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Characterisation of biotinylated liposomes for in vivo targeting applications

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Liposomes containing monosialoganglioside (G_{M1}) or polyethylene glycol (PEG) lipid derivatives have prolonged circulation in the blood. This favours liposome extravasation to tumour sites. In this report it is shown that inclusion of G_{M1}, PEG_{550}-DPPE or PEG_{2000}-DPPE in liposomes containing biotin-DPPE significantly diminished the ability of vesicles to bind to streptavidin in vitro. Steric inhibition due to the bulky head group of these lipids was least for biotin-DPPE liposomes containing G_{M1}. Biodistribution studies in C26 tumour-bearing mice showed that G_{M1}-liposomes containing small amounts of biotin-DPPE have long circulation life-times in the blood. Using fluorescent microscopic techniques, liposomes containing both G_{M1} and biotin-DPPE were detected within extra-vascular spaces in tumours. In addition it was shown that biotin-DPPE in G_{M1}-liposomes bound streptavidin in situ. These results suggest that G_{M1}-liposomes containing biotin-DPPE have potential use as diagnostic or therapeutic reagents in pre-targeting applications dependent on the high-affinity interaction of biotin with streptavidin.

Liposome; Streptavidin; Biotin; Ganglioside; Polyethyleneglycol; Targeted liposome

1. INTRODUCTION

Significant progress has been achieved in the development of liposomes as carriers of therapeutic or diagnostic agents [1,2]. The first liposome formulations were designed such that uptake by the reticuloendothelial system was minimised [3]. These liposomes circulate for extended times and as a consequence extravasate and reach tumour sites [4]. This property (also referred to as 'stealth'; LTI, Menlo Park, CA) is achieved by incorporation of lipids such as G_{M1} or PEG derivatives of phosphatidylethanolamine in liposome preparations. It has been suggested that sterically stabilised liposome formulations which have the ability to bind to target ligands in situ have potential applications for drug targeting to tumour tissues [5].

Tumour-specific targeting of liposomes can be achieved in vivo in two ways. A targeting molecule may be attached directly to liposomes by non-covalent [6] or covalent strategies [7–10] prior to administration. Alternatively tumours may be labelled in vivo indirectly by exploiting high-affinity interactions based on reagents such as biotin and streptavidin. In this case, labelling of selected tissues may be achieved in three steps by administration of biotinylated antibody, a bridging molecule, such as avidin or streptavidin, and lastly biotinylated liposomes. In a recent study, a step-wise approach to targeting was used successfully to localise ^{111}In-labelled biotin to tumours [11]. Substitution of radioactively labelled biotin with biotin-derivatised vesicles containing large amounts of radioactive nuclides (or anti-cancer drugs) may further aid in early detection and treatment of cancer.

The ability to target biotinylated liposomes to tumour sites in vivo will depend on a number of factors. Access of liposomes to avidin- or streptavidin-coated cells within the tumour is a pre-requisite. Secondly biotin lipid derivatives must remain stably associated with liposomes in vivo. In addition, lipid components incorporated into liposome systems should not hinder the high-affinity binding of streptavidin to biotin-liposomes in vivo. In this report we first describe studies aimed at optimising the binding of streptavidin to biotinylated-liposomes in vitro. We then describe the in vivo behaviour of liposomes containing biotin lipid derivatives in tumour-bearing mice. This information should aid in

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Abbreviations: G_{M1}, monosialoganglioside; PEG, polyethyleneglycol; biotin-DPPE, *N*-biotinoyl dipalmitoyl phosphatidyl-ethanolamine; EPPS, *N*-(2-hydroxyethyl) piperazine-*N*'-3-propanesulphonic acid; HEPES, *N*-(2-hydroxyethyl) piperazine-*N*'-2-ethanesulphonic acid; DTPA, di-ethylene-tetraamino pentaacetic acid; HBS, 25 mM HEPES, 150 mM NaCl, pH 7.5; PBS, phosphate buffered saline.

the development of liposome based pre-targeting protocols for drug targeting applications.

