Biochimica et Biophysica Acta, 1068 (1991) 133–141
© 1991 Elsevier Science Publishers B.V. All rights reserved 0005-2736/91/$03.50
ADONIS 000527369100338K

BBAMEM 75359

Pharmacokinetics of stealth versus conventional liposomes: effect of dose

T.M. Allen and C. Hansen

Department of Pharmacology, University of Alberta, Edmonton (Canada)

(Received 5 March 1991)

Key words: Pharmacokinetics; Liposome; Reticuloendothelial system; Mononuclear phagocyte system; Poly(ethylene glycol); Monosialoganglioside

Liposomes which substantially avoid uptake into the mononuclear phagocyte system (MPS), termed Stealth® liposomes, have recently been formulated (Allen, T.M. and Chonn, A., (1987) FEBS Lett. 223, 42–46). The pharmacokinetics of stealth liposomes as a function of liposome dose and a comparison to conventional liposome pharmacokinetics, was the subject of the present study. We have examined the tissue distribution of two different formulations of stealth liposomes, i.e., sphingomyelin : egg phosphatidylcholine : cholesterol : monosialoganglioside GM_1 (SM : PC : CHOL : GM_1) 1 : 1 : 1 : 0.2 and SM : PC : CHOL : polyethylene glycol distearoylphosphatidylethanolamine (PEG(1900)-DSPE) 1 : 1 : 1 : 0.2, and compared them with the tissue distributions seen for a liposomal formulation which is avidly removed from circulation by the cells of the MP system (PC : CHOL, 2 : 1). Tissue distribution in mice was examined over a 100-fold concentration range (0.1 to 10 μmol phospholipid / mouse) and at several time points over a 48 h time period. Liposome size ranged from 92–123 nm in diameter for all compositions. Clearance from blood of PC : CHOL liposomes following intravenous administration showed a marked dose dependence (i.e., saturation-type or Michaelis-Menten kinetics), with MPS uptake decreasing and% of injected dose in blood increasing as dose increased, over the entire dosage range. Injection of stealth liposomes, on the other hand, resulted in % of injected doses of liposomes in MPS, blood and carcass which were dose-independent and log-linear (first order kinetics) over the entire dosage range. The doses of stealth liposomes containing PEG(1900)-DSPE required for MPS saturation was higher than 10 μmol phospholipid / mouse or 400 μmol / kg. The dosage-independence of the pharmacokinetics of stealth liposomes and their lack of MPS saturation within the therapeutic dose range are two more assets, in addition to the prolonged circulation half-lives, leading towards their eventual use as drug delivery systems in the clinic.

Introduction

Liposomes of novel formulations, with long circulatory half-lives and low uptake by mononuclear phagocyte cells (Stealth® liposomes), have recently been described [1–6]. These liposomes have greatly decreased uptake into the mononuclear phagocyte system (MPS, also termed the reticuloendothelial system) as compared to liposome formulations which have been widely used in the past. Because of their pronounced tendency to rapidly localize in and saturate the MPS, liposomes of conventional formulations can alter the ability of this important host defense system to remove foreign particulate material from circulation, causing MPS impairment [7,8].

A number of studies have examined tissue uptake of liposomes as a function of liposome dose [7,9–13], liposome size [10], time post-injection [7], pre-dosing with liposomes [12,14] or other particulate material [15] and the presence of tumour cells [11]. In each study, decreased amounts of liposomes in liver and increased amounts of liposomes in spleen and blood were observed with increasing liposome dose. These observations have been interpreted as demonstrating that saturation of the ability of liver macrophages to bind to and endocytose liposomes occurs at higher lipid doses, with a concomitant increase in blood levels of liposomes and subsequent 'spillover' into spleen. Mauk and Gamble [9] have reported a liver saturating dose in mice of approx. 0.5 mg/mouse (0.28 μmol lipid/mouse) for

Correspondence; T.M. Allen, Department of Pharmacology, University of Alberta, Edmonton, Canada T6G 2H7.

small unilamellar liposomes (SUV) composed of distearoylphosphatidylcholine : cholesterol, 2 : 1 at 3 h after intravenous injection. Since regeneration of MPS binding or uptake sites for liposomes and other particulate matter is occurring continually over time, a detailed understanding of liposomal pharmacokinetics and the MPS saturating effects of liposomes can be only obtained from experiments where data points are collected over a period of many h, for a wide range of liposome doses. No such detailed experiments have been conducted to date.

We have examined the tissue distribution of liposomes over a 100-fold concentration range, for 48 h following intravenous injection into mice, in order to determine, if possible, values for MPS saturating doses for MPS-seeking liposomes and for MPS-avoiding (Stealth) liposomes and the pharmacokinetics of each formulation with increasing dose. As liposomal drug delivery systems approach the time when they will be used in therapeutic applications, studies such as these will help us to establish guidelines for appropriate liposome compositions, doses and time between doses, in order to avoid, as much as possible, complications which may arise because of MPS impairment.

