Biochimica et Biophysica Acta, 1061 (1991) 56–64
 0005-2736/91/$03.50
ADONIS 000527369100055E

BBAMEM 75086

Uptake of liposomes by cultured mouse bone marrow macrophages: influence of liposome composition and size

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(Received 21 June 1990)
(Revised 24 September 1990)

Key words: Macrophage; Bone marrow; Liposome; Reticuloendothelial system; Ganglioside; Poly(ethylene glycol)

A wide range of liposome compositions have previously been examined in vivo for their ability to affect the uptake of liposomes into cells of the reticuloendothelial (RE, mononuclear phagocyte) system (Allen, T.M. and Chonn, A. (1987) FEBS Lett. 223, 42–46; Allen et al. (1989) Biochim. Biophys. Acta 981, 27–35). In this study we have examined the ability of cultured murine bone marrow macrophages to endocytose liposomes of various compositions and have looked for correlations between the in vivo and the in vitro observations. Compounds which substantially decreased RE uptake of liposomes in vivo, such as monosialoganglioside (G_{M1}) and a novel synthetic lipid derivative of polyethyleneglycol (PEG-PE), also greatly decreased liposome uptake by bone marrow macrophages in a concentration-dependent manner. Lipids which increase bilayer rigidity, such as sphingomyelin (SM) and cholesterol (CHOL), decreased both in vivo and in vitro uptake of liposomes. Likewise, positive correlations were observed between the in vivo behavior of liposomes containing phosphatidylserine (PS) or various gangliosides and the ability of these liposomes to be taken up by bone marrow macrophages. Total liposome uptake by macrophages increased with incubation time at 37°C while very little liposome association with the macrophages was observed at 4°C. Liposome uptake increased with liposome concentration and for liposomes composed of egg phosphatidylcholine (PC) uptake plateaued at 40 nmol lipid per mg cell protein. There was an inverse correlation between liposome size of extruded large unilamellar vesicles and their uptake by macrophages.

Introduction

Liposomes of conventional formulations are avidly removed from circulation by the cells of the reticuloendothelial (RE) system (mononuclear phagocyte system) [1–3]. We [4–6] and others [7] have recently formulated liposomes with greatly decreased uptake by the RE system. It is of interest to compare the uptake of such novelly composed liposomes by macrophages in culture with that of liposomes of previous formulations in the hope that such studies will provide insights into the mechanism of uptake of liposomes by macrophages, which will in turn enable us to design liposomes better suited to the various application which we can envision for them. Several investigators have reported in the past on the uptake of liposomes by resident [8–10] elicited [11–13], peritoneal, liver [14–16] or culture-derived macrophages [17,18].

In previous experiments, liposomal uptake by macrophages was found to be higher if the liposome surface contained negative charge [11,15], fetal bovine serum opsonic factors [14], IgG [11,16], fibronectin [11] or malondialdehyde-treated LDL [18]. Photopolymerized vesicles were taken up more rapidly and more extensively than nonpolymerized liposomes [12]. The total number of particles taken up varied inversely with liposome size [9,11] but the total lipid taken up was independent of liposome size [11]. Addition of cholesterol [13] or phosphatidylinositol [8] to the liposomes decreased their uptake by macrophages.

In this paper we tested the hypothesis that the ability of liposomes to be removed from circulation by the cells of the RE system in vivo can be correlated with the uptake of liposomes by macrophages in vitro and examined the kinetics of uptake. Illum and co-workers [19] have shown a direct correlation between phagocytic uptake of polystyrene microspheres by the RE system in vivo and by peritoneal macrophages in vitro. They found that substances which reduced uptake in vivo

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(non-ionic surfactants) also reduced macrophage uptake in vitro. We have chosen to work with bone marrow macrophages because of their very low contamination with other cell types in culture [20], their convenience and the ease of manipulation of culture conditions for kinetic studies.

We examined the effect of liposome composition, liposome size, liposome concentration and incubation time on the uptake of liposomes by murine bone marrow macrophages. Several lipid labels and one aqueous space label were employed in the studies. We have provided, for the first time, evidence for a direct correlation between in vivo uptake of liposomes by the RE system and in vitro uptake of similarily composed liposomes by bone marrow macrophages.

