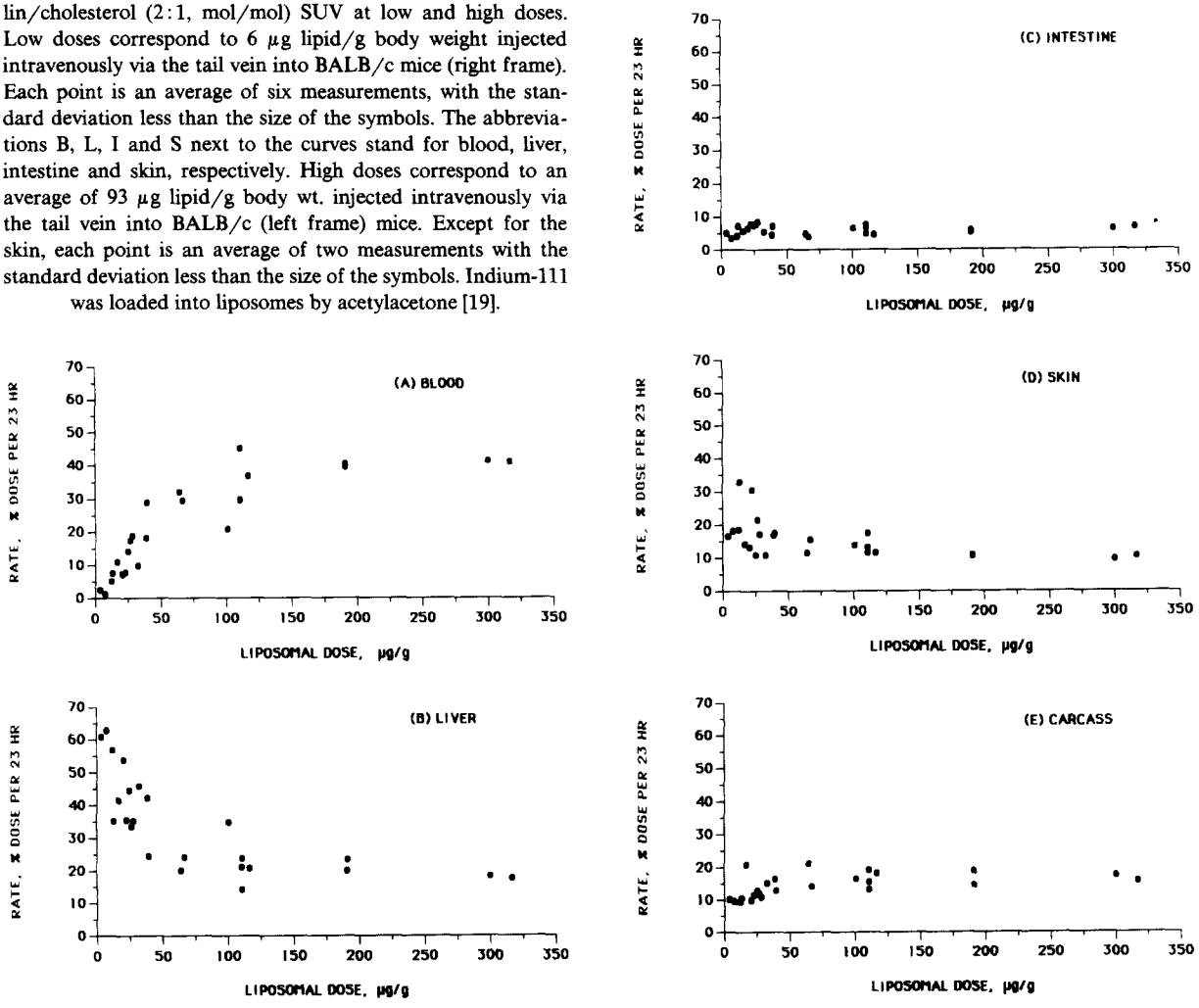

Fig. 3. Effect of liposomal lipid dose on the rate of elimination from the blood and the rates of accumulation in tissues. Various liposomal lipid doses of sphingomyelin/cholesterol (2:1, mol/mol) SUV were injected intravenously via the tail vein into BALB/c mice. The rates were expressed as the percentage of the total injected dose accumulated in a tissue or remaining in the blood per 23 h. The injected liposomal lipid doses were expressed as μg total lipid per g mouse body weight. In (E), the activity is the sum of the activities from the carcass and legs. Indium-111 was loaded into liposomes by 8-hydroxyquinoline [19].

TABLE II

TIME-COURSE OF BIODISTRIBUTION OF BOVINE BRAIN SPHINGOMYELIN/CHOLESTEROL (2:1, MOL/MOL) SUV ENTRAPPING NITRILOTRIACETIC ACID-$^{111}In^{3+}$ IN BALB/c MICE

The data are percentage ± S.D. of total administered dose present in indicated tissue at designated time post-injection. The liposomal dose is 35–75 μg total lipid/g mouse body weight. The liposomes were loaded with In-111 by 8-hydroxyquinoline-$^{111}In^{3+}$

Tissue	0.25 h ($n=6$)	1.0 h ($n=6$)	3.0 h ($n=10$)	5.0 h ($n=6$)	23.0 h ($n=6$)
Blood	73.8±2.4	72.5±2.3	64.8±1.9	62.0±1.3	15.7±7.2