2. MATERIALS AND METHODS

2.1. *Materials*

Egg phosphatidylcholine (EPC) was obtained from Avanti Polar Lipids (Alabaster, USA). *N*-((monomethoxy-polyethyleneglycol$_{2000}$) succinoyl)-2-oleoyl-1-palmitoyl-*sn*-glycero-3-phospho-ethanolamine (MePEG$_{2000}$-S-POPE) and *N*-((monomethoxy-polyethyleneglycol$_{550}$) succinoyl)-2-oleoyl-1-palmitoyl-*sn*-glycero-3-phospho-ethanolamine (MePEG$_{550}$-S-POPE) were gifts from Lipex Lipids Inc. (Vancouver, Canada). *N*-Biotinoyl dipalmitoyl phosphatidylethanolamine (biotin-DPPE), cholesteryl 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-dodecanoate (cholesteryl-BODIPY FL C12) and rhodamine 123 were purchased from Molecular Probes (Eugene, USA). Monosialoganglioside (G_{M1}) was a gift from Fidia (Abano Terme, Italy). Cholesterol, Texas red-labelled streptavidin, Tween 20, A23187, diethylene-tetraamino pentaacetic acid (DTPA), HEPES and citric acid were purchased rom Sigma Chemical Co. All other chemicals were of standard grade. Balb/c female mice (18–20 g) were obtained from Charles River (Calco, Italy). C-26 colon carcinoma cells were kindly supplied by Dr. G. Parmiani of the Istituto Nazionale Tumori in Milano.

2.2. *Preparation of liposomes*

Large unilamellar vesicles (LUVs) were prepared as described by Hope et al. [12]. Briefly, appropriate amounts of lipid mixtures dissolved in chloroform were deposited in a tube and dried to a lipid film under a stream of nitrogen followed by high vacuum for 2 h. Lipid samples were hydrated in the appropriate buffer, frozen and thawed 5 times and extruded 10 times through two stacked 100 nm polycarbonate filters. Liposome size, as estimated by Quasi-elastic light scattering (Brookhaven, Holtsville, USA) for all liposome preparations studied was between 90–115 nm.

2.3. *Remote loading of liposomes with aqueous markers*

Rhodamine 123-loaded liposomes (EPC 59.9–59 mol%; cholesterol 40 mol%) containing biotin-DPPE (0.1–1 mol%) were prepared by a modification of the procedure of Mayer et al. [13,14]. For sterically stabilised liposome samples G_{M1}, PEG_{550} or PEG_{2000} (5 mol%) were included in the lipid film. In brief, liposomes prepared in 300 mM citric acid buffer, pH 4.0, were exchanged on PD-10 columns (Pharmacia) equilibrated with 25 mM EPPS, 150 mM NaCl, pH 8.5. Rhodamine 123 (100 nmol) was incubated with chromatographed liposomes (5 mM total lipid) for 15 min at 60°C. Unencapsulated rhodamine 123 was separated from liposomes by gel filtration on PD-10 columns equilibrated with 25 mM HEPES, 150 mM NaCl, pH 7.5 (HBS). Rhodamine 123 was estimated by monitoring fluorescence at 536 nm using a Perkin Elmer L5 spectrofluorimeter with an excitation wavelength of 512 nm. Phospholipid was determined by the colorimetric method of Fiske and Subbarrow [15].

^{111}In-loaded vesicles were prepared by the method of Mauk and Gamble [16]. Briefly, liposomes composed of EPC (40–35.25 μmol), cholesterol (20 μmol), GM_1 (4 μmol), biotin-DPPE (0.16–3.2 μmol) and the ionophore, A23187 (0.16 μmol), were prepared by hydrating the lipid film in HBS containing the chelator DTPA (10 mM). Liposomes were incubated with $^{111}InCl_3$ (30–40 μCi) in sodium citrate buffer, pH 7.4, after removal of unentrapped DTPA by column chromatography on Sephadex G-25 equilibrated with HBS. The molar ratio of DTPA to $InCl_3$ was 5:0.15 and the log [citrate]/[$InCl_3$] was 2.5 in the incubation mixture. After 1 h incubation at 55–60°C, vesicles were chromatographed on PD-10 columns equilibrated with HBS to remove unentrapped ^{111}In. Aliquots were counted in a LKB gamma counter (Bromma, Sweden) to estimate ^{111}In levels. Efficiency of ^{111}In encapsulation in liposomes varied between 60–89%.

