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Rapid Report

The presence of G_{M1} in liposomes with entrapped doxorubicin does not prevent RES blockade

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The incorporation of ganglioside G_{M1} or phosphatidylethanolamine-polyethyleneglycol conjugates into liposomes can result in extended circulation lifetimes in vivo. This has been attributed to an ability to avoid uptake by the reticuloendothelial system (RES), specifically the phagocytic cells of the liver and spleen. Here we examine whether a representative large unilamellar vesicle (LUV) formulation which contains G_{M1} (distearoylphosphatidylcholine/cholesterol/G_{M1}, 45:45:10 mol/mol), actually does avoid the RES. It is shown that a pre-dose of LUVs which contain G_{M1} and entrapped doxorubicin blocks the accumulation of subsequently injected empty distearoylphosphatidylcholine/cholesterol liposomes in liver. It is therefore concluded that liposomes exhibiting extended circulation lifetimes can induce RES blockade and do not avoid uptake by liver phagocytes.

The use of liposomes as drug delivery vehicles requires extended blood residence times to allow optimal delivery to target tissue. Typically, 100 nm diameter large unilamellar vesicles (LUVs) composed of long-chain, saturated lipids such as distearoylphosphatidylcholine (DSPC) in combination with cholesterol are used as a basic delivery vehicle [1,2]. The circulation lifetimes of these systems can be improved by incorporation of the ganglioside G_{M1} [3]. Recent work has also focused on the use of phosphatidylethanolamine-polyethyleneglycol derivatives as an alternative means of providing the hydrophilic coat apparently required to prolong circulation lifetimes [4–8]. Generally, these "extended lifetime" formulations yield dose-independent clearance kinetics, with 5 to 30% of the injected dose remaining in the (mouse) circulation at 24 h. It has been suggested that these formulations exhibit an ability to avoid uptake by the phagocytic cells of the reticuloendothelial system (RES) found predominantly in the liver and spleen [3–8].

In this work we examine the validity of this hypothesis. The approach taken is based on previous work from this laboratory concerning the characterization and biodistribution of doxorubicin-loaded liposomes [1]. It was shown that the RES function was strongly influenced by liposomes containing doxorubicin as such liposomes were cleared from the circulation at a much slower rate than empty liposomes. Further, it was found that pre-dosing with liposomes containing doxorubicin resulted in greatly extended circulation lifetimes of a subsequent injection of empty liposomes. These results were attributed to an ability of doxorubicin-loaded liposomes to impair or "blockade" RES function. The experiments performed here compare the influence of G_{M1} on the ability of a pre-dose of liposomes containing doxorubicin to blockade RES function, as expressed by the extended circulation lifetimes exhibited by a subsequent injection of empty liposomes. One of two possible results would be expected. If G_{M1}-containing liposomes with entrapped doxorubicin do in fact avoid the RES, the liver and spleen function should not be affected and the subsequent injection of empty liposomes should be cleared normally. On the other hand, if G_{M1}-containing liposomes do not avoid the RES, we would expect to see an impaired ability of the RES to clear liposomes and thus extended circulation lifetimes for subsequent injections.

The liposome preparations and doxorubicin entrapment procedures employed in this study have been

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Abbreviations: RES, reticuloendothelial system; LUV, large unilamellar vesicle; G_{M1}, monosialoganglioside; DSPC, distearoylphosphatidylcholine; HBS, HEPES-buffered saline; EDTA, ethylenediaminetetra-acetic acid.