Materials and Methods

Egg phosphatidylcholine (PC) and bovine brain sphingomyelin (SM) were purchased from Avanti Biochemicals (Birmingham, AL). Monosialoganglioside (GM_1) was purchased from Makor Chemical (Jerusalem). Cholesterol (CHOL) and *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) was purchased from Sigma Chemical (St. Louis, MO). Polyethylene glycol distearoylphosphatidylethanolamine (PEG(1900)-DSPE) was a gift from Liposome Technology (Menlo Park, CA); the synthesis of this compound is described in Ref. 6. Lα-3-Phosphatidylcholine 1,2-di[1-^{14}C]palmitoyl ([^{14}C]DPPC) was purchased from Amersham International (U.K.). Tyraminylinulin was synthesized and ^{125}I-tyraminylinulin was prepared according to the technique of Sommerman et al. [16]. Na^{125}I was obtained from the Edmonton Radiopharmaceutical Center. Pyrogen-free saline for injection (0.9%, USP) was obtained from Travenol Canada (Mississauga, Ontario). All test-tubes, syringes etc. used in the experiments were sterile and pyrogen-free.

Liposome preparation

Liposomes were composed of either PC : CHOL, 2 : 1, SM : PC : CHOL : GM_1, 1 : 1 : 1 : 0.2 or SM : PC : CHOL : PEG(1900)-DSPE, 1 : 1 : 1 : 0.2. Liposomes were prepared by vortexing dried lipid films in sterile, pyrogen-free 0.9% saline buffered with 10 mM TES (pH 7.4) (buffered saline) containing ^{125}I-tyraminylinulin. Lipid concentrations were normally 10μmol/ml. Liposomes were extruded 10-times through 0.1μm Nuclepore filters according to Olsen et al. [17]. This procedure has been shown to result in primarily unilamellar vesicles [18]. The resulting liposomes were sized by dynamic light scattering using a Brookhaven BI90 particle sizer (Brookhaven Instrument, Holtsville, NY) and trapped volumes were determined. Liposome size ranged from 92–123 nm and trapped volumes, as determined from the specific activity of entrapped ^{125}I-TI, averaged around 2 l/mol phospholipid.

For experiments in which higher doses were required for injection, large volumes of extruded liposomes were concentrated by placing the liposomes in 50000 Da exclusion-limit dialysis tubing (Spectra/Por) and coating the outside with dry Sephadex G-50 (Pharmacia, Sweden), according to the procedure of Abra and Hunt [10]. Non-entrapped ^{125}I-tyraminylinulin was removed by passing the liposomes over Ultragel AcA 34 (IBF Biotechnics, France) or by dialysing the samples against buffered saline.

Animal experiments

Female ICR (outbred) mice in the weight range of 23–27 g were obtained from the Animal Breeding Unit of the University of Alberta and maintained in standard housing. Mice (three per group) were given a single bolus injection in the tail vein with 0.2–0.3 ml of liposomes containing 10^5–10^6 cpm of entrapped ^{125}I-tyraminylinulin and 0.1–10μmol phospholipid, as desired. At selected times post-injection, i.e., 0.08, 0.25, 0.5, 1, 2, 6, 12, 24 and 48 h, the mice were sacrificed and tissues excised and counted for radiolabel in a Beckman 8000 gamma-counter. Tissues sampled included liver, spleen, lung, heart, blood and carcass (remainder of the animal). Blood-correction factors, having previously been determined [2], were applied to tissues and carcass. The ^{125}I-tyraminylinulin label is an excellent marker for intact liposomes as the label, when released from liposomes in body fluids, is rapidly eliminated from the body via kidney filtration and the label is also metabolically inert [1,16]. The data is presented as % of in vivo cpm, which corrects for leakage of the label and represents intact liposomes remaining in the body at given time points, allowing for a more accurate picture of the pharmacokinetics to be obtained [7]. The extent of leakage of label from the circulating liposomes at any given time point is proportional to the ratio of cpm which remain in the whole animal (remaining cpm = sum of cpm in all dissected tissues plus total blood volume plus residual carcass) to injected cpm at time 0.

Pharmacokinetic analyses

Pharmacokinetic parameters were determined for phospholipid doses of 0.1, 0.5, 1, 2, 5 and 10μmol/

mouse, using the JANA (Statistical Consulting, Lexington, KY) and the PCNONLIN, v3.0 (Statistical Consulting, Lexington, KY) pharmacokinetic programs.

Results

Liposomes composed of PC: CHOL, 2:1 are readily recognized and removed from circulation by the cells of the MPS. The blood, liver plus spleen and carcass levels of 0.1 μm diameter liposomes of this composition, as a function of time and dose, is shown in Fig. 1A–C. Although liver and spleen data were collected and analyzed separately, this did not contribute in any substantial way to the understanding or discussion of the results so they were added together *.

The elimination of PC:CHOL liposomes from blood shows non-linear pharmacokinetics (Fig. 1A), indicating saturation of some component of uptake into the MPS (Fig. 1B). The shift in the curves, with increasing dose, away from a rapid component of elimination at the early times suggests that there may be two mechanisms of elimination, possibly involving two different processes one of which has low capacity and is easily saturated at doses of less than 0.5 μmol mouse and probably closer to 0.1 μmol/mouse and the other of which has a high capacity. As can been seen in Fig. 1B, as time post-injection increases, % MPS uptake of liposomes increases and as dose increases, % uptake of liposomes at any time point decreases. The highest levels of MPS uptake, i.e., 80% of in vivo cpm, were observed only for the lowest liposome doses (0.1 μmol/mouse), which plateaued in the MPS at these levels by 6 h post-injection (Fig. 1B and Table I). At the same time point, less than 5% of liposomes were still circulating in blood (Fig. 1A), the remainder of liposomes being found in carcass tissues (Fig. 1C). This is probably the only liposome dose where the blood clearance and MPS uptake is dominated by the low capacity process.