2.4. *Binding of biotin-DPPE liposomes to streptavidin in vitro*

Rhodamine 123 was loaded into liposomes as described. Binding of liposome preparations containing biotin-DPPE to streptavidin was studied on microtitre plates [14]. First, 96-well plates (Nunc) were coated with streptavidin by incubating protein (1 μg/well in 50 mM carbonate buffer, pH 9.6) with plates at 37°C for 1 h. Plates were then washed with Tween wash solution (0.05% Tween 20 in 150 mM NaCl) 3 times and then once with HBS to remove unbound streptavidin. Biotin-DPPE liposomes (0–200 nmol in 200 μl of HBS) containing rhodamine 123 (80–90 nmol/μmol total lipid) were added to wells and plates were incubated for a further hour at 37°C. After 4 washes of plates with HBS, levels of plate-associated liposomes were determined after addition of ethanol (300 μl) by estimating rhodamine 123 fluorescence as described above. Non-specific binding of biotin-DPPE liposomes to plates was estimated in the presence of excess free biotin (1,000 molar excess to the biotinylated lipid derivative in liposomes).

2.5. *Biodistribution studies in tumour-bearing mice*

Balb/c female mice were injected with C-26 colon carcinoma cells ($1–5 \times 10^5$). 2–3 weeks post-injection mice bearing tumours (0.7–0.8 cm in diameter) were injected i.p. or i.v. with liposomes (EPC 62–61.25 mol%; cholesterol 31 mol%; G_{M1} 6.2 mol%; biotin-DPPE 0.25 mol%; 0.5 μmol total lipid in 0.2 ml) containing trapped ^{111}In (15–20 μCi). At indicated times, blood was collected under ether anesthesia by cardiac puncture in heparinised tubes. For studies on the biodistribution of liposomes, animals were sacrificed by cervical dislocation. Organs were removed, washed with heparinised saline, weighed, and levels of ^{111}In liposomes in tissues and blood were estimated by counted samples in a LKB gamma counter (Bromma, Sweden). Results are expressed as a percentage of the injected dose normalised per g of tissue [11].

2.6. *Fluorescence spectroscopy experiments*

Liposomes (EPC/cholesterol/GM_1, molar ratio 10:5:1) containing the non-exchangeable fluorescent cholesterol ester derivative, cholesteryl-BODIPY FL C12 (0.5 mol%), with or without biotin-DPPE (0.25 mol%), were prepared as described above. C26 tumour-bearing mice were injected with liposomes (1 μmol in 0.2 ml) by the tail vein. At 24 h, mice were sacrificed, tumours were removed and fixed in 4% paraformaldehyde overnight. Thin sections of about 22–25 μm were prepared. In situ accessibility of the biotin moiety of DPPE in liposomes was examined using Texas red-labelled streptavidin. Non-specific binding sites were blocked by pre-incubation of sections with PBS containing 1% BSA for 20 min. After several washes, sections were incubated with streptavidin labelled with Texas red (10 μg/ml in PBS) for 30 min at room temperature. Slices were washed several times with PBS prior to fluorescent microscopic analysis.

Fluorescence emission spectra of tumour sections were recorded under epi-illumination conditions by means of a Leitz microspectrograph (Wetzlar, Germany) equipped with an optical multichannel analyser (Princeton Applied Research, Princeton, USA) mounted with an intensified silicon photodiode array detector (model 1420/512). As the excitation source, a 100 W mercury lamp (Osram, Germany) was used, in combination with KG1 and BG38 anti-thermic filters. A UG1 filter (2 mm, 350–405 nm pass band) was used to select the excitation wavelengths. These wavelengths do not correspond to the excitation peaks of BODIPY and Texas red (479 and 490 nm, respectively). They were selected to obtain an appreciable fluorescence signal in order to analyse both fluorochromes simultaneously in stained samples.

Fluorescent image analysis in tumour sections was performed under epi-illumination conditions by means of a photon-counting processor (Hamamatsu Photonic, Germany) equipped with a Hamamatsu C2400-09 ISIT camera attached to a Leitz Orthoplan fluorescence microscope. As the excitation source, a 100 W mercury lamp (Osram, Germany) combined with KG1 and BG38 anti-thermic filters was used. A UG1 filter (2 mm, 350–405 nm pass band) was used to select the excitation wavelengths. The 455 and 515 interference filters and the 630 cut-off filters were used to select the wavelengths corresponding to background, BODIPY and Texas red emissions, respectively. Fluorescence images were shown on a Sony Trinitron colour video monitor (Sony Corp., Tokyo, Japan), digitally stored on mass mag-

netic memory and printed as photographs or slides by means of a Freeze-frame video (Polaroid, Cambridge, MA, USA). Measurements were performed with Leitz 25 × (NA 1,32) oil-immersion objectives.