Materials and Methods

Materials

Egg phosphatidylcholine (egg PC), phosphatidylserine (PS), bovine brain sphingomyelin (SM), egg phosphatidylethanolamine (PE) and phosphatidic acid (PA) were purchased from Avanti Polar Lipids (Birmingham, AL). Cholesterol, galactosylceramides (GAL) and glucosylceramides (GLU) were purchased from Sigma Chemical (St. Louis, MO). Gangliosides were purchased from Supelco, Bellefonte, PA (G_{M1}, asialo G_{M1} (ASG_{M1}), G_{D1a}, G_{T1b}), from Makor Chemicals, Jerusalem (G_{M1}) and from Calbiochem, San Diego, CA (G_{M1}, G_{M2}, G_{M3}, G_{D1a} and G_{T1b}). Globosides (GLOB) were purchased from Supelco. Dulbecco's MEM culture media was purchased from Gibco Canada (Burlington, Ont.) and fetal bovine serum (FBS) and horse serum (HS) was purchased from Hyclone Laboratories (Logan, UT) or Gibco Laboratories (Burlington, Ont.). L-3-phosphatidylcholine,1,2-di[1-^{14}C]palmitoyl ([^{14}C]DPPC, 3.7–4.4 GBq/mmol) and cholesteryl [1-^{14}C]oleate [^{14}C]CHOL, 1.85–2.2 GBq/mmol) were purchased from Amersham Canada (Oakville, Ont.). [1,2(n)-^{3}H]-Cholesteryl hexadecyl ether [^{3}H]CHE, 1.48–2.22 TBq/mmol) was purchased from DuPont Canada (Mississauga, Ont.). Tyraminylinulin was synthesized and ^{125}I-tyraminylinulin (^{125}I-TI) was prepared according to the technique of Sommerman et al. [21]. Polyethylene glycol distearoylphosphatidylethanolamine (PEG-PE), a compound in which a carbamate derivative of PEG-1900 is linked to PE through the NH_2 group, was a gift of Liposome Technology, Menlo Park, CA.

Cultivation of bone marrow-derived macrophages

Briefly, culture was done essentially as described by Lee and Wong [20]. Bone marrow was extracted from tibia and fibula of 6–12-week-old DBA/2J or BALB/cCr//ALE (BALB/c) mice by flushing the bones with 1–2 ml of Puck's saline (pH 7.4). Single cell suspensions of marrow cells were plated at $1.5 \cdot 10^6$ cells per petri dish (non-tissue culture, Falcon-Optilux 1005) in 25 ml of Dulbecco's MEM containing 2% FCS, 18% HS, 50 mg/l gentamycin or 1% of antibiotic-antimycotic solution (Sigma Chemical) and 10% L-cell conditioned medium. The cultures were incubated for 6 days at 37°C in a humidified atmosphere of 5% CO_2 in air. The macrophages were then at late logarithmic phase and greater than 98% pure as judged from phagocytic measurements of polystyrene beads (0.8 μm) and the presence of Fc(IgG) receptors, morphology and esterase staining [20]. Macrophages were harvested by vigorously flushing the petri dishes with PBS or PBS-EDTA (0.02% EDTA). All subsequent incubations were carried out in the above Dulbecco's medium, containing 3% FCS and 1% antibiotic-antimycotic solution and 2% L-cell conditioned media to maintain optimal cell viability.

Preparation of liposomes

Large multilamellar liposomes were prepared by vortexing dried lipid films in buffer (10 mM Mes/Mops, 150 mM NaCl (pH 7.4), 290 mOsm), at a lipid concentration of 3 mM. Sufficient [^{14}C]DPPC, [^{14}C]CHOL or [^{3}H]CHE was added to the liposomes to result in a minimum of 2000 cpm of cell-associated radioactivity per 10^6 cells. In experiments using the aqueous space label ^{125}I-TI, sufficient counts were added during liposome formation to also result in 2000 cpm of cell-associated radioactivity per 10^6 cells. Entrapped ^{125}I-TI was separated from free ^{125}I-TI by chromatography over Ultragel AcA 34. Gangliosides or glycolipids were dissolved in chloroform/methanol (2 : 1) and aliquots were added to appropriate lipid mixtures in chloroform prior to evaporation of organic solvent. Except where indicated, liposomes were extruded 10 times through 0.1 μm Nuclepore filters [22]. This procedure has been shown to result in primarily unilamellar vesicles [23]. The average size of liposomes, as measured by dynamic light scattering using a Brookhaven BI90 particle sizer (Brookhaven Instrument Corp., Holtsville, NY) was from 102 to 128 nm. Large unilamellar liposomes (LUV) were prepared according to the reverse evaporation (REV) technique of Szoka and Papahadjopoulos [24] and extruded through the appropriate sized Nuclepore filters.