The % uptake into the MPS of higher liposome doses was approx. 20% lower than that seen for doses of 0.1 μmol/mouse, averaging 61.4 $\pm$ 5.8% for liposome doses of 0.5 to 10.0μmol phospholipid/mouse at 48 h post-injection (Table I). For these doses, the blood clearance and MPS uptake is dominated by the high capacity process. The amount of time that it took to reach the maximum intact liposome uptake of approx. 60% increased with increasing dose. For doses of 0.5, 1.0 and 2.0 μmol phospholipid/mouse, maximum MPS uptake of liposomes occurred at approx. 12 h post-injection with little further uptake occuring up to 48 h. For doses of 5.0 μmol/mouse maximum uptake occurred at 24 h and maximum MPS uptake did not occur until 48 h post-injection for doses of 10.0 μmol/mouse. In other words, it required less time for the lower liposome doses to be removed from circulation, but given sufficient time the MPS could remove approx. 60% of the dose, independent of dose.

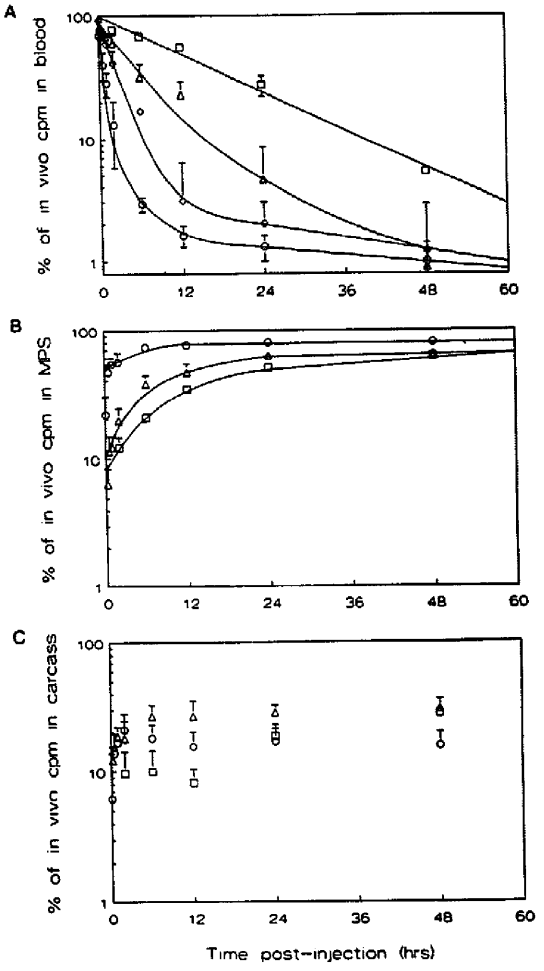

Fig. 1. Tissue distribution of PC:CHOL, 2:1 liposomes (MLV extruded through 0.1μm Nuclepore filters), labelled with ^{125}I-tyraminylinulin, in mice as a function of increasing time post-injection following i.v. injection in 0.2 to 0.3 ml buffer. (A) % of cpm remaining in vivo which are circulating in blood. (B) % of cpm remaining in vivo which have been taken up by liver and spleen. (C) % of cpm remaining in vivo which are associated with remaining carcass tissues. (○) 0.1 μmol/mouse, (◇) 0.5 μmol/mouse, (△) 1.0 μmol/mouse, (□) 10.0 μmol/mouse. Results are given as mean $\pm$ S.D., $n = 3$.

* In our experiments we find consistently that liver plus spleen uptake account for the majority of uptake into MPS organs [4,6] and therefore define MPS uptake for the purposes of this paper as uptake into liver plus spleen, recognizing that uptake into liver hepatocytes or into bone marrow may also be taking place.