3. RESULTS

3.1. *In vitro studies*

Binding of biotin-DPPE liposome samples to streptavidin was studied on streptavidin-coated microtitre plates [15]. Lipid was estimated from measurements of fluorescence due to vesicle-associated rhodamine 123. Results are expressed as the percentage of lipid bound to total lipid added, for a range of lipid concentrations (0–100 nmol added per well). As shown in Fig. 1, the % of lipid bound to plates decreased as the amount of EPC/cholesterol/biotin-DPPE liposomes added to wells was increased. This was due to saturation of streptavidin binding sites on plates with biotin-DPPE-liposomes at concentrations greater than 50 nmol lipid. Lipid binding was dependent on incorporation of biotin-DPPE in vesicles as minimal amounts of biotin-DPPE liposomes bound to plates in the presence of excess free biotin (1,000 molar excess to the biotinylated lipid derivative in liposomes). This microtitre protocol was used to study the influence of lipid composition on the binding of biotinylated liposomes to streptavidin.

The effect of varying the mol% of biotin-DPPE in liposomes on levels of lipid bound to streptavidin-coated plates was first studied. The results in Fig. 2 show a clear correlation between amounts of lipid

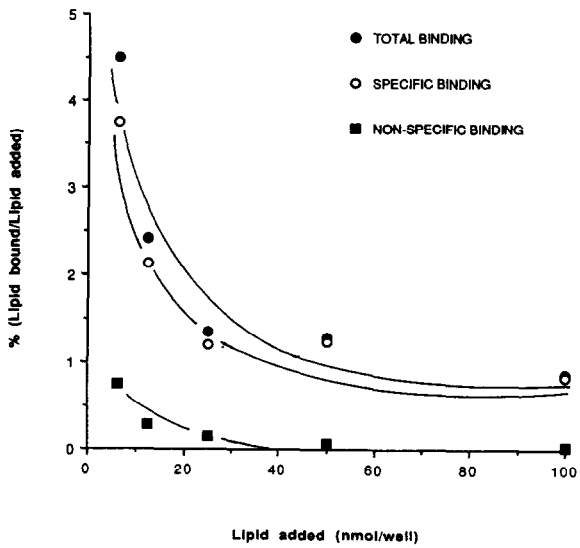

Fig. 1. Binding of biotin-DPPE liposomes to streptavidin coated microtitre plates. Liposomes (EPC 59.75 mol%; cholesterol 40 mol%; biotin-DPPE 0.25 mol%) containing rhodamine 123 (90 nmol/μmol lipid) were added to streptavidin-coated plates at various concentrations (0.50 nmol/well in 200 μl). Plates were incubated for 1 h at 37°C, washed, and lipid remaining bound was estimated as described in section 2. For non-specific lipid binding estimates, biotin-DPPE liposomes were incubated in the presence of excess free biotin (1,000 molar excess).

