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Phospholipid composition of small unilamellar liposomes containing melphalan influences drug action in mice bearing PC6 tumours

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Studies with solid tumour bearing animals injected intravenously with drug-containing liposomes suggest that uptake of drugs by the tumour occurs after their release into the circulation [1, 2]. For instance, small liposomes were found to be more efficient than large liposomes in mediating uptake of ^{111}In-labelled bleomycin [3, 4] and ^{99m}Tc [5] by a number of implanted tumours, presumably because the former persist in the blood longer [3, 6] and thus release their contents over prolonged periods of time [7, 8]. Release of drugs as a mode of their uptake by tumours is also supported by the successful chemotherapy of ascites tumours with phase-specific drugs entrapped in vesicles from which they can leak out slowly [9, 10].

Recent *in vivo* work [7, 8, 11–14] indicates that solute release from circulating liposomes can be controlled by adjusting their cholesterol content and/or phospholipid component, both of which determine the extent of phospholipid removal by high-density lipoproteins and ensuing leakiness of the bilayers. The scope of such control on solute release has been expanded further by the finding [8, 13, 14] of an inverse relationship between liposomal leakiness and half-life in the circulation. Elsewhere [15] we have shown that appropriate adjustments in the lipid composition of liposomes containing phase-specific vincristine and melphalan can influence drug clearance rates from the blood of injected mice. Here we examine the effect of liposomal lipid composition on melphalan localization in the tissues of PC6 tumour bearing mice and chemotherapeutic activity. Melphalan was chosen because of the sensitivity of the PC6 tumour to it.

Materials and methods

The sources and grades of egg L-α-phosphatidylcholine (PC), L-α-dimyristoyl phosphatidylcholine (DMPC), sphingomyelin (SM), cholesterol, melphalan (Alkeran), ^{14}C-labelled melphalan ([^{14}C]melphalan) (labelled in the ethyl side chains, sp. act. 12.35 mCi/mmole, 98% pure by radioautography) and Sepharose CL-6B have been described elsewhere [15]. $^{99m}TcO_4$ was obtained from a 250-mCi molybdenum–technetium generator (Radiochemical Centre, Amersham, U.K.).

Preparation of liposomes. Small unilamellar PC, DMPC and SM liposomes (30–60 nm dia.) containing melphalan and its radiolabelled derivative were prepared [15] from 40 μmoles phospholipid with and without cholesterol. For chemotherapy experiments liposomes were prepared from equimolar (34 μmoles) PC or SM and cholesterol in the presence of up to 85 mg melphalan and 0.1–0.2 μCi [^{14}C]-melphalan dissolved in 50 μl Alkeran solvent [15] which was then diluted to 1.7 ml with 0.1 M phosphate-buffered saline (PBS) [15]. When needed, the vol. of the final liposome suspensions containing 1.8–3.8% of the melphalan used in 2 ml was reduced to about 1 ml by placing the preparation in dialysis tubing in contact with polyethylene glycol 6000 (British Drug Houses Chemicals Ltd, Poole, U.K.). Liposomes were subsequently dialysed for at least 4 hr before use.