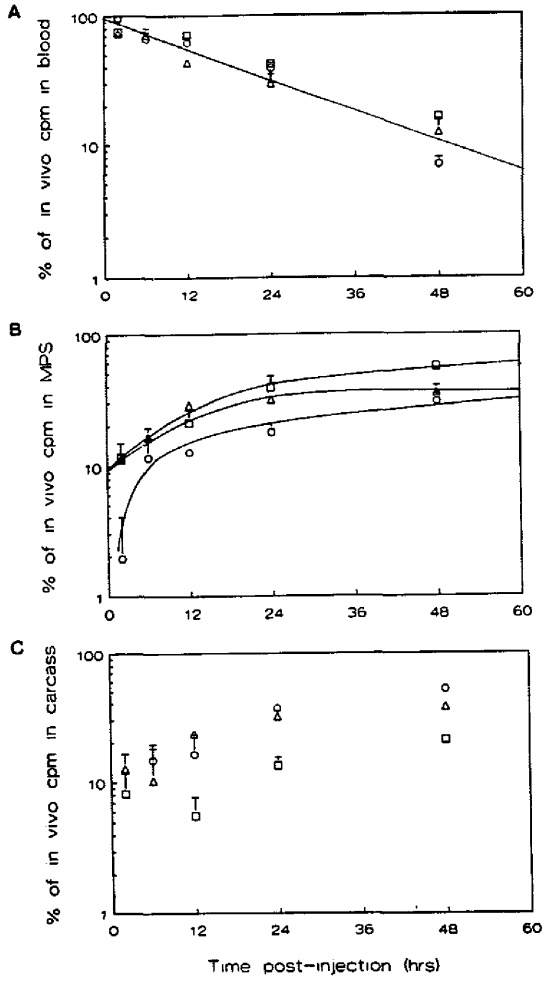

Fig. 2. Tissue distribution of SM:PC:CHOL:GM_1, 1:1:1:0.2 liposomes (MLV extruded through 0.1 μm Nuclepore filters), labelled with ^{125}I-tyraminylinulin, in mice as a function of increasing time post-injection following i.v. injection in 0.2 to 0.3 ml buffer. (A) % of cpm remaining in vivo which are circulating in blood. (B) % of cpm remaining in vivo which have been taken up by liver and spleen. (C) % of cpm remaining in vivo which are associated with remaining carcass tissues. (○) 0.1 μmol/mouse, (△) 1.0 μmol/mouse, (□) 10.0 μmol/mouse. Results are given as mean ± S.D., $n = 3$.

Carcass levels of PC:CHOL liposomes tended to increase slowly with time over 48 h (Fig. 1C), reaching levels of approx. 30% of in vivo cpm by 48 h after injection. Previous experiments have shown equivalent carcass levels of PC:CHOL liposomes with the liposomes associated at low levels with most carcass tissues sampled [4]. Carcass levels showed considerable variability at the earlier time points (Fig 1C).

When liposomes were composed of SM:PC:CHOL:GM_1, 1:1:1:0.2 (first generation Stealth® liposomes) blood clearance was log-linear and did not change with dose (Fig. 2A). The linear nature of the data provides no evidence for a high affinity, low capacity uptake system for these liposomes similar to that seen for PC:CHOL liposomes, even at injected doses of 0.1 μmol/mouse. Although total uptake of liposomes into the MPS increased with increasing time post-injection, as also seen with PC:CHOL liposomes, the extent of liposome uptake was greatly diminished relative to the preceding formulation (Fig. 2B vs. Fig. 1B). By 24 h post-injection an average of only 28% of in vivo cpm was in liver and spleen for all doses. The MPS uptake of PC:CHOL liposomal phospholipid, for liposomes of similar size, was from double to quadruple that seen for GM_1-containing liposomes, depending on injected dose. There was a tendency for MPS uptake of GM_1-containing liposomes to increase with increasing dose, which may be related to the tendency of the average liposome size to creep up at the higher doses (from 92 nm at the two lowest doses, 101 nm at the two intermediate doses and 123 nm at the two highest doses). The observations with stealth liposomes are distinctly different from the pronounced tendency of PC:CHOL liposomes to show decreased MPS levels with increasing dose.

Blood levels decreased with time in a dosage-independent manner, with an apparent circulation half-life of 16.4 ± 3.1 h (Table II) and by 48 h post-injection an average of 10% of intact GM_1-containing liposomes still remained in circulation (Fig. 2A). Liposome levels in carcass increased considerably with time, although a higher percentage was associated with carcass at the lowest dose, than at higher doses. Maximum carcass

TABLE I

A comparison of total MPS uptake (liver plus spleen) of intact liposomal phospholipid, as a function of time post-injection and dose, for liposomes composed of PC:CHOL, 2:1 (MLV extruded through 0.1 μm Nuclepore filters). Mean ± S.D., $n = 3$.

Dose (μmol/mouse)	MPS uptake (μmol phospholipid)				
	2 h	6 h	12 h	24 h	48 h
0.1	0.04±0.01	0.07±0.0	0.07±0.01	0.08±0.01	0.08±0.0
0.5	0.10±0.03	0.21±0.02	0.28±0.04	0.37±0.05	0.34±0.05
1.0	0.16±0.10	0.36±0.09	0.58±0.08	0.63±0.07	0.66±0.03
2.0	0.3 ±0.1	0.5 ±0.2	1.0 ±0.05	1.0 ±0.1	1.1 ±0.2
5.0	0.67±0.1	1.3 ±0.2	2.2 ±0.2	2.9 ±0.2	2.8 ±0.2
10.0	1.2 ±0.2	2.1 ±0.1	3.4 ±0.3	5.0 ±0.4	6.2 ±0.2

TABLE II

Pharmacokinetic parameters

Liposome composition	Injected dose (μmol)	Volume of distribution (ml)	Elimination rate constant (h^{-1})	AUC (nmol h/ml)	Half-life (h)		Total body clearance (ml/h)
					$T_{1/2\alpha}$	$T_{1/2\beta}$	
PC:CH	0.1	2.7	0.39	93	0.7	1.8	1.08
2:1	0.5	2.2	0.24	924	2.0	2.9	0.54
	1.0	2.4	0.16	2591	3.2	4.4	0.39
	2.0	2.1	0.10	9573		6.9	0.21
	5.0	2.3	0.06	34512		11.1	0.15
	10.0	2.1	0.05	89295		13.0	0.11
SM:PC:CH:GM_1	0.1	1.9	0.05	1068		13.9	0.09
1:1:1:10%	0.5	1.9	0.04	6208		16.5	0.08
	1.0	2.4	0.04	10282		16.9	0.10
	2.0	2.1	0.05	17443		12.8	0.11
	5.0	2.2	0.04	53662		16.6	0.09
	10.0	2.2	0.03	138754		21.6	0.07
SM:PC:CH:	0.1	2.3	0.04	1202		19.2	0.08
PEG-DSPE	0.5	2.1	0.05	5255		15.1	0.10
1:1:1:10%	1.0	2.2	0.04	11156		17.5	0.09
	2.0	2.1	0.03	29225		22.2	0.07
	5.0	2.3	0.03	65126		21.3	0.08
	10.0	2.1	0.03	170150		24.8	0.06

levels obtained by 48 h post-injection averaged 43% of in vivo cpm (Fig. 2C), a few percent higher than carcass levels seen for the higher doses of PC:CHOL liposomes (Fig. 1C) and liposomes were distributed between all carcass tissues samples [4].