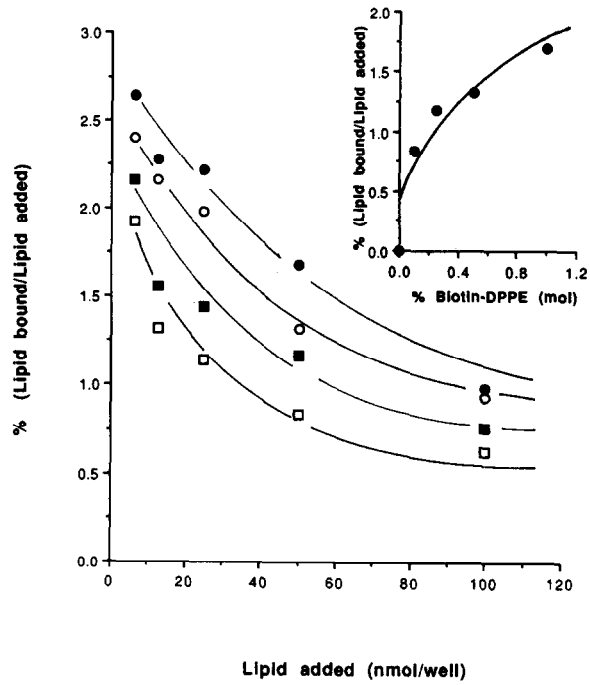

Fig. 2. Effect of varying mol% of biotin-DPPE in EPC/cholesterol liposomes. Binding of liposomes (EPC 59.9–59.75 mol%; cholesterol 40 mol%; biotin-DPPE 0.1–1 mol%) to streptavidin-coated plates was determined as described in Fig. 1. Data are presented as specific binding of liposomes to plates. (Inset) Relationship between amount of biotin-DPPE in liposomes and % of lipid bound to plates.

bound and the mol% of biotin-DPPE present in liposomes. For example, as the mol% of biotin-DPPE in liposomes was increased from 0.1 to 1 mol%, the % levels of lipid bound to plates increased from 0.8 to 1.7% when 50 nmol lipid was added to wells. As shown in the inset in Fig. 2, there was a non-linear relationship between the mol% of biotin-DPPE in liposomes and% lipid bound to plates. Levels of lipid bound to plates tended to reach saturation when liposomes were prepared containing 1 mol% biotin-DPPE.

Considerable effort has been made to design liposomes which have the ability to extravasate and reach tumour sites in vivo. The most common formulations require the incorporation of G_{M1} or PEG derivatives of PE in the lipid membrane [4]. We investigated the effect of these lipids when included in vesicles on the binding of biotin-DPPE liposomes to streptavidin. The results in Fig. 3 illustrate that inclusion of G_{M1}, PEG_{550} or PEG_{2000} (5 mol%) caused a decrease in levels of lipid bound to streptavidin plates. As the length of the PEG moiety was increased from 550 to 2,000, levels of lipid bound to streptavidin plates decreased accordingly (41 and 67%, respectively, of control biotin-DPPE liposomes when 50 nmol lipid was added per well). Steric hindrance was less significant for biotin-DPPE liposomes containing G_{M1} (31% of control biotin-DPPE liposomes bound when 50 nmol lipid was added/well).

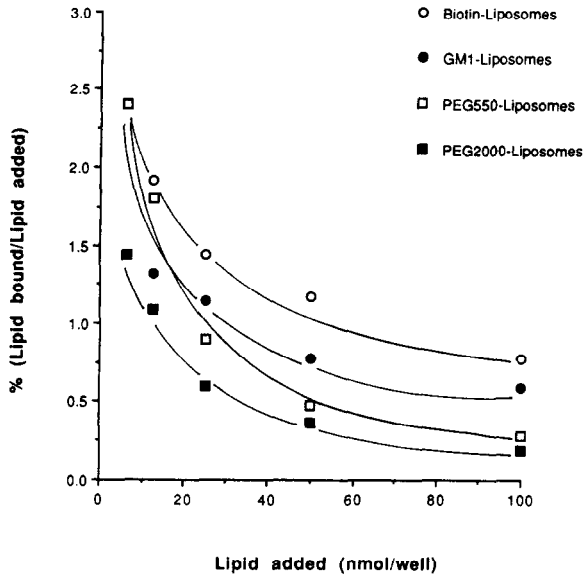

Fig. 3. Influence of stealth lipids on biotin-DPPE liposome binding to streptavidin. Liposomes (EPC 54.75–59.75 mol%; cholesterol 40 mol%; biotin-DPPE 0.25 mol%) containing G_{M1}, PEG_{550} or PEG_{2000} (5 mol%) were prepared as described in section. Binding of liposome samples to streptavidin plates was determined as described in Fig. 1.