Incubation of liposomes with macrophages

Macrophages were plated in triplicate onto Linbro 6 well plates at 10^6 cells/well or Linbro 24 well trays at $2 \cdot 10^5$ cells/well and left to adhere and recover overnight at 37°C in a humidified atmosphere of 5% CO_2 in air. To each test well was added 35–100 nmol of phospholipid/10^6 cells. The amounts of phospholipid were deliberately kept at the low end of the range in order to avoid saturation of uptake for any of the several liposome compositions employed in the experiments. Plates to be incubated at 4°C were sealed in a plastic bag

containing 5% CO_2 in air. Both the 37°C and the 4°C samples were rocked on a plate rocker (Bellco) at approx. 10 cycles/min. After incubation (usually for 4 h), the cells were washed three times with cold PBS to remove non-adherent and non-phagositized liposomes and non-viable macrophages, if any, which do not adhere to the dish. The cells were then dissolved in 0.5 ml of 0.1 M NaOH, placed in ACS scintillation fluid (Fisher Scientific) and counted in a Beckman LS-6800 counter. Cell protein was determined on representitive wells by a modified Lowry procedure [25] or by the Bio-Rad procedure [26] (Bio-Rad Canada, Mississauga, Ont.) and liposome phosphate was determined according to the method of Bartlett [27].

Uptake of liposomes was expressed as nmol lipid/mg cell protein. In earlier experiments the uptakes were normalized to 100 nmol lipid added per 10^6 cells following lipid phosphate determination. In later experiments total liposomal phosphate was determined prior to addition of liposomes to cells and exactly 100 nmol of lipid was added per 10^6 cells. Specific uptake was calculated as the percentage uptake at 37°C minus the uptake at 4°C. The reference liposomes, PC liposomes (MLV extruded through 0.1 μm Nuclepore filters), were run concurrently with each separate macrophage culture to allow appropriate compensation for variability in different bone marrow preparations. Results are expressed as mean ± S.D.

Results

Uptake of reference liposomes, composed only of egg PC (0.1μm extruded), is shown in Fig. 1 as a function of temperature, time and liposome concentration. Liposome uptake at 37°C represents a combination of vesicle adsorption to the cell surface and active cellular endocytosis, whereas at 4°C endocytosis is inhibited and surface adsorption is the main mode of liposome association with the macrophages as demonstrated earlier [12,28]. As can be seen from Fig. 1A, liposome association with macrophages at 4°C was considerably lower than at 37°C and remained low over the time course of the experiments. Liposome uptake (100 nmol lipid added/10^6 cells) at 37°C increased linearly with time and showed no sign of saturating for up to 16 h of incubation. When liposome uptake was examined as a function of liposome concentration (4 hour incubations) uptake of PC liposomes was linear up to approx. 400 nmol phospholipid/mg cell protein* with saturation of uptake occurring at approx. 1500 nmol phospholipid/mg cell protein phospholipid/mg cell protein.

Table I gives the results for macrophage uptake of

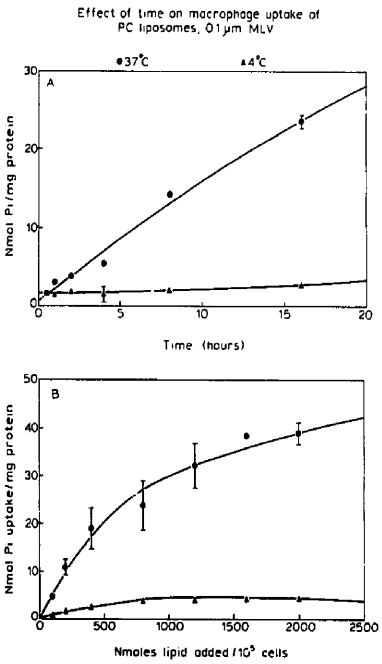

Fig. 1. Uptake of PC liposomes (MLV extruded through 0.1 μm Nuclepore filters) by murine bone marrow macrophages. Incubations were at 37°C (●) and at 4°C (▲). (A) Uptake as a function of incubation time. Liposomes were labelled with [^{14}C]DPPC and incubated with 100 nmol of lipid per 10^6 cells. (B) Uptake as a function of liposome concentration for 4 h incubations. Uptake is expressed at nmol phospholipid/mg cell protein. Mean ± S.D., $n = 3$.

PC liposomes labelled with three different lipid labels and one aqueous space label. Uptake of liposomes by macrophages at 4°C was not significantly different for

TABLE I

Uptake by bone marrow macrophages of PC liposomes (REV extruded through 0.1 μm Nuclepore filters) labelled with the lipid labels [^{14}C]cholesterol oleate, [^{14}C]dipalmitoylphosphatidylcholine or [^{3}H]-cholesteryl hexadecyl ether or labelled with the aqueous space label ^{125}I-tyraminylinulin at 37°C or 4°C. The amount of phospholipid add was 100 nmol/10^6 cells and incubation times were 4 h. Control dishes were incubated with liposomes (labelled with [^{14}C]DPPC or [^{14}C]CHOL) in the absence of macrophages. Results are expressed as nmol phospholipid/mg cell protein ± S.D. (n = number of assays).