Liver	5.2±0.5	5.4±1.0	7.3±1.2	9.1±0.7	44.3±8.0
Spleen	0.0±0.0	0.0±0.0	0.3±0.2	0.5±0.1	1.8±0.1
Intestine	1.4±0.2	1.8±0.9	3.0±0.3	3.3±0.3	5.7±1.2
Skin	2.4±0.3	2.0±0.3	4.2±0.9	4.7±1.0	9.3±2.7
Carcass	9.0±2.9	8.0±3.1	10.8±0.9	9.8±0.6	10.7±2.3
Legs	2.1±0.5	3.1±0.7	2.9±0.3	2.9±0.6	4.9±0.8
Tail	2.2±0.6	2.0±0.9	1.9±0.5	1.9±0.5	2.2±0.5

TABLE III

TIME-COURSE OF BIODISTRIBUTION OF BOVINE BRAIN SPHINGOMYELIN/CHOLESTEROL (2:1, MOL/MOL) SUV ENTRAPPING NITRILOTRIACETIC ACID-$^{67}Ga^{3+}$ IN BALB/c MICE

The data are percentage ± S.D. of total adminstered dose present in indicated tissue at designated time post-injection. The liposomal dose is 35–75 μg total lipid/g mouse body weight. The liposomes were loaded with Ga-67 by 8-hydroxyquinoline-$^{67}Ga^{3+}$.

Tissue	0.25 h ($n=4$)	1.0 h ($n=2$)	3.0 h ($n=4$)	5.0 h ($n=4$)	23.0 h ($n=4$)
Blood	70.8±1.0	72.5±1.3	63.2±2.6	56.5±3.0	16.7±3.2
Liver	7.8±2.9	5.5±0.9	7.4±0.9	11.7±4.7	41.5±2.9
Spleen	0.2±0.1	0.0±0.1	0.2±0.3	0.5±0.1	1.4±0.2
Intestine	1.4±0.1	1.6±0.3	2.9±0.4	4.1±0.8	8.0±2.3
Skin	2.2±0.6	3.2±0.4	4.5±0.9	4.9±0.7	9.0±1.6
Carcass	8.3±1.3	7.9±2.7	12.0±1.0	11.1±0.9	11.3±1.1
Legs	2.2±0.9	3.5±1.1	3.4±0.2	3.2±0.7	4.5±0.7
Tail	2.3±0.5	2.0±0.9	1.8±0.5	2.3±0.7	2.4±0.3