The dosage-independence of blood clearance and MPS uptake of stealth liposomes could also be convincingly demonstrated with the newest generation of stealth liposomes composed of SM : PC : CHOL : PEG(1900)-DSPE (Fig. 3A-C). Blood clearance showed log-linear pharmacokinetics, for all doses, with an apparent circulation half-life of 20.0 ± 3.5 h (Table II), which is not a statistically significant difference from the value found for GM_1-containing liposomes. MPS uptake of these liposomes averaged 27% of in vivo cpm at 24 h post-injection, a figure very similar to that found for GM_1-containing liposomes. Carcass levels at 48 h (average of 46%) were also very similar to that seen for liposomes containing GM_1. In these experiments, there were no statistically significant differences in the rates at which liposomes of all three compositions lost their contents in vivo. At 48 h post-injection, 44.9 ± 4.9% of PC:CHOL liposomes remained *in vivo*, while comparative figures for SM:PC:CHOL:GM_1 and SM : PC : CHOL : PEG(1900)-DSPE liposomes were 50.0 ± 3.9% and 46.7 ± 3.7% respectively (not shown). Therefore, differences in the amount of *in vivo* label could not explain any of the aforementioned observations, not to mention that the method of data analysis automatically corrects for different leakage rates between different formulations [7].

At very low doses of liposomes it is possible that the liposomes could be converted all or in part to lipoproteins and the clearance of the lipoproteins could be very different from that of intact liposomes. We have also used a [^{14}C]DPPC phospholipid label, in PC:CHOL, 2:1 liposomes, for experiments at 0.5 μmol/mouse, in order to understand how lipid exchange and/or transfer to plasma proteins might be contributing to our clearance data. At the early time points, up to approx. 12 h post-injection, the results for 0.5 μmol/mouse labelled with either lipid label or aqueous space label were very similar (not shown). However, a comparison of lipid label at 0.01 μmol/mouse and aqueous label at 0.05 μmol/mouse (the lowest dose we could give of aqueous label due to the limiting specific activity of the label) shows that the lipid label is removed from blood dramatically slower than the aqueous space label at a 5-fold higher dose, suggesting that the liposomal phospholipids at the very low dose of 0.01 μmol/mouse are immediately and completely transferred to and/or exchanged with plasma lipoproteins and the clearance rate is that for lipoproteins, rather than for intact liposomes (Fig. 4). The use of an aqueous space label which measures intact liposomes clearly is advantageous in our pharmacokinetic studies, as the results would not be expected to be complicated by exchange/transfer to lipoproteins. It is obvious that all of the liposomes have not disintegrated completely at the higher dose of 0.05 μmol/mouse, otherwise no label would be left in vivo after one-half hour post-injection. However, we do

have evidence for approx. 20% increased label leakage at this low dose, suggesting that a portion of the liposomes have been converted to lipoproteins.

Pharmacokinetic parameters, as generated using the PCNONLIN pharmacokinetic program, are given in Table 2 for all three liposome compositions, for all injected doses. The volume of distribution for all three liposome compositions is only slightly higher than the blood volume of the mice used in the experiments suggesting that none of the liposomes are capable of

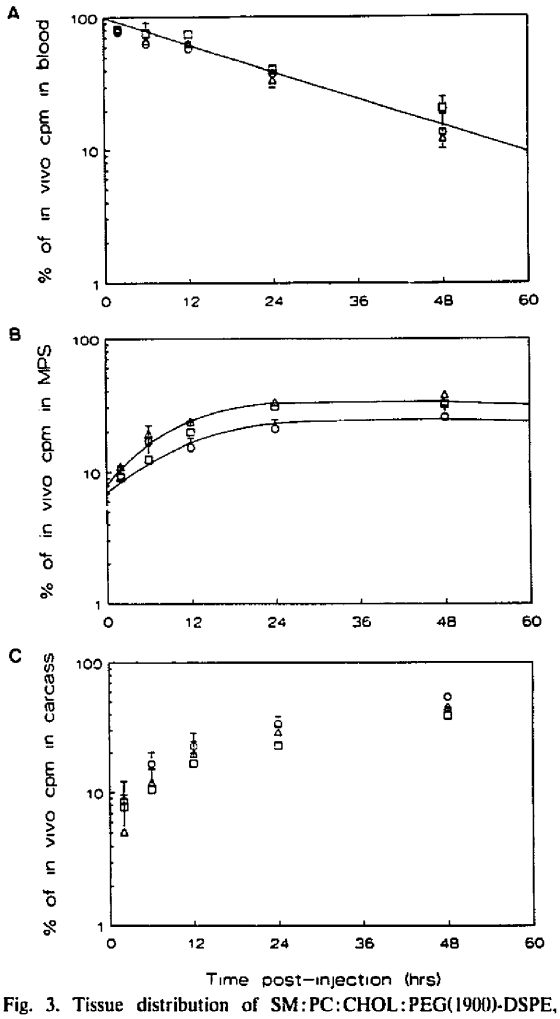

Fig. 3. Tissue distribution of SM:PC:CHOL:PEG(1900)-DSPE, 1:1:1:0.2 liposomes (MLV extruded through 0.1 μm Nuclepore filters) in mice as a function of increasing time post-injection following i.v. injection in 0.2 to 0.3 ml buffer. (A) % of cpm remaining in vivo which are circulating in blood. (B) % of cpm remaining in vivo which have been taken up by liver and spleen. (C) % of cpm remaining in vivo which are associated with remaining carcass tissues. (○) 0.1 μmol/mouse, (△) 1.0 μmol/mouse, (□) 10.0 μmol/mouse. Results are given as mean ± S.D., $n = 3$.