Liposome label	Uptake (37°C, nmol/mg (n))	Uptake (4°C, nmol/mg (n))
[^{14}C]CHOL + macrophages	6.6 ± 1.5 (12)	1.2 ± 1.1 (6)
[^{14}C]DPPC + macrophages	5.0 ± 1.0 (12)	1.0 ± 0.7 (6)
[^{3}H]CHE + macrophages	3.3 ± 0.6 (12)	1.5 ± 0.4 (6)
^{125}I-TI + macrophages	8.2 ± 0.5 (6)	1.7 ± 0.2 (6)
[^{14}C]CHOL control	0.3 ± 0.1 (6)	0.4 ± 0.1 (3)
[^{14}C]DPPC control	0.4 ± 0.2 (6)	0.6 ± 0.2 (3)
Free ^{125}I-tyraminylinulin + macrophages	0.6 ± 0.1 (6)	0.5 ± 0.01 (6)

* 10^6 bone marrow macrophages contain 0.16 ± 0.06 mg cell protein (n = 14).

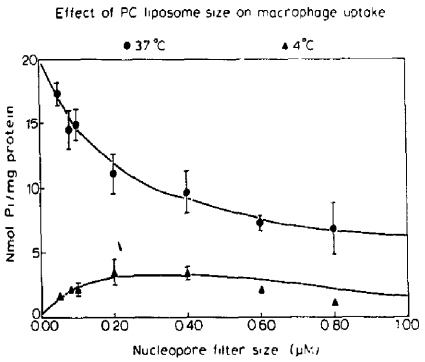

Fig. 2. Uptake of PC liposomes (100 nmol lipid/10^6 cells, labelled with [^{14}C]DPPC) by bone marrow macrophages for 4 h incubations at 37°C (●) and at 4°C (▲). Large unilamellar liposomes made by the REV method were extruded through decreasing pore size Nuclepore filters. Uptake is expressed at nmol phospholipid/mg cell protein. Mean ± S.D., $n = 3$.

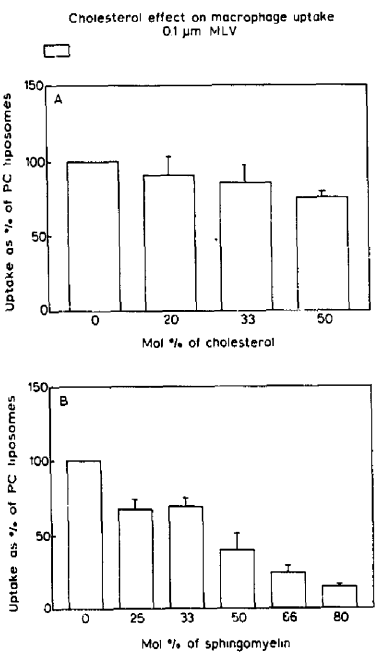

Fig. 3. Effect of increasing concentrations of cholesterol (A) and sphingomyelin (B) on uptake of PC liposomes (MLV extruded through 0.1 μm Nuclepore filters), labelled with [^{14}C]DPPC, by bone marrow macrophages for 4 h incubations at a liposome concentration of 100 nmol phospholipid/10^6 cells. Liposome uptake was calculated as specific uptake (uptake at 37°C minus uptake at 4°C) as a percentage ratio of specific uptake for PC reference liposomes. Individual test results were divided by the mean of the control results and then averaged to give a mean ± S.D., $n = 3$.

any of the four labels. Uptake was lowest for liposomes labelled with [^{3}H]CHE, higher for [^{14}C]DPPC and highest for [^{14}C]CHOL. Liposomes labelled in the aqueous compartment with ^{125}I-TI had uptakes in the same range for that of [^{14}C]CHOL (Table I). Labelled liposomes incubated with cell culture dishes in the absence of macrophages and treated identically to the experimental dishes were not associated with the dishes to any degree (Table I). This measurement is most likely a significant overestimate of liposome binding to culture dishes, as liposome 'binding' sites on the culture dishes would normally be occupied by macrophages during our experiments which are conducted at or near macrophage confluence.

When large unilamellar PC liposomes were extruded through decreasing pore size Nucleopore filters, we observed increasing uptake of phospholipid at 37°C as the filter pore size decreased (Fig. 2). Pore sizes were 0.05, 0.08, 0.1, 0.2, 0.4, 0.6 and 0.8 μm and the corresponding sizes of the liposome preparations, as measured by dynamic light scattering, were 0.078, 0.090, 0.125, 0.196, 0.289, 0.438 and 0.570 μm, respectively. Association of liposomes with the macrophages at 4°C did not appear to be size-dependent (Fig. 2).