Animal experiments. Tumour-bearing animals were prepared by subcutaneous implantation of PC6 tumour fragments (approximately 1 mm^3; from 2–3 g tumours of routinely passaged BALB/c mice) into BALB/c female mice weighing 25–27 g. After 3 weeks, when according to preliminary experiments tumours were expected to weigh 2–3 g, mice were divided into groups and injected in the tail vein with 0.2 ml liposomes (3–4 μmoles phospholipid) containing [^{14}C]melphalan (0.02–0.05 μCi). At time intervals, 50-μl blood samples were taken from the tail and assayed for ^{14}C [15]. In other experiments similar tumour-bearing BALB/c mice were divided into groups, injected with liposomes as earlier and killed by cervical dislocation at 4 and 24 hr. ^{14}C radioactivity was then measured in blood plasma and tissues (see Table 1). To assess and correct for blood-associated radioactivity in the tissues animals were injected intravenously 5 min before death with 0.1 ml ^{99m}Tc-labelled mouse erythrocytes prepared as follows. One millilitre of twice washed packed fresh cells were mixed with 200 μg/l of $SnCl_2 \cdot 2H_2O$ in 0.15 ml PBS and incubated at 18° for 5 min. Subsequently, 500 μCi of $^{99m}TcO_4$ in 0.5 ml 0.9% NaCl was added and after 5 min incubation the cells were washed 4 times with PBS, diluted with 0.9% NaCl to 2×10^6 cpm/ml and used immediately. As each mouse was killed, 100 μl of blood was retained for the assay of ^{14}C and 50 μl for the assay of ^{99m}Tc. Tissues were removed, weighed, counted for ^{99m}Tc to estimate blood present, homogenized together with water to a final vol. of 4 ml (liver) or 2 ml (all other tissues) using an MSE homogenizer and duplicate 0.4-ml aliquots transferred to vials containing 2.4 ml of NCS tissue solubilizer. Samples were allowed to digest overnight at 18°. Unreacted NCS was partially neutralized by the addition of 70 μl of glacial acetic acid and, subsequently, 17 ml of scintillant (7.06 g PPO and 88.24 mg POPOP in 1 litre toluene) was added to each vial. The contents were stored at 18° for 5 days for complete decay of ^{99m}Tc and chemiluminescence and ^{14}C radioactivity was measured [15]. Results were then corrected for ^{14}C radioactivity contributed by blood. Because [^{14}C]melphalan (labelled in the ethyl side chains) is not metabolised *in vivo* but only hydrolyzed to the mono- and dihydroxyethyl inactive derivatives [16], no attempts were made to distinguish between parent drug and any radioactive metabolic products that might be formed.

In chemotherapy experiments, 3 weeks after inoculation of BALB/c female mice weighing 16–20 g with PC6 tumour fragments, mice with tumours weighing 2–3 g received a single intravenous injection of 0.2 ml of melphalan in the free form or entrapped in liposomes composed of equimolar PC or SM and cholesterol (see Table 2). Animals were killed 8 days after treatment and tumour wt was taken as a measure of chemotherapeutic activity. In other experiments, similarly treated mice were observed for tumour regression and killed when tumour wts approached 5 g after regression failed to occur or upon regrowth following partial regression. In control experiments mice received "empty" liposomes alone or mixed with the free drug.

Results and discussion

Clearance rates of [^{14}C]melphalan from the blood of BALB/c tumour-bearing mice injected with the free or liposome-entrapped drug were nearly identical (results not shown) to those already observed [15] in similar experiments with normal T.O. mice: there was a very rapid clearance of melphalan injected in the free form and, again, administration of the drug in liposomes reduced the drug rate of clearance in direct relation to their cholesterol content. The greatest reduction in clearance rates was attained with cholesterol-rich SM liposomes [see, for example, melphalan levels in blood at 4 hr (Table 1)]. It would, therefore, appear that the presence of a PC6 tumour (2–3 g) in mice did not alter appreciably drug leakage from circulating liposomes or clearance. As discussed already [15], clearance rates of melphalan given in liposomes represent the sum total of clearance rates of drug that is still entrapped or otherwise associated with liposomes and of drug that has leaked or dissociated from liposomes and is present within the circulation in the free or protein-bound form.

Table 1 shows the content (corrected for blood radio-

Table 1. Radioactivity levels in tissues of PC6 tumour bearing mice injected with free or liposome-entrapped [^{14}C]-melphalan*

Melphalan preparation	Tumour 4 hr	Tumour 24 hr	Liver 4 hr	Liver 24 hr	Spleen 4 hr	Spleen 24 hr	Blood plasma 4 hr
Free drug	1.8 ± 0.1	0.8 ± 0.1	0.5 ± 0.1	0.3 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	2.9 ± 0.3
PC	2.2 ± 0.3	0.9 ± 0.2	5.4 ± 0.7	0.8 ± 0.1	1.8 ± 0.2	0.9 ± 0.0	8.2 ± 4.1
PC, C (1:0.28)	2.8 ± 0.9	0.8 ± 0.1	5.4 ± 0.6	0.8 ± 0.1	2.0 ± 0.3	1.2 ± 0.0	9.2 ± 2.3
PC, C (1:1)	4.7 ± 1.7†	1.6 ± 0.2	9.6 ± 0.9	1.4 ± 0.6	3.6 ± 0.4	1.6 ± 0.0	11.9 ± 2.2
SM, C (1:1)	3.0 ± 0.0†	1.1 ± 0.1	2.8 ± 0.2	0.3 ± 0.0	1.4 ± 0.2	0.9 ± 0.0	46.1 ± 3.4
DMPC, C (1:1)	3.2 ± 0.5†	1.2 ± 0.1	2.9 ± 0.6	0.9 ± 0.1	0.7 ± 0.2	0.7 ± 0.0	19.2 ± 2.7