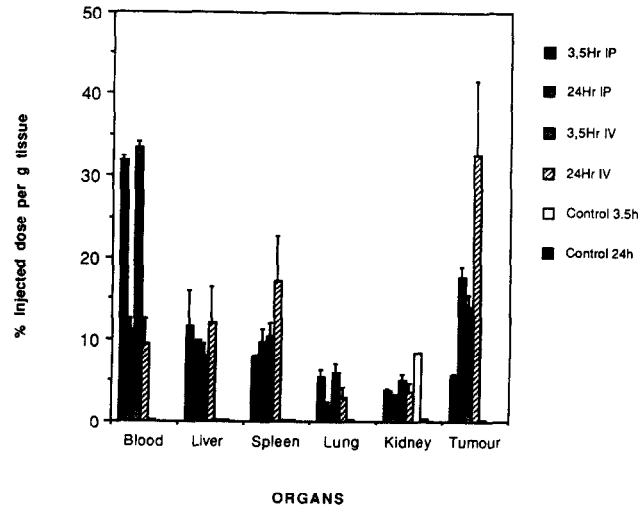

Fig. 4. In vivo behaviour of G_{M1} liposomes containing biotin-DPPE in C26 tumour-bearing mice. Liposomes (EPC 62 mol%; cholesterol 31 mol%; G_{M1} 6.2 mol%; biotin-DPPE 0.25 mol%; 0.5 μmol total lipid in 0.2 ml) containing trapped ^{111}In (15–20 μCi) were injected i.p. or i.v. into balb/c female mice bearing tumours (0.7–0.8 cm in diameter). At 3.5 and 24 h liposome biodistribution in various organs was estimated as described in section 2.

3.2. *In vivo behaviour of biotinylated liposomes*

Our interest is to use biotin-DPPE liposomes in targeting applications in vivo. We first examined the biodistribution of EPC/cholesterol/G_{M1} liposomes containing biotin-DPPE in female mice bearing C26 tumours (Fig. 4). These liposome preparations had long circulation times in the blood. Up to 30–35% of the injected dose was detected in the blood 3.5 h post-injection when liposomes were administered i.v. or i.p. After 24 h, levels of liposomes in tumours were high (15–30%). Tumour-associated radioactivity was due to liposome-encapsulated ^{111}In-DTPA, as free ^{111}In-DTPA when injected i.v. was not detected in solid tumours 3.5 or 24 h post-injection (Fig. 4). These results show that inclusion of biotin lipid derivatives in G_{M1}-liposomes did not affect their ability to circulate for extended periods in the blood and extravasate to tumour sites. Increasing biotin-DPPE in vesicles up to 1 mol% did not affect this behaviour of liposomes in C26 tumour-bearing mice (data not shown).

The ability of streptavidin to cross-link biotinylated targeting ligands with biotinylated liposomes within tumours will determine the success of diagnostic or therapeutic applications based on pre-targeting protocols. With this aim in mind, we examined the accessibility of streptavidin to bind biotin-DPPE in liposomes at extra vascular sites within tumours. EPC/cholesterol/G_{M1} liposomes containing the fluorescent lipid marker cholesterol-BODIPY (0.5 mol%) and in some cases biotin-DPPE (0.25 mol%), were injected into tumour-bearing mice by the tail vein. Slices prepared from tumours 24 h post-injection were probed for biotin binding reactivity with Texas red-labelled streptavidin. The fluorescence emission spectra of tumour slices are shown in Fig. 5. An emission band at 515 nm due to BODIPY-derivatised cholesterol ester, was detected when tumour slices were prepared from mice injected with either biotinylated or non-biotinylated G_{M1}-liposomes. A second signal at 600 nm was detected when tumour slices prepared from mice injected with G_{M1}-liposomes containing biotin-DPPE, were probed with Texas red-labelled streptavidin. Under equivalent conditions, negligible levels of fluorescence were obtained at 600 nm when

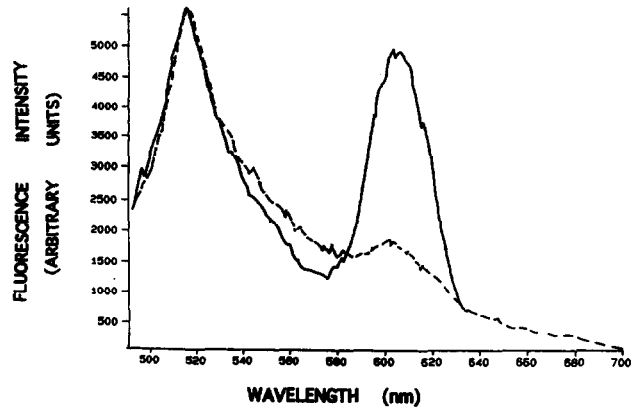

Fig. 5. Microspectrofluorimeter analysis of tumour sections. C26 tumour-bearing mice were injected with cholesterol-BODIPY liposomes containing G_{M1} and in some cases biotin-DPPE. 24 h post-injection, tumours were isolated, thin sections were prepare and probed for reactivity with Texas red streptavidin. Fluorescent spectra were obtained from tumour sections isolated from mice injected with (dashed line) G_{M1}-liposomes or (solid line) G_{M1}-liposomes containing biotin-DPPE.

biotin-DPPE was not included in the liposome formulation.