Inclusion of increasing amounts of cholesterol in the PC liposomes resulted in a decrease in the uptake by macrophages (Fig. 3a), but the results only reach significance at 50 mol% cholesterol ($P > 0.01$). Likewise, inclusion of another lipid which increases membrane rigidity, sphingomyelin, in increasing amounts in the PC liposomes resulted in substantial reduction in uptake,

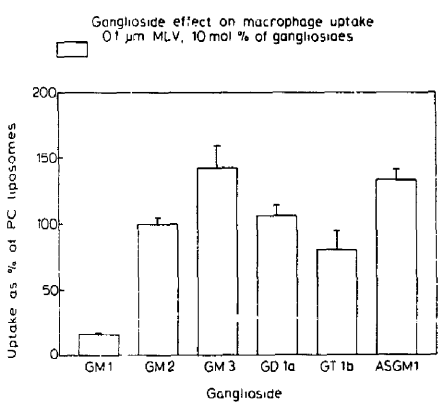

Fig. 4. Effect of various gangliosides or asialoganglioside at 10 mol% in PC liposomes (MLV extruded through 0.1 μm Nuclepore filters), labelled with [^{14}C]DPPC, on liposome uptake by bone marrow macrophages for 4 h incubations at a liposome concentration of 100 nmol phospholipid/10^6 cells. Liposome uptake was calculated as specific uptake (uptake at 37°C minus uptake at 4°C) as a percentage ratio of specific uptake for PC reference liposomes. Individual test results were divided by the mean of the control results and then averaged to give a mean ± S.D., $n = 3$.

with liposomes containing 80 mol% SM experiencing an 85% reduction in uptake (Fig. 3b).

Fig. 4 shows the results for macrophage uptake of PC liposomes containing 10 mol% each of various gangliosides. Monosialoganglioside G_{M1} was the only ganglioside which resulted in a substantial reduction of uptake of PC liposomes by bone marrow macrophages. Other gangliosides of the G_M series, G_{M2} and G_{M3}, resulted in either no reduction in uptake (G_{M2}) or an increase in uptake (G_{M3}). Disialoganglioside, G_{D1a}, and trisialoganglioside, G_{T1b}, were also not capable of substantially reducing uptake. Removal of the sialic acid from G_{M1}, resulting in asialoganglioside (ASG_{M1}), appeared to stimulate uptake of liposomes by macrophages (Fig. 4). These results correlate well with those reported by us for the effect of gangliosides on the *in vivo* uptake of liposomes by the mouse RE system [4,6].

Increasing the concentration of G_{M1} in PC liposomes progressively decreased uptake by macrophages (Fig. 5A). The inclusion of 10 mol% G_{M1} in the liposomes reduced the macrophage uptake by as much as 90%. Again, this correlates well with the decreases in RE

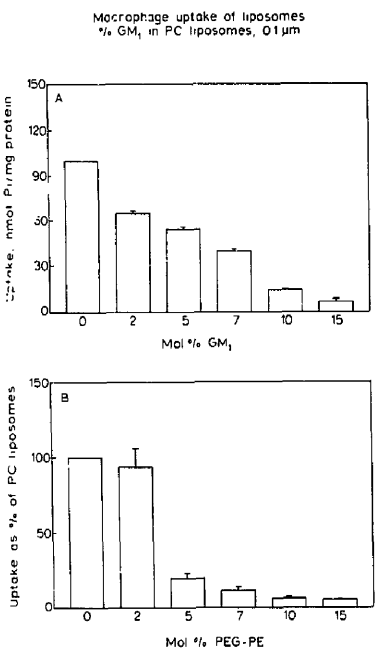

Fig. 5. Uptake of PC liposomes, labelled with [^{14}C]CHOL, by bone marrow macrophages as a function of increasing concentration of ganglioside G_{M1} (A) or PEG-PE (B) in liposomes (MLV extruded through 0.1 μm Nuclepore filters). Incubations were for 4 h at a liposome concentration of 100 nmol phospholipid/10^6 cells. Liposome uptake was calculated as specific uptake (uptake at 37°C minus uptake at 4°C) as a percentage ratio of specific uptake for PC reference liposomes. Individual test results were divided by the mean of the control results and then averaged to give a mean ± S.D., n = 3.

TABLE II

Effect of phospholipids and glycolipids on uptake of liposomes (MLV extruded through 0.1 μm Nuclepore filters, labelled with [^{14}C]DPPC) by mouse bone marrow macrophages. Results from different experiments are normalized tc the uptake of PC liposomes (MLV extruded through 0.1 μm Nuclepore filters) and are expressed as the ratios of specific uptake, i.e., 37°C minus 4°C (n ≥ 3).

Liposome composition, molar ratio	Specific uptake (%)
PC	100
PC:GLOB, 1:0.1	38.1
PC:GLOB, 1:0.2	21.1
PC:GLU, 1:0.1	43.4
PC:GLU, 1:0.2	30.7
PC:GAL, 1:0.1	106.0
PC:GAL, 1:0.2	163.6
PC:PS, 1:0.1	120.4
PC:PS, 1:0.2	134.6
PC:PS, 1:0.4	187.7

uptake of liposomes containing G_{M1} which have been reported by us in in vivo experiments [4,6].