TABLE IV

TIME-COURSE OF BIODISTRIBUTION OF DSPC/CHOLESTEROL (2:1, MOL/MOL) SUV ENTRAPPING NITRILOTRIACETIC ACID-$^{67}Ga^{3+}$ IN BALB/c MICE

The data are percentage ± S.D. ($n=2$) of total administered dose present in indicated tissue at designated time post-injection. The marker Ga-67 were entrapped in the SUV by sonicating the lipid thin film in 1 mM nitriloacetic acid-$^{67}Ga^{3+}$/0.106 M sodium phosphate (pH 7.4) (Materials and Methods).

Tissue	0.25 h	1.0 h	3.0 h	23.0 h
Blood	77.1±1.9	68.1±0.2	56.3±2.2	10.9±0.3
Liver	4.1±0.9	6.4±0.3	13.9±1.7	39.7±2.9
Spleen	0.0±0.0	0.4±0.0	0.9±0.1	2.3±0.4
Intestine	1.0±0.0	2.0±0.5	3.8±0.6	8.5±0.1
Skin	2.9±0.2	2.7±0.6	4.6±0.1	10.3±2.5
Carcass	7.5±1.0	10.4±0.1	10.8±0.2	14.1±0.1
Legs	2.2±0.1	3.3±0.7	3.3±0.6	6.3±0.3
Tail	1.8±0.0	3.2±1.0	2.8±0.4	2.7±0.0

TABLE V

TIME-COURSE OF BIODISTRIBUTION OF DPPC/CHOLESTEROL (2:1, MOL/MOL) SUV ENTRAPPING NITRILOTRIACETIC ACID-$^{111}In^{3+}$ IN BALB/c MICE

The data are percentage ± S.D. ($n=4$) of total administered dose present in indicated tissue at designated time post-injection. The liposomes were loaded with In-111 by 8-hydroxyquinoline-$^{111}In^{3+}$.

Tissue	0.25 h	1.0 h	3.0 h	5.0 h	23.0 h
Blood	58.8±4.3	47.8±3.8	42.9±4.1	36.0±1.6	8.4±2.0
Liver	5.9±2.0	8.7±1.7	11.5±1.4	15.0±2.0	30.3±1.3
Spleen	0.3±0.1	0.6±0.3	1.0±0.1	1.4±0.1	2.2±0.3
Intestine	1.9±0.4	2.5±0.3	4.4±0.8	5.4±0.2	9.2±0.6
Skin	6.5±2.2	9.3±2.0	9.3±1.6	11.1±2.2	11.4±1.7
Carcass	13.6±1.8	15.4±0.7	15.4±0.7	16.0±1.2	18.7±1.6
Legs	3.9±0.8	4.6±1.8	5.1±0.9	5.3±1.0	6.9±1.0
Kidneys	2.9±0.2	4.5±0.9	4.5±0.9	4.5±0.7	6.6±0.7
Tail	2.4±2.1	2.4±1.5	2.4±1.0	2.2±1.8	1.9±0.0

TABLE VI

TIME-COURSE OF BIODISTRIBUTION OF NITRILOTRIACETIC ACID-$^{111}In^{3+}$ AND NITRILOTRIACETIC ACID-$^{67}Ga^{3+}$ IN BALB/c MICE

The data are percentage ± S.D. ($n = 4$) of total administered dose present in indicated tissue at designated time post-injection.