* BALB/c mice (5 in each group) bearing PC6 tumours were divided into 6 groups of 10 mice each and injected intravenously with (2–5) × 10^{-2} μCi of [^{14}C]melphalan in its free form or entrapped in various types of liposomes as shown. From each group, five mice were killed at 4 hr and five mice at 24 hr. ^{14}C radioactivity was then determined in tissues and corrected for blood radioactivity (see text). Values are expressed as % ± S.E.M. of the dose per gram tissue or per total blood plasma [15]. Numbers in parentheses denote phospholipid:cholesterol (C) molar ratios. For other details see Materials and methods. Radioactivity content of total blood plasma at 24 hr was 12.5% of the injected dose for SM, C liposomes and less than 1% for all other preparations. Radioactivity content of lungs, kidney, brain and skeletal muscle at 4 hr was below 2% of injected dose/g.

† Significantly different ($P < 0.05$–0.01) from value obtained with free melphalan.

Table 2. Body wt changes and tumour wts in PC6 tumour bearing BALB/c mice injected with free or liposome-entrapped melphalan*

Melphalan preparation	Dose (mg/kg body wt)	Body wt (g ± S.E.M.) Day 1	Body wt (g ± S.E.M.) Day 8	% mean change in body wt	Tumour wt on day 8 (g ± S.E.M.)
Control (PBS)	—	23.2 ± 0.2	27.3 ± 0.2	+17	5.60 ± 0.10
Free drug	0.36	23.5 ± 1.3	26.2 ± 1.9	+11.5	3.55 ± 0.16
	0.73	23.8 ± 2.4	24.8 ± 1.6	+4	1.46 ± 0.35
	1.10	25.3 ± 1.5	25.2 ± 1.7	0	0.74 ± 0.23
	1.45	24.5 ± 1.3	24.3 ± 1.0	1	0.62 ± 0.06
	5.10	24.3 ± 1.3	21.9 ± 0.8	−10	0.21 ± 0.07
	11.80	21.5 ± 0.5	20.1 ± 0.6	−7	0.10 ± 0.02
PC, C (1:1)	0.20	23.2 ± 1.3	24.0 ± 1.3	+3	4.42 ± 0.23
	0.65	23.0 ± 1.0	24.0 ± 0.5	+4	2.27 ± 0.33
	0.91	22.2 ± 0.4	21.3 ± 0.6	−4	0.65 ± 0.06
	3.50	23.0 ± 0.9	21.2 ± 0.6	−7	0.46 ± 0.09
	3.90	24.6 ± 0.9	23.3 ± 0.8	5	0.68 ± 0.27
	4.30	24.8 ± 1.1	23.0 ± 0.8	7	0.64 ± 0.33
	12.90	25.3 ± 0.8	20.9 ± 0.7	−17	0.24 ± 0.08
SM, C (1:1)	0.40	22.2 ± 0.7	23.8 ± 0.2	+7	1.61 ± 0.10
	1.50	26.3 ± 1.4	24.3 ± 0.4	−7	0.04 ± 0.01
	1.80	24.8 ± 3.0	22.5 ± 0.3	−9	0.06 ± 0.02
	2.00	23.8 ± 0.2	22.3 ± 0.3	6	0.04 ± 0.01
	2.20	23.5 ± 2.0	21.5 ± 0.9	−9	0.04 ± 0.00
	3.20	26.8 ± 1.3	24.3 ± 0.4	−9	0.04 ± 0.01