A synthetic polyethylene glycol derivative of phosphatidylethanolamine has been tested in vivo in mice and has been found to markedly reduce uptake of liposomes by the RE system in a concentration-dependent manner (Allen et al., in preparation). We have tested this derivative for its ability to affect the uptake of PC liposomes by macrophages (Fig. 5B). There was a dramatic reduction in uptake, beginning at approx. 5 mol% of PEG-PE and reaching 95% reduction with 10 mol% of PEG-PE (Fig. 5B).

The effect of some other lipids and glycolipids on uptake of liposomes by macrophages, as compared to PC liposomes, are given in Table II. Galactosylceramide stimulated liposome uptake, particularly at 20 mol% in PC liposomes. Glucosylceramide, on the other hand, decreased liposome uptake. A negatively charged phospholipid, PS, when incorporated into PC liposomes, substantially increased liposome uptake by macrophages in a concentration-dependent manner (Table II).

Because we suspected that there could be strain differences between bone marrow macrophages from different mouse strains, perhaps related to chronic infections in the mouse colonies for different strains, we compared bone marrow macrophages from three different mouse strains, BALB/c, ICR and C57Bl/6J x DBA/2J (B6D2F1) for their ability to phagocytize liposomes at various concentrations. We also examined bone marrow macrophages from a BALB/c strain of mouse which had been maintained since birth in a bacterial and virus free environment. Macrophages from both the BALB/c (inbred) mice and the ICR (outbred) mice both showed good ability to take up liposomes, with values for specific uptake of 5.0 ± 0.4 and 4.3 ± 0.2 nmol lipid/mg protein, respectively, for 100 nmol

lipid added and 4 h incubations. Macrophages grown from the hybrid strain (B6D2F1) showed poorer ability to phagocytize liposomes (1.6 ± 0.1 nmol/mg protein). The macrophages from the specially maintained BALB/c strain had levels of liposome uptake nearly equivalent to the normal BALB/c mice (4.3 ± 0.2 nmol/mg protein), but were more difficult to culture with the numbers of macrophages obtained being consistently lower than for the normally maintained mice.

Discussion

We have demonstrated for the first time that there is a direct correlation between the uptake of liposomes by culture-derived murine bone marrow macrophages and the uptake of liposomes by the RE system in vivo in mice. Novel liposome compositions which have been shown to dramatically reduce RE uptake in vivo [4,6,7], had similar pronounced effects in reducing macrophage uptake in vitro, providing experimental evidence that the mechanism for reduction of liposomes uptake in vivo is through the ability of these new liposome formulations to avoid recognition and uptake by macrophages.

Radiolabelled liposomes may become associated with macrophages by several different pathways, including binding of liposomes to the cell surface, phagocytosis of liposomes and transfer of lipid molecules from the liposome to the cell membrane [30]. We have compared results obtained at two incubation temperatures for uptake experiments where liposomes were labelled with three different lipid labels and one aqueous space label, which should allow us to distinguish between uptake and binding and/or lipid exchange. Controls for liposome association with the culture dishes and for uptake of free aqueous space label by the macrophages were included.

The free aqueous space label, ^{125}I-TI, which is metabolically inert, was added to the macrophages at concentrations which would occur if all of the liposome label leaked out of the liposomes under the influence of the macrophages or the media. This is undoubtedly a substantial overestimate of the actual situation. The free label was taken up by the macrophages to a very small extent, therefore the uptake of ^{125}I-TI seen in our experiments must have been due primarily to either surface association or endocytosis of intact liposomes. Endocytosis is inhibited at 4°C and radiolabel associated with the cells at this temperature is a good measure of surface adsorption of liposomes [12,28]. All three lipid labels and the aqueous space label, showed very low levels of association with the macrophages at 4°C as compared with that seen at 37°C and did not significantly differ from each other, suggesting that surface association of liposomes at 37°C is not the major contributor to the high levels of cell-associated radioactivity at this temperature. Uptake of aqueous space label at 37°C was not significantly different to uptake seen for liposomes labelled with [^{14}C]CHOL and only slightly higher than that seen for the [^{14}C]DPPC label. Therefore, extensive exchange of the lipid labels with the cell membranes does not appear to be taking place during the time course of our experiments. If this were the case, then uptake of the aqueous space label would be significantly lower than uptake of the lipid label. However, the fact that liposomes labelled with [^{3}H]CHE (which is non-metabolizable and non-exchangeable [31]) showed 34 to 50% less uptake by macrophages than the other two lipids ($P > 0.05$), suggests that low levels of exchange or metabolism of [^{14}C]CHOL and [^{14}C]DPPC may have been taking place. It is possible that the [^{14}C]CHOL and the [^{14}C]DPPC labels are more easily exchangeable than the [^{3}H]CHE label, or that the presence of the [^{14}C]CHE label, which is not a naturally-occurring lipid, in some way slightly inhibits uptake of liposomes by macrophages.