Tissue	In-111				Ga-67			
	0.17 h	1 h	12 h	24 h	0.17 h	1 h	12 h	24 h
Blood	36.7±5.7	29.1±0.7	8.3±0.6	3.3±4.0	32.0±4.0	28.4±2.1	6.6±0.5	3.7±0.6
Liver	4.8±0.7	4.8±0.3	7.0±0.8	8.9±0.8	4.0±0.7	4.4±0.1	6.2±0.5	10.1±1.3
Intestine	7.9±2.1	9.7±1.2	11.7±2.2	9.4±0.6	6.1±1.4	6.4±1.4	6.2±1.3	9.3±0.8
Skin	14.0±1.4	18.8±2.0	7.7±0.5	4.8±0.3	18.3±2.0	18.8±1.9	13.3±1.3	12.3±0.1
Carcass	39.7±3.0	37.0±2.3	25.5±2.9	18.3±2.8	38.2±3.0	34.5±3.3	22.9±0.7	19.6±2.4
Leg muscle	5.5±1.7	4.0±0.3	2.5±1.5	3.6±0.5	5.4±1.3	4.2±0.4	3.0±1.4	2.8±0.6
Kidneys	2.4±0.2	1.8±0.6	2.3±0.1	1.8±0.2	5.1±1.3	4.7±1.2	12.0±2.2	10.7±2.2
Excretion	0.0±0.0	3.8±3.5	34.2±2.4	45.6±4.4	0.0±0.0	7.6±6.9	28.4±4.7	25.1±5.7
Tail	4.1±1.6	2.3±1.5	2.3±1.5	2.4±0.7	3.9±1.6	2.8±0.5	2.5±2.0	2.8±0.6

pared to the biodistribution of sphingomyelin/cholesterol (2 : 1, mol/mol) SUV or DSPC/cholesterol (2 : 1, mol/mol) SUV, DPPC/cholesterol (2 : 1, mol/mol) SUV showed no additional In-111 activity in the intestine or skin, despite the fact that DPPC/cholesterol (2 : 1, mol/mol) SUV are leaky, although concentrations in the kidneys and carcass were elevated. Furthermore, in contrast to the time-dependent accumulation of the activity of SUV in the intestine and skin (Fig. 1), the activities of the free markers in these two tissues either decrease with time or remain relatively constant from 10 min to 24 h post-injection (Table VI).

Discussion

The lack of uptake of large MLV by non-reticuloendothelial tissues is primarily a result of the physical confinement of the large liposomes to the vascular bed. On the other hand, the accumulation of the SUV-encapsulated radioactive marker could be accounted for in two ways: one characterized by the distribution of intact SUV and the other due to the distribution of the released marker. When the liposomes are leaky in the blood, as in the case of DPPC/cholesterol (2 : 1, mol/mol) SUV [24], the biodistribution data of the liposomes delineate a composite distribution of the intact liposomes and the released marker. On the other hand, if the liposomes are stable in the blood, such as sphingomyelin/cholesterol (2 : 1, mol/mol) SUV or DSPC/cholesterol (2 : 1, mol/mol) SUV, the data of biodistribution principally reflects the distribution of intact liposomes.

Several lines of evidence suggest that the activity entrapped in sphingomyelin/cholesterol (2 : 1, mol/mol) SUV or DSPC/cholesterol (2 : 1, mol/mol) SUV in the intestine, skin, and perhaps the carcass is delivered via intact liposomes. First, these two types of liposome remained intact in vitro and in vivo during the entire course (24 h) of the study, as indicated by the gamma-ray perturbed angular correlation studies (Fig. 1). Second, if the biodistribution were accounted for by released In-111, the activity of the SUV-entrapped In-111 in the skin should decrease with time, as the free markers would behave (Table IV). The apparent pattern of the time-dependent accumulation of SUV-encapsulated activity in the skin (Fig. 2, Tables II and III) favors the mechanism of SUV-mediated uptake of entrapped markers in the skin.