* BALB/c mice weighing 16–20 g were inoculated subcutaneously with PC6 tumour as described in Materials and methods. Twenty-one days later when the tumours weighed 2–3 g, mice were divided at random into 20 groups. One group of 12 mice, served as control (PBS-treated) and 19 groups of 5 mice were given a single intravenous injection (0.2 ml) of various doses of melphalan in its free form (6 groups) or entrapped in cholesterol-rich PC (7 groups) or SM (6 groups) liposomes. Each liposome-treated mouse received 320 μmoles phospholipid/kg body wt. All animals were weighed on the day of the treatment (day 1) and on the day of their death (day 8). Tumour weight on day 8 was taken as a measure of the therapeutic activity of melphalan. Numbers in parentheses denote phospholipid:cholesterol (C) molar ratios.

activity) of the PC6 tumour in [^{14}C]melphalan or metabolite radioactivity after injection of mice with the free or entrapped drug: radioactivity values as seen in the tumours of mice injected with free melphalan (1.8% of the injected dose/g tissue at 4 hr), increased only modestly (up to 2.6-fold) by administering the drug in cholesterol-rich PC (4.75%), DMPC (3.3%) and SM (3.1%) liposomes (Table 1). In contrast, differences in drug uptake were much greater in the liver and spleen, which are known [17, 18] to endocytose liposomes. At 4 hr there was a 6–20-fold radioactivity increase (to 2.8–9.6%) in the liver and a 4–18-fold increase (to 0.7–3.6%) in the spleen over the values obtained with the free drug (Table 1). With both tissues, uptake was greatest with cholesterol-rich PC liposomes, presumably because: (a) these liposomes are less leaky to melphalan than cholesterol-free of cholesterol-poor PC liposomes [15] and, therefore, lose less drug en route to the tissues; and (b) are taken by the liver and spleen to a greater extent than similar DMPC and SM liposomes [8]. The decline in drug levels in the liver after 24 hr could be attributed to biliary excretion [19].

The effect of such liposome-induced changes in the clearance and tissue distribution of melphalan on its chemotherapeutic activity was investigated (Table 2). Tumour wt 8 days after a single intravenous injection of melphalan, free or entrapped in cholesterol-rich PC and SM liposomes, decreased in all treated mice (5.6 g in untreated animals) with increasing amounts of administered drug. However, whereas treatment with as little as 1.5 mg melphalan/kg body wt given in SM liposomes reduced the tumour wt to 0.04 g, such a reduction could not be achieved with free melphalan or with melphalan given in PC liposomes even when 11.8–12.9 mg of the drug/kg body wt was used. Promotion of drug effectiveness by SM liposomes is also reflected in PC6 tumour bearing mice treated with 0.5–2.6 mg per/kg body wt of free or liposome-entrapped melphalan and then observed for tumour regression: total regression and 100% survivors were achieved only with melphalan (all doses) entrapped in SM liposomes (Table 3 and legend). We were unable to establish the LD_{50} (and derive the therapeutic index) for melphalan entrapped in either of the liposomal preparations and even with twice the highest doses of Table 2 all tested mice survived. Still higher doses would have necessitated injection volumes that could not be tolerated by the animals. Others [20] have also failed to observe toxicity in PC6 tumour bearing mice injected intravenously with small liposomes containing a lipophilic alkylating agent, even at the highest dose.

The melphalan-containing PC and SM cholesterol-rich liposomes tested for chemotherapeutic activity were chosen for their widely different rates of melphalan release in the presence of blood [15] and vesicle clearance from the circulation [8, 13, 14]. Both a slower drug release from, and the long half-life (16 hr in mice [8]) of SM liposomes may have been instrumental in their pronounced chemotherapeutic activity (Tables 2 and 3). In conjunction with this it is of interest that free melphalan given by the intraperitoneal route to PC6 tumour bearing mice can be curative at the low dose of 0.1 mg/kg (Dr T. A. Connors, personal communication) but, apparently, not when given intravenously at a much higher dose (Table 3). It is probable that gradual entry of the drug from the peritoneal cavity into the periphery leads to its sustained presence in the circulation whereas intravenously given drug is removed very rapidly (less than 5% of the dose recovered in the blood after 1 hr [15]). It also seems that, under the present conditions, increased levels of drug in the tumour as seen with PC and SM liposomes (Table 1) are not necessarily linked with good chemotherapeutic performance for both preparations. It may be that in the case of SM liposomes tumour drug levels are maintained in an *active* form for extended periods of time as a result of the prolonged