This comparison of several different labels at two different temperatures gives us some confidence that the the majority of the radioactivity associated with the macrophages at 37°C was as a result of phagocytosis of liposomes and not primarily as a result of another mechanism.

We have previously reported on the effects of a number of gangliosides and glycolipids on the uptake of liposomes by the RE system [4,6,29]. We can now compare the results of the in vitro experiments using bone marrow macrophages with the in vivo results. The in vivo results in our previous papers are reported as RE uptake as perentage of radiolabel remaining in vivo in the whole animal at the time of tissue sampling and are therefore corrected for the leakage of the radiolabel (^{125}I-TI) from the liposomes. ^{125}I-TI, when released from liposomes in vivo is rapidly removed from the body by filtration [4,21]. The effect of gangliosides in the in vitro experiments correlated well with results found in vivo, with ganglioside G_{M1} reducing macrophage uptake of liposomes in a concentration-dependent manner in both experimental systems. Other gangliosides, asialoganglioside and galactosylceramide, which were not capable of reducing RE uptake of liposomes in vivo [6,29], also did not decrease macrophage uptake of liposomes in vitro. On the other hand, the presence in liposomes of globosides and glucosylceramides reduced marcophage uptake of liposomes in vitro, but had either no effect (glucosylceramides) or stimulated (globosides) RE uptake of liposomes in vivo. Of all the lipids tested, these two lipids were the only ones for which we did not see a correlation between the in vivo and the in vitro results. The reason for these differences are not presently known, but may relate to the presence or absence of different receptors on murine Kupffer cells as compared to bone marrow macrophages. Uptake by bone

marrow in vivo cannot be directly compared for PC liposomes and G_{M1}-containing liposomes, as PC liposomes are avidly taken up by liver and spleen and have little or no chance to get to bone marrow. G_{M1}-containing liposomes, by virtue of their long circulation half-lives in vivo, actually have higher bone marrow uptake than PC liposomes [6], but it is an invalid comparison. Bone marrow macrophages, therefore, allow us to make a direct comparison, under conditions of equivalent access, between the two liposome compositions.

We have observed that a synthetic phospholipid derivative of polyethylene glycol, PEG-PE, which is capable of substantially reducing RE uptake of liposomes in a concentration-dependent manner (Allen et al., in preparation) is also capable of dramatically reducing uptake of liposomes by bone marrow macrophages in the concentration range of 5–20 mol% in liposomes. Illum et al. [19] have previously reported that microspheres coated with hydrophilic materials (poloxamer 338) experienced reduction in uptake into the RE system in rabbits. They hypothesize that increasing surface hydrophilicity will generally lead to decreased uptake by the RE system. Both G_{M1} and PEG-PE are likely acting to reduce uptake of liposomes by both cultured macrophages and by the RE system through this mechanism. The surfaces of liposomes containing either G_{M1} or PEG are most likely resistant to opsonisation with plasma components which render them recognizable by phagocytic cells through the dual mechanism of increased surface hydrophilicity and stearic hinderance.

Uptake of liposomes by mouse bone marrow macrophages as a function of time and liposome concentration appears to follow similar kinetics to uptake of liposomes by mouse peritoneal macrophages [11,30] and rat Kupffer cells [14,15]. Although exact comparisons cannot be made because of differing experimental conditions between different groups of researchers, it appears that the extent of lipid uptake (nmol lipid/mg cell protein) by mouse bone marrow macrophages is less than that for peritoneal macrophages, which in turn is less than that for Kupffer cells. At 90 min incubation with 100 nmol lipid/10^6 bone marrow macrophages, uptake of PC:CH, 1:1 (0.1 μm) liposomes averaged approx. 1.5 nmol lipid/mg cell protein. For peritoneal macrophages a figure of approx. 5 nmol/mg can be extrapolated for 0.6 μm PC:CH, 1:1 liposomes [11], while for rat Kupffer cells the corresponding figure for PC:CH, 1:1 liposomes (0.4 μm) is approx. 20 nmol/mg protein [15]. Using a similar size of liposomes for the comparison would amplify the difference, as we have found that bone marrow macrophages experience a reduction in uptake of lipid as liposome size increases. The differences between bone marrow macrophages and Kupffer cells would be reduced, however, if the comparisons were made on the basis of nmol lipid/10^6 cells, rather than on the basis of cell protein, as the bone marrow macrophages contain on average 160 μg protein/10^6 cells, while Kupffer cells contain on average only 100 μg protein/10^6 cells [16].