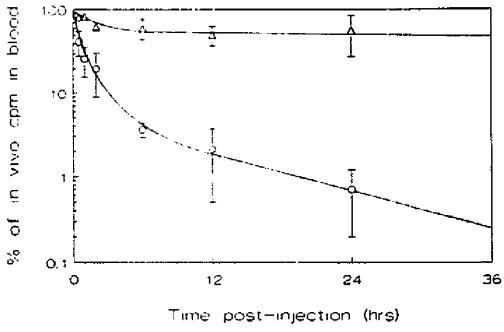

Fig. 4. A comparison of blood levels as a function of time for liposomes composed of PC:CHOL, 2:1 (MLV extruded through 0.1 μm Nuclepore filters) labelled with [^{14}C]DPPC (△), or ^{125}I-tyraminylinulin (○). Mice received 0.01 μmol phospholipid in experiments using the [^{14}C]DPPC label and 0.05 μmol of phospholipid in experiments using ^{125}I-tyraminylinulin label. Mean ± S.D., $n = 3$.

exiting from the central compartment to any great extent, except perhaps in tissues like liver with discontinuous endothelia. The elimination rate constants, the circulatory half-lives and the total body clearance for the two stealth compositions do not change with dose, while the same parameters for the PC:CHOL liposomes change noticeably with increasing dose, becoming similar to those seen for stealth liposomes only at the highest dose. The areas under the curve (AUC, nmol phospholipid h/ml) increase with increasing dose for all liposome compositions but are dramatically higher, particularly at lower doses, for both stealth liposome compositions as compared to PC:CHOL liposomes and the AUC for the PC:CHOL liposomes, even at the highest dose is only 52 to 64% of that seen for stealth liposomes. The AUC for the PC:CHOL liposomes increased disproportionately to increasing dose (960-fold over a 100-fold dose range), while the AUC for the stealth formulations increased in proportion to the increasing dose (130 to 140-fold), additional evidence for saturation of uptake of PC:CHOL, but not stealth, liposomes.

PC:CHOL liposomes at the lower doses were analyzed via a two compartment model with the $T_{\frac{1}{2}\alpha}$ component being postulated as due to a low capacity, high affinity uptake. Accurate estimates of the $T_{\frac{1}{2}\alpha}$ half-life is likely possible only at the lowest dose as we already begin to see evidence of saturation of this component at 0.5 μmol/mouse. The $T_{\frac{1}{2}\beta}$ component is postulated to be due to a low affinity, high capacity uptake system, which probably has a number of contributing factors, as discussed below. At doses above 1 μmol/mouse, a one compartment model was adequate for data analysis. Both $T_{\frac{1}{2}\alpha}$ and $T_{\frac{1}{2}\beta}$ increased with increasing dose, with the $T_{\frac{1}{2}\beta}$ beginning to approach that for stealth liposomes at the highest dose.

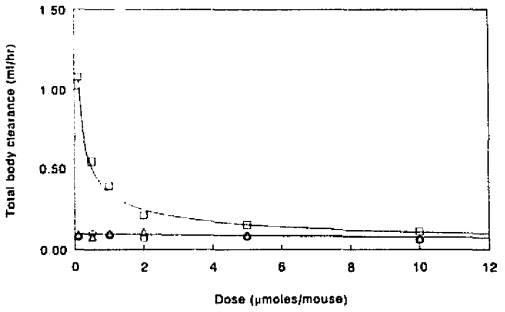

Fig. 5. A plot of total body clearance versus injected dose for liposomes (MLV extruded through 0.1 μm Nuclepore filters) composed of PC:CHOL, 2:1 (□), SM:PC:CHOL:GM_1, 1:1:1:0.2 (△) or SM:PC:CHOL:PEG(1900)-DSPE, 1:1:1:0.2 (○).

Total body clearance for both stealth liposome compositions was constant with increasing dose, while the same parameter decreased dramatically for PC:CHOL liposomes, particularly at the lower doses, again providing evidence for a saturable uptake system (Fig. 5).

Discussion

The blood clearance data can be easily and simply explained by the process of removal of liposomes from circulation by uptake into the MPS. At long time points post-injection, association of liposomes with other carcass tissues (by a mechanism as yet not clearly understood) may also be playing a role.