Table 3. Tumour regression in PC6 tumour bearing BALB/c mice injected with free or liposome entrapped melphalan*

Melphalan preparation	Dose (mg/kg body wt)	% mean change in body wt	Tumour regression
Control (PBS)	—	+16	No regression
Free drug	0.5	+16	Partial, then regrowth
	1.0	+12	Partial, then regrowth
	1.4	+8	Partial, then regrowth
	1.9	−4	Partial, then regrowth
PC, C (1:1)	1.0	+7	Partial, then regrowth
	1.2	−4	Partial, then regrowth
	1.4	−3	Partial, then regrowth
	2.0	−3	Partial, then regrowth
SM, C (1:1)	1.0	−5	Total regression†
	1.6	−10	Total regression
	1.9	−13	Total regression
	2.6	−9	Total regression

* BALB/c mice bearing PC6 tumours as in the legend to Table 2 were divided 21 days after inoculation with the tumour into 13 groups. One group of 8 mice served as control (PBS-treated) and the remainder 12 groups (5 mice in each) were given a single intravenous injection (0.2 ml) of comparable doses of melphalan in its free form (4 groups) or entrapped in cholesterol-rich PC (4 groups) or SM (4 groups) liposomes. All animals were weighed on the day of treatment (day 1) and on day 8 and wt differences were recorded as % (see table). When tumour regression failed to occur or tumour regrowth occurred after partial regression, mice were killed when tumour wts approached 5 g. In supplementary similar experiments there was no regression in mice (4 in each group) treated with "empty" (PBS-loaded) cholesterol-rich PC or SM liposomes and tumour regression was partial followed by regrowth when mice (4 in each group) were treated with "empty" cholesterol-rich PC or SM liposomes mixed with melphalan (1.0 mg/kg body wt). Each liposome-treated mouse received 320 μmoles phospholipid/kg body wt. Numbers in parentheses denote phospholipid : cholesterol (C) molar ratios.

† Mice with total tumour regression survived over 30 days.

presence of the drug in the circulation [15] and, therefore, its continuous entry into the tumour. Evaluation of data becomes even more complex when one considers that the kinetics of drug metabolism by the tumour will probably differ according to the drug's rate of entry into the cells which, in turn, could be influenced by the rate of the drug's leakage from liposomes and association with plasma proteins [15].

The therapeutic efficacy of liposomal drugs in tumour-bearing animals has varied [9, 10, 20–26] according to the type of liposomes, the drug and the tumour model used. Our findings suggest that further work with alternative liposomal compositions and sizes which provide for a wider spectrum of rates of drug leakage and vesicle clearance in selected tumour models should improve our understanding of liposomal drug mode of action in experimental cancer chemotherapy and help to establish conditions for its optimization. Unfortunately, SM liposomes, which were so effective under the present conditions, induce, as such, spleen and liver enlargement in chronically treated mice [27]. It would therefore be of interest to see whether cholesterol-rich distearoyl phosphatidylcholine liposomes with a half-life of 20 hr in mice [14] are as effective.

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The action of histamine on *p*-nitrophenyl phosphatase activity in cardiac microsomes

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It is generally agreed that the enzyme system $(Na^+ + K^+)$ATPase is involved in the transport of Na^+ and K^+ across cell membranes [1]. The ATPase reaction is a two-step process; the Na^+-dependent formation of a phosphoenzyme, followed by its breakdown in the presence of K^+ [2, 3]. Phosphatase activity associated with the $(Na^+ + K^+)$-ATPase system has been demonstrated using the substrate *p*-nitrophenyl phosphate (pNPP), whose breakdown is stimulated by K^+ and inhibited by ouabain [4, 5]. It seems likely that K^+-activated *p*-nitrophenyl phosphatase (pNPPase) represents the terminal dephosphorylation phase of the $(Na^+ + K^+)$ATPase enzyme system on