The differences in liposome uptake between different macrophage populations are probably a reflection of macrophages in different stages of differentiation or maturation. The bone marrow macrophages may contain reduced numbers of receptors recognizing liposomal phospholipids or their opsonins, or lack some classes of these receptors completely. In an ongoing series or experiments we are examining the effect of pre-opsonizing liposomes with individual, or combinations of, plasma proteins on uptake by macrophages in serum-free media. In serum-free medium, uptake of PC liposomes (MLV extruded through 0.1 μm Nuclepore filters) was approx. 70% lower than uptake in the serum-containing media. Uptake can be stimulated dramatically by opsonizing the liposomes with some human or mouse plasma proteins (T. Allen, unpublished data). Clearly, therefore, opsonins play an important role in the uptake of liposomes by mouse bone marrow-derived macrophages. In many important ways, uptake of liposomes by bone marrow macrophages reflects what is seen in the more differentiated macrophage populations like peritoneal macrophages and Kupffer cells as well as the situation in vivo.

Several researchers have reported that negative charge, usually PS, increases uptake of liposomes [11,15,30] by other types of macrophages. We can now report that the situation is similar for bone marrow macrophages demonstrating that sensitivity to PS is a property of the most primitive macrophages. Addition of PS to PC liposomes increased the uptake by bone marrow macrophages (Fig. 1) or peritoneal macrophages by approx. 4-fold [11]. The ability of macrophages to recognize PS may have functional significance in the removal of senescent red blood cells from circulation [5,32].

Rabbit peritoneal macrophages experienced a reduction in liposome uptake upon addition of cholesterol to PC liposomes [13], as we have observed for mouse bone marrow macrophages. However, Schroit et al. [30] report that cholesterol increases uptake of PC liposomes. Our results are correlated with in vivo observations in which inclusion of cholesterol reduces uptake of liposomes by the RE system [4,33–35], the basis of which is most likely the ability of cholesterol to bring about bilayer rigidification in fluid liposomes and decrease opsonization of the liposomal surface. A similar mechanism may be acting in the ability of cholesterol to reduce uptake of liposomes by bone marrow macrophages.

Inclusion of sphingomyelin in liposomes decreased macrophage uptake by bone marrow macrophges, an observation which is again correlated with in vivo results [36–39]. A similar observation has been made for

the effect of sphingomyelin on liposome uptake by Kupffer cells by Dijkstra et al. [40]. The mechanism is again probably due to the ability of sphingomyelin to rigidify the liposomal bilayer.

We have observed that uptake of liposomal lipid by bone marrow macrophages is negatively correlated with liposome size for extruded REV liposomes, which are primarily unilamellar or oligolamellar [23]. A similar observation has been made for unilamellar liposome uptake by rat peritoneal macrophages by Schwendener et al. [9]. Hsu and Juliano, on the other hand, reported that small unilamellar liposomes, in terms of ng lipid/μg protein, were taken up to a lesser extent than MLV and REV (0.6 μm) by mouse peritoneal macrophages [11]. This may be a reflection of the increased amount of lipid per particle in larger liposomes due to their multilamellarity. However, in terms of particle numbers the above experiments are all in agreement with each other, with the number of particles taken up decreasing with increasing liposome size. Liposomes are therefore behaving differently than particulate substrates (Percoll or polystyrene beads) where uptake by rat peritoneal macrophages was found to be positively correlated with particle size [10]. The behavior of the particulate substrates was more closely correlated with the behavior of particulate matter, including liposomes in vivo, where uptake is also positively correlated with size. The negative correlation which we have observed between bone marrow macrophages and liposome size is the opposite to that which is normally observed in vivo, for reasons which are not presently understood.

In summary, we have shown that a number of strong correlations exist between the ability of cultured bone marrow macrophages in vitro to take up liposomes of various lipid compositions and the ability of the RE system to recognize and remove liposomes of similar compositions from circulation. The uptake of liposomes by bone marrow macrophages could therefore be used as a preliminary screening assay to search for novel liposome compositions which avoid RE uptake. As well, these macrophages could be employed as an assay system for understanding factors which lead to recognition and uptake of liposomes by RE and other macrophages. Insights gained from experiments such as these will ultimately lead to the design of liposomes which show further improvements in circulation half-lives and this will further expand the therapeutic applications of liposomes as drug delivery systems.

Acknowlegements

This work was supported by grants from the Medical Research Council of Canada (MA-9127) and the National Cancer Institute of Canada. We thank Liposome Technology Inc. of Menlo Park, CA for their generous gift of polyethylene glycol distearoylphosphatidylethanolamine and Dr. Bill Colmers for the use of his LaserJet II printer.

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