The blood clearance data and pharmacokinetic parameters for PC:CHOL liposomes suggest that there are two phases of elimination involved in the removal of these liposomes from circulation. We propose this biexponential process represents two mechanisms of elimination i.e., the binding process and the recycling process, respectively. The ability of Kupffer and other MPS cells to remove particulate matter from circulation over a period of time depends, on the one hand, on the rate at which recognition, binding and uptake sites are occupied and become unavailable for further liposome uptake and on the other hand, on the rate of recycling or resynthesis of the binding and uptake sites. A large number of past studies (reviewed in Ref. 19), as well as this study, provide evidence that the recognition, binding and uptake processes for liposomal formulations commonly used in the past are relatively rapid, with half-lives on the order of a few minutes or longer, inversely related to liposome size. We propose that the recognition and binding event represents a high affinity, low capacity process and that recycling of binding sites, and possibly recruitment of new MPS cells, is involved in the high capacity process.

Recycling of MPS binding sites and regeneration of MPS function, is undoubtedly a result of a number of processes, including lipid metabolism [20–22], recruitment of monocytes from bone marrow [23,24], proliferation of Kupffer cells in situ [25] and possibly derivation of MPS cells from blood monocytes [26]. Of the processes involved in MPS recycling, phospholipid metabolism appears to be the most rapid process, occurring over a period of 2–3 h after liposome ingestion following a lag period of approx. 30 min [22], while Kupffer cell turnover is a slow process taking approx. 21 days for total replacement of the Kupffer cell population [23]. With single doses of liposomes of compositions which are relatively non-toxic to cells in culture, such as the PC:CHOL liposomes used in these experiments [27], it is likely that recycling of binding sites is playing a major role in the high capacity process. For liposomes that are toxic to MPS cells, either by virtue of their lipid composition or their contents, recruitment and proliferation of MPS cells probably plays the major role.

At the lowest phospholipid doses of 0.1 μmol/mouse, almost the total liposome dose is rapidly taken up by high affinity, low capacity sites. At the highest doses, the rate of MPS uptake of liposomes after the first few minutes post-injection, when the high affinity, low capacity uptake sites become saturated, is probably dominated by the rate of recycling of binding sites and possibly recruitment of macrophages, rather than by the rate of recognition, binding and uptake.

Depression of MPS activity could be due either to saturation of binding sites and uptake mechanisms with liposomes or to depletion of plasma opsonins by liposomes. The latter explanation has been rejected as playing a role in depression of MPS activity by Abra and Hunt [10] and by Ellens et al. [12], therefore saturation of MPS uptake is the most likely explanation of the non-linear pharmacokinetics seen for PC:CHOL liposomes.

Because we are using small liposomes (92–123 nm), it is possible that some of the liposome uptake is by liver parenchymal cells as well as by macrophages, particularly at the higher doses, where liposomes are circulating for longer periods of time. For small unilamellar liposomes, which are half or less of the size of those employed in these experiments, the uptake by the total parenchymal cell population in liver can be equivalent to the uptake of the Kupffer cell population [21]. However, we have previously reported that we do not begin to see increased uptake of liposomes into whole liver, presumably due to liposomes accessing parenchymal cells, until the size of the liposomes falls below 80 nm [2,4]. Therefore, it is unlikely that uptake by liver parenchymal cells of the liposomes in these experiments is contributing in a substantial way to our observations.

One of the more striking observations from these experiments is that the blood clearance rates of the

two stealth liposome compositions was independent of dose over the entire dosage range tested. Blood, lung, heart, kidney and remaining carcass levels of liposomes likewise showed little dependence on dose. It would appear that we have not reached MPS saturation levels at doses up to 10 μmol/mouse (400 μmol/kg) for the PEG(1900)-DSPE-containing and the GM_1-containing liposomes, which is 20-fold or more higher than the liposome doses where we began to see changes in the elimination rate constants for PC:CHOL liposomes. The elimination rate constants for stealth liposomes are constant over the range of concentrations which one would expect to encounter clinically, with the absolute rate of elimination of liposomes directly proportional to their concentration. This is in contrast to the situation for PC:CHOL liposomes where elimination rate constants and total body clearance varied with the concentration of liposomes (Fig. 5 and Table II). Such non-linear (Michaelis-Menten) pharmacokinetics is an indication of saturation of MPS binding sites.

The half-life in circulation for liposomes containing PEG(1900)-DSPE averages 20.0 ± 3.5 h and for GM_1 averages 16.4 ± 3.1 h and the primary tissues for liposome association at long time points reside in carcass, not in liver and spleen. Carcass tissues have been dissected and analyzed separately for liposomes levels in mice injected with GM_1-containing liposomes [4] and PEG(1900)-DSPE-containing liposomes [6] and the liposomes appear to be spread widely over all the sampled carcass tissues, although it cannot presently be determined if they have exited the central compartment or have bound to, or otherwise associated with, capillary endothelia within the central compartment. The volume of distribution data suggest the latter explanation.

In a separate set of experiments we have demonstrated that 0.1 μm diameter liposomes containing 10 mol% PEG(1900)-DSPE had a 95% decreased uptake by murine bone marrow macrophages as compared to PC liposomes and those containing 10 mol% GM_1 had an 85% decrease in uptake [5], clearly demonstrating that decreased macrophage recognition of stealth liposomes is contributing to the slower blood clearance rates seen for these liposome compositions. It remains, however, to explain what is the mechanism for removal of stealth liposomes from the circulation. Three possible explanations suggest themselves. Stealth liposomes may be recognized by the same MPS binding sites as the non-stealth liposomes, but with a lower binding affinity. Stealth liposomes may be recognized by a different low affinity binding site. The removal of stealth liposomes from circulation is via the same two processes as non-stealth liposomes, but the rate of removal is governed by the rate at which the stealth component is removed from the liposomes and they are converted to non-stealth liposomes. We favour the later explanation, as we have previously reported that PEG attached to single fatty acyl chains or cholesterol, although experiencing high levels in the circulation at early time points post-injection, fall to low blood levels by 24 h post-injection likely due to a greater ease of removal of the stealth component from the liposomes because of decreased hydrophobic interaction of the anchor with the liposomal lipids [6]. Experiments are planned to measure the rate of removal of PEGylated lipid from liposomes. If this hypothesis is shown to be valid, then MPS saturation will not be reached as long as the rate of removal of the stealth component from the liposomes is slower than the rate of MPS binding and recycling.