described in detail previously [1]. Briefly, lipid mixtures in chloroform were dried to a film under a stream of nitrogen gas, then further dried under high vacuum for a minimum of 4 h. For the pre-dose compositions, the lipid was hydrated with 300 mM citric acid (pH 4.0), frozen and thawed five times, and then extruded at 65°C ten times through three stacked polycarbonate filters (Nuclepore, Canada) of 100 nm pore size employing an extrusion device (Lipex Biomembranes, Canada). An approx. 100 nm mean diameter for the resultant LUVs was determined employing a Nicomp 370 particle sizer. A transmembrane pH gradient was established by passing the LUV preparations down a Sephadex G-50 column equilibrated with 150 mM sodium carbonate buffer (pH 7.5) and collecting the LUVs in the void volume. For entrapment of doxorubicin, an aliquot of preheated doxorubicin in saline was then added to preheated liposomes at 65°C to achieve the indicated drug/lipid ratios (mol/mol) and incubated at this temperature for a further 10 min. Entrapment efficiencies were in excess of 95%. For the empty LUVs employed for the subsequent injection, the lipid film was hydrated in HBS (20 mM HEPES, 150 mM NaCl, pH 7.4) and extruded as described above. DSPC was obtained from Avanti Polar Lipids, doxorubicin was obtained from Adria Laboratories, and cholesterol, G_{M1}, and all other chemicals were obtained from Sigma.

Female BDF-1 mice (20–23 g, Charles River, Canada) were injected with the specified dose of empty and drug-loaded liposomes via the lateral tail vein. Pre-doses consisted of 100 nm diameter DSPC/cholesterol (55:45, mol/mol) LUVs and 100 nm diameter DSPC/cholesterol/G_{M1} (45:45:10) LUVs, with or without entrapped doxorubicin. These LUVs were injected at a dose of 0.33 μmol lipid per mouse (10 mg/kg lipid dose for DSPC/cholesterol) delivered in a volume of 200 μl. A trace amount of [^{3}H]cholesteryl hexadecyl ether (NEN, Canada) was used as a non-metabolizable and non-exchangeable lipid marker [9] for determining the biodistribution of this pre-dose at 24 h. Blood was collected by heart puncture and placed in EDTA-treated microtainers (Becton-Dickinson, Canada). Plasma was prepared by centrifuging (200 × g) the blood samples for 10 min. Liver and spleen were extracted, weighed, and digested as required for estimation of tissue-associated radioactivity. For the biodistributions of the subsequent injections, the pre-doses described above (but not containing any radiolabeled lipid marker) were given, and then a subsequent injection of empty liposomes was administered 24 h later. This later injection was composed of 100 nm diameter DSPC/cholesterol (55:45) LUVs with a trace amount of [^{3}H]cholesteryl hexadecyl ether, injected at a dose of 3.3 μmol lipid per mouse (100 mg/kg lipid dose) delivered in 200 μl. One control group of mice received no pre-dose. The mice were sacrificed 24 h later and the liposome biodistribution in blood, liver, and spleen was determined. All biodistribution results were analyzed using a two-tailed Student's t-test.

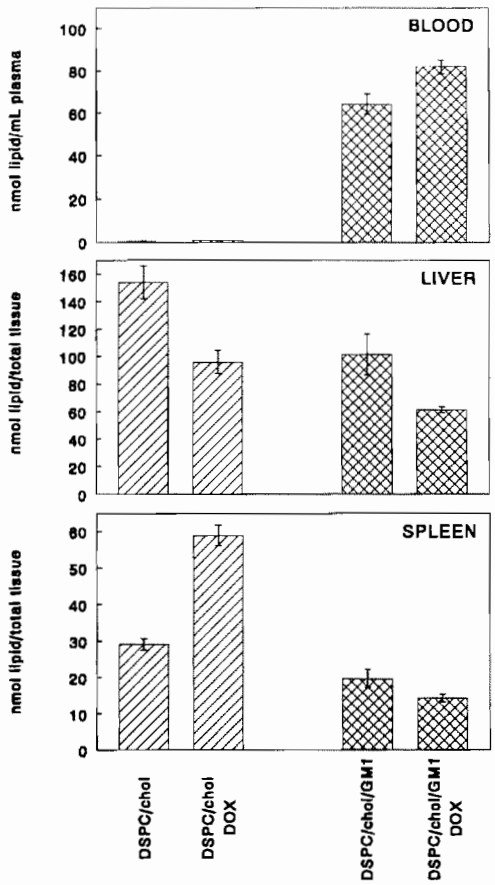

Fig. 1. Biodistribution of the pre-dose. Large unilamellar vesicles of various compositions were injected via lateral tail vein at a dose of 0.33 μmol lipid/mouse. At 24 h, the mice were sacrificed and tissue samples indicated were taken. Lipid compositions used were DSPC/cholesterol (55:45 mol/mol), and DSPC/cholesterol/G_{M1} (45:45:10). Where employed, doxorubicin was entrapped at a drug/lipid ratio of 0.2 (mol/mol). Values shown represent the mean of results from 8 animals ± S.E. of the mean.