The fluorescence patterns corresponding to the distribution of liposomes in tumours, before and after streptavidin labelling, are shown in Fig. 6. Natural fluorescence due to tumour tissue was subtracted from images. Two distribution patterns of BODIPY-labelled liposomes were present in the tumour sections: Fig. 6A shows diffuse fluorescence in extracellular spaces; Fig. 6B shows groups of fluorescent granules localised near vessel-like structures. In the case of biotinylated liposomes, the fluorescent signal due to Texas red streptavidin overlayed that attributed to cholesterol-BODIPY in liposomes (Fig. 6B and D). This was particularly obvious in tumour regions displaying a clustered liposome distribution, and confirmed the interaction between biotin residues on vesicle surfaces and streptavidin. Overall, tumour morphology suggests a distribution of liposomes in extracellular spaces and near blood vessels.

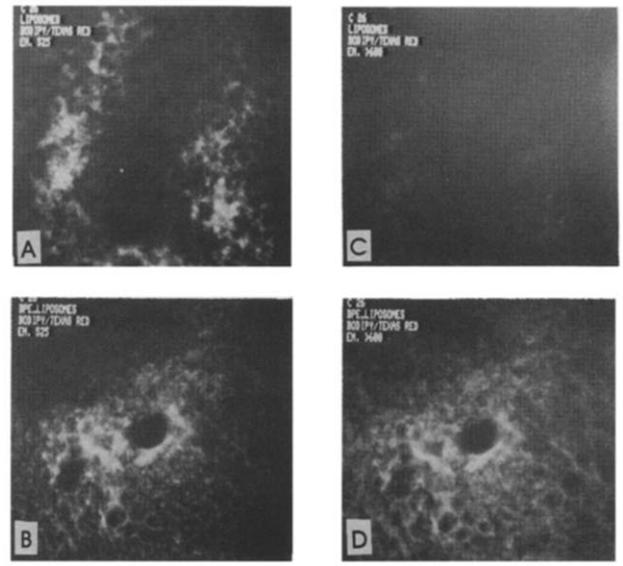

Fig. 6. Image analysis of tumour sections. Fluorescence patterns of tumour sections from animals treated with G_{M1}-liposomes containing cholesterol-BODIPY and in some cases biotin-DPPE after staining with Texas red streptavidin are shown. Data were acquired at 515 nm (corresponding to BODIPY emission) and 630 nm (corresponding to Texas red emission) and is corrected for natural fluorescence.

4. DISCUSSION

Targeted delivery of liposomes containing radionuclides or anti-cancer agents to tumours has significant diagnostic and therapeutic potential [1]. Our interests have concentrated on pre-targeting strategies which exploit the high-affinity binding of streptavidin to biotinylated molecules [5,6,9]. A possible strategy could be to sequentially label tumours with biotinylated antibody, streptavidin and finally biotinylated liposomes. Accessibility of streptavidin to bind both biotinylated antibodies and biotinylated liposomes at tumour sites is essential for optimal tumour targeting. In this report we concentrated on optimising binding of biotin-DPPE liposomes to streptavidin. First we show that liposome composition can influence the ability of biotinylated liposomes to bind streptavidin in vitro. Next we addressed the in vivo properties of liposomes containing both G_{M1} and biotin-DPPE in tumour-bearing mice. It is shown that sterically stabilised liposome preparations containing biotin-DPPE remain intact in vivo and extravasate at tumour sites. In addition we demonstrate that streptavidin has access to the biotin moiety in liposomes within the extracellular spaces in solid tumours. This information supports the use of biotinylated liposomes in indirect targeting protocols.