Third, if the tissue uptake were due to the released In-111, the released $^{111}In^{3+}$ should bind tightly to serum or tissue proteins, giving a low time-integrated perturbation factor, $\langle G_{22}(\infty)\rangle$ [15,16]. Mauk and Gamble [24] reported that the activity of DSPC/cholesterol (2 : 1, mol/mol) SUV-encapsulated $^{111}In^{3+}$ observed in the skin, intestine and the carcass at 3 h post-injection was

due primarily to free nitrilotriacetic acid-$^{111}In^{3+}$ entrapped in the liposomes, as indicated by the high integrated perturbation factor, $\langle G_{22}(\infty)\rangle$. Finally, the gradual increase of the liposomal activity in the intestine and skin with a decreasing concentration of liposomes in the blood shown in Fig. 2 and Table II suggests that the liposomal activities observed in these two tissues are truly liposomally delivered.

It has been shown that liposomal lipid can be transferred and reutilized by cells that do not involve in the original process of the uptake of liposomes [5]. The uptake of MLV by skin, intestine and carcass remains consistently low over a long period up to at least 24 h (Table I). This observation is true for MLV prepared either by membrane extrusion or by bath sonication. Thus, the SUV-encapsulated activity shown in the intestine, skin and carcass does not appear to be from the redistribution of marker after the SUV have been degraded by other tissues such as liver.

The biodistribution of DSPC/cholesterol SUV in mice has been reported previously [24,29]. However, the published data appeared to be conflicting. Using DSPC/cholesterol (2 : 1, mol/mol) SUV entrapping nitrilotriacetic acid-$^{111}In^{3+}$ in the aqueous phase, Mauk and Gamble [24] reported a tissue distribution quite similar to the data shown in Table III. Using DSPC/cholesterol (1 : 1, mol/mol) SUV containing $^{111}In^{3+}$-bleomycin, which can be embedded in the lipid bilayer of the liposomal membrane and is also soluble in the inner aqueous compartment, Senior et al. [29] showed a significant (about 35% of the injected dose) localization of the activity of In-111 in the carcass. As indicated by the results of the scintigraphic imaging of the same type of liposomes in rats, the activity in the carcass was shown to be primarily due to the uptake of In-111 by the bone marrow. The exact cause of the discrepancy between these two reports is not known.

It has been shown by many investigators that, after the injection of an excess lipid dose of liposomes to experimental animals, the blood level of liposomes rises and the extent of uptake of large liposomes by the liver and spleen is temporarily suppressed [5,25–28]. This indicates that the uptake of large liposomes by the phagocytic cells in the reticuloendothelial system is a saturable process. Mathematically, a saturable process may be described by a non-linear, capacity-limited Michaelis-Menten process.

On the other hand, we have previously shown that the kinetics of the uptake of sphingomyelin/cholesterol (2 : 1, mol/mol) SUV by the liver in the mouse are compatible with a model involving two parallel pathways [16]. One pathway is a non-linear, capacity-limited Michaelis-Menten process contributed primarily by the phagocytosis of SUV by the phagocytic Kupffer cells in the liver (the initial, saturating phase in Fig. 3A and B), the other pathway is a linear, non-saturable process, which follows first-order kinetics (the subsequent, saturated phase in Fig. 3A and 3B). It has been postulated that pinocytosis (or fluid-phase endocytosis) of the parenchymal cells in the liver is the predominant contribution of the non-saturable process of the hepatic uptake of SUV. The linear, non-saturable kinetics shown in Fig. 3C, D and E suggest that the uptake of intact sphingomyelin/cholesterol (2 : 1, mol/mol) SUV or DSPC/cholesterol (2 : 1, mol/mol) SUV by the intestine, skin and the carcass are not due to phagocytic cells.

Using the technique of gamma-ray perturbed angular correlation, it was shown that the DSPC/cholesterol (2 : 1, mol/mol) SUV taken up by the skin, intestine and carcass were mostly degraded by 12–24 h post injection, while the SUV remaining in the blood were still intact [24]. This suggests that certain cellular components are actively involved in the degradation of the SUV taken up by these tissues. Neither the cells involved in the uptake of SUV by these non-reticuloendothelial tissues nor the site (intracellular or extracellular sites) of the degradation is known at this time. Further characterization at the cellular level is required.

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