The biodistribution of the pre-dose at 24 h after injection is shown in Fig. 1. In the blood, the liposomes which did not contain G_{M1} are present in very low levels (< 1% of injected dose) whereas those containing G_{M1} are present at levels corresponding to approx. 25% of the injected dose at 24 h. The difference is largely accounted for by reduced liver and (to a smaller extent) spleen uptake for the G_{M1}-containing formulation. Both DSPC/cholesterol liposomes and G_{M1}-containing liposomes exhibit significantly reduced liver uptake when the liposomes contain entrapped doxorubicin ($P < 0.05$ for both groups). This results in greatly increased uptake in the spleen for the DSPC/cholesterol LUVs which did not contain G_{M1}. However this effect is not seen for G_{M1}-containing liposomes

where the two spleen panels are not significantly different ($P > 0.05$).

The biodistribution 24 h after the subsequent injection of empty DSPC/cholesterol LUVs is shown in Fig. 2. The biodistribution observed in a group of mice which received no pre-dose is shown in the left panels. This pattern of blood clearance and liver and spleen uptake is taken as a primary control. Pre-doses of liposomes which did not contain drug results in no significant difference in the uptake into the liver and spleen from their respective controls (P values all > 0.05) and indicates that pre-injection of a low dose of lipid alone does not alter the pattern of uptake into these tissues. A slight increase in blood levels for the pre-dose containing G_{M1} is observed. However, pre-injection of drug-loaded liposomes which did not contain

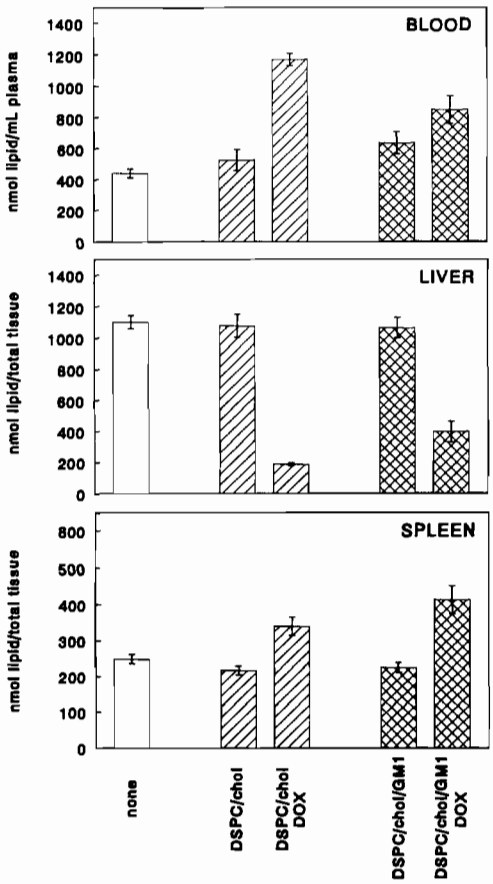

Fig. 2. Biodistribution of the subsequent injection of empty DSPC/cholesterol large unilamellar vesicles. At 24 h after injection of the pre-doses indicated at bottom, LUVs composed of DSPC/cholesterol (55:45 mol/mol) were injected via lateral tail vein at a dose of 3.3 μmol lipid/mouse. The mice were sacrificed 24 h later and lipid levels of this subsequent injection were determined for the tissues indicated. Pre-dose compositions were none, DSPC/cholesterol, DSPC/cholesterol/doxorubicin, DSPC/cholesterol/G_{M1}, and DSPC/cholesterol/G_{M1}/doxorubicin, as described in Fig. 1. Where employed, doxorubicin was entrapped at a drug/lipid ratio of 0.2 (mol/mol). Values shown represent the mean of results from 8 animals ± S.E. of the mean.