The capacity of biotinylated liposomes to bind efficiently to streptavidin at tumour sites will be dictated by first the ability of liposomes to reach the tumour and secondly accessibility of streptavidin to biotin in liposomes. Significant progress has been made in the design of liposome formulations which avoid the reticuloendothelial system, circulate for long times in the blood and extravasate at tumour sites [3,4]. Few studies have addressed how liposome components included in vesicles for optimal in vivo drug delivery applications affect the ability of liposomes to bind to target antigen. This study shows that inclusion of low amounts (0.25–1 mol%) of biotin-derivatised lipid in EPC/cholesterol liposomes is sufficient for optimal binding to streptavidin. This corresponds to between 110–440 biotin molecules present in the outer monolayer for vesicles with a diameter of 100 nm. The ability of biotin-DPPE liposomes to bind to streptavidin is however dramatically influenced by inclusion of lipids possessing 'stealth' properties in vivo. For example, a 70% decrease in levels of lipid bound to streptavidin-coated plates was detected for biotin-DPPE liposomes containing DPPE-PEG_{2000} when compared to control biotin-DPPE liposomes. This inhibitory effect was alleviated in part by reducing the length of PEG coupled to DSPE (PEG_{550}) or substitution with G_{M1} (31% and 41% decrease in % of lipid bound, respectively, when compared to control biotin-DPPE liposomes).

To maximise binding of biotin-DPPE liposomes containing G_{M1} to streptavidin, it may be beneficial to introduce a spacer arm between the biotin moiety and the liposome surface. Recently we observed that a 6 carbon spacer arm biotin derivative of DPPE (termed biotin-X-DPPE) alleviates steric hindrance attributed to G_{M1} [15]. However, inclusion of PEG_{2000}-DSPE significantly inhibited binding of biotinylated liposomes to streptavidin-coated plates. Using a liposome-based agglutination assay similar results were recently reported [17]. These findings suggest that the design of sterically stabilised liposome formulations for targeting applications in vivo may require a compromise between optimal cir-

culation times in the blood and optimal association of streptavidin with biotin-DPPE liposomes.

Targeting of biotin-DPPE liposomes to streptavidin-coated tumour cells in vivo will depend first of all on their ability to access tumour sites. In this study we show that stealth liposomes containing 0.25 mol% biotin-DPPE have long circulation half-lives in the blood. At extended times substantial levels of biotin-DPPE liposomes were detected in tumours (15–30%). Levels reported here are significantly higher than previous reports for G_{M1}-liposomes (12% of injected dose/g tissue, [3]) or for DSPC/cholesterol liposomes containing biotin-X-DSPE (7% of injected dose/g tissue, [18]). The reason for this discrepancy is not clear. One possibility is that high grade G_{M1} vs. commercially available product was used in these studies. Overall we conclude that inclusion of small amounts of biotin-DPPE in G_{M1}-liposomes does not appear to affect vesicle stability or the stealth nature of the liposome preparation in vivo.

In order to direct biotin-DPPE liposomes to streptavidin-coated antibodies within the tumour mass, biotin-DPPE should remain as an integral component of the lipid membrane. In addition biotin in liposomes must have access to streptavidin in extra-vascular spaces within the tumour. Previous studies have shown, using both aqueous and lipid markers, that G_{M1}-containing liposomes have access to extra-vascular tumour spaces [3,4,19]. Using fluorescent microscopy we confirm that biotin-DPPE liposomes containing G_{M1} and the fluorescent liposome marker, cholesterol-BODIPY, localise within the vasculature of the tumour. We further demonstrate that the biotin moiety of DPPE in liposomes can be detected within tumours by specific interaction with Texas red-labelled streptavidin. Biotinylated liposomes may therefore bind to target cells when tumours are pre-labelled with biotinylated ligands and coated with streptavidin.

In summary our work clearly supports the use of G_{M1} liposomes containing biotin-DPPE in indirect targeting applications in vivo. This liposome system should be capable of binding to tumours coated with streptavidin-labelled biotinylated antibodies. At present the use of G_{M1} liposomes containing biotin-DPPE in vivo would represent a compromise between optimum circulation half-lives in the blood and maximum capacity of streptavidin to bind biotin in liposomes. The development of novel biotinylated lipid derivatives which, when incorporated into liposomes, confer stealth properties in vivo and in addition retain the ability to bind streptavidin efficiently, would increase the potential application of this approach to tumour targeting in vivo.

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