In summary, we have demonstrated that, over a clinically relevant dosage range, the blood clearance of stealth liposomes follows first-order kinetics and MPS saturating doses have not yet been reached at doses of 400 μmol/kg. In addition to their prolonged circulation times, therefore, this provides further advantages for the use of stealth formulations in a variety of therapeutic applications, by simplifying the pharmacokinetics from non-linear to linear and by greatly reducing the chances of adverse consequences as a result of MPS impairment.

Acknowledgements

This work was supported by the Medical Research Council of Canada (MA-9127) and by Liposome Technology Inc., who supplied the PEG(1900)-DSPE used in these experiments. We would like to thank Dr. Y.K. Tam, Faculty of Pharmacy, University of Alberta and Dr. George Torok-Both for helpful suggestions and Dr. Tam for the generous use of his computors and pharmacokinetic programs.

References

1 Allen, T.M. and Chonn, A. (1987) FEBS Lett. 223, 42–46.
2 Allen, T.M. (1988) in Liposomes in the Therapy of Infectious Diseases and Cancer. UCLA Symposium on Molecular and Cellular Biology. (Lopez-Berestein, G. and Fidler, I., eds.), Vol. 89, pp 405–415, Alan R. Liss, New York.
3 Gabizon, A. and Papahadjopoulos, D. (1988) Proc. Natl. Acad. Sci. USA 85, 6949–6953.
4 Allen, T.M., Hansen, C. and Rutledge, J. (1989) Biochim. Biophys. Acta 981, 27–35.
5 Allen, T.M., Austin, G.A., Chonn, A., Lin, L. and Lee, K.C. (1991) Biochim. Biophys. Acta 1061, 56–64.
6 Allen, T.M., Hansen, C., Martin, F., Redemann, C. and Yau-Young, A. (1991) Biochim. Biophys. Acta, 1066, 29–36.
7 Poste, G. (1983) Biol. Cell 47, 19–38.
8 Allen, T.M., Murray, L., McKeigan, S. and Shah, M. (1984) J. Pharmacol. Exp. Therap. 228, 267–275.
9 Mauk, M.R. and Gamble, R.C. (1979) Proc. Natl. Acad. Sci. USA 76, 765–769.
10 Abra, R.M. and Hunt, C.A. (1981) Biochim. Biophys. Acta 666, 493–503.

11 Ellens, H., Morselt, H.W.M., Dontje, B.H.J., Kalicharan, D., Hulstaert, C.E. and Scherphof, G.L. (1983) Cancer Res. 43, 2927–2934.
12 Ellens, H., Mayhew, E. and Rustum, Y.M. (1982) Biochim. Biophys. Acta 714, 479–485.
13 Hojo, H., Hoshino, Y., Kurita, T. and Hashimoto, Y. (1985) Res. Commun. Chem. Path. Pharmacol. 47, 373–385.
14 Abra, R.M., Bosworth, M.E. and Hunt, C.A. (1980) Res. Commun. Chem. Path. Pharmacol. 29, 349–360.
15 Patel, K.R., Li, M.P. and Baldeschwieler, J.D. (1983) Proc. Natl. Acad. Sci. USA 80, 6518–6522.
16 Sommerman, E.F., Pritchard, P.H. and Cullis, P.R. (1984) Biochem. Biophys. Res. Commun. 122. 319–324.
17 Olsen, F., Hunt, C.A., Szoka, R.C., Vail, W.J. and Papahadjopoulos, D. (1979) Biochim. Biophys. Acta 557, 9–23.
18 Mayer, L.D., Hope, M.J. and Cullis, P.R. (1986) Biochim. Biophys. Acta (858) 161–168.
19 Allen, T.M. (1988) in Liposomes as Drug Carriers (Gregoriadis, G., ed.), pp. 37–50. John Wiley & Sons, New York.
20 Ellens, H., Morselt, H. and Scherphof, G. (1981) Biochim. Biophys. Acta 674, 10–18.
21 Roerdink, R., Regts, J., Van Leeuwen, B. and Scherphof G. (1984) Biochim. Biophys. Acta 770, 195–202.
22 Dijkstra, J., Van Galen, M., Regts, D. and Scherphof, G. (1985) Eur. J. Biochem. 148, 391–397.
23 Crofton, R.W., Diesselhoff-den-Dulk, M.M.C. and Van Furth, R. (1978) J. Exp. Med. 148, 1–17.
24 Gale, R.P., Sparkes, R.S. and Golde, D.W. (1978) Science 201, 937–938.
25 Wisse, E. (1980) Blood Cells 6, 91–92.
26 Van Furth, R. (1980) Blood Cells 6, 87–91.
27 Allen, T.M., McAllister, L., Mausolf, S. and Gyorffy, E. (1981) Biochim. Biophys. Acta 643, 1–17.