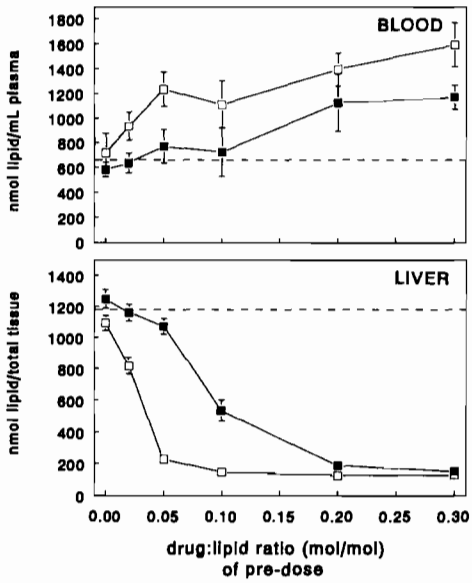

Fig. 3. Dose-titration of entrapped doxorubicin in the pre-dose: biodistribution of the subsequent injection. Pre-doses with entrapped doxorubicin at drug/lipid ratios of 0.00, 0.02, 0.05, 0.10, 0.20, and 0.30 (mol/mol) were injected via lateral tail vein at a dose of 0.33 μmol total lipid/mouse. At 24 h after injection of these pre-doses, a subsequent injection of empty liposomes composed of DSPC/cholesterol (55:45 mol/mol) was given at a dose of 3.3 μmol per mouse. The mice were sacrificed 24 h later and lipid levels of this subsequent injection were determined for the tissues indicated. Pre-dose compositions were (□), DSPC/cholesterol, and (■) DSPC/cholesterol/G_{M1}, with entrapped doxorubicin as indicated. Values shown represent the mean of results from 4 animals ± S.E. of the mean. The dashed lines with no symbols represent the mean of results from mice which received no pre-dose.

G_{M1} substantially blocks liver uptake of the later injection of empty liposomes, resulting in elevated blood levels and spleen uptake. Pre-doses of doxorubicin-loaded G_{M1}-containing LUVs also results in dramatic blockade of liver uptake as well as elevated spleen uptake. The reduction of liver uptake is slightly less than that observed in the absence of G_{M1}. Interestingly, there is no significant difference for blood levels for G_{M1}-containing LUVs. It is important to note that these results represent the effect of liposomally entrapped doxorubicin; the administration of free doxorubicin prior to injection of empty liposomes has been shown not to alter the liposome clearance [1].

These studies were extended to determine the minimum dose of doxorubicin required in both control and G_{M1}-containing liposomes to induce significant RES blockade. These studies were performed by injection of the same lipid pre-doses which contained varying amounts of doxorubicin, and then determining the biodistribution of a subsequent injection of empty liposomes. The drug/lipid ratios of the pre-doses were 0.00, 0.02, 0.05, 0.10, 0.20, and 0.30 (mol/mol). The mean values obtained for mice which received no pre-dose are indicated by the dashed lines. In both the

blood and liver, the effect of entrapped doxorubicin in liposomes without G_{M1} on the biodistribution of the subsequent injection are readily apparent even at very low drug levels (drug/lipid ratios < 0.02 mol/mol), whereas for the G_{M1}-containing pre-dose, higher drug doses (drug/lipid ratios of 0.10 mol/mol) are required to induce the same effect. It should be noted that these dose levels are very small in comparison to the doses required to result in therapeutic benefit. A drug/lipid ratio of 20 would be required to achieve the maximum tolerated doxorubicin dose of 20 mg/kg [1,10] for example. Thus, at any reasonable dose of doxorubicin in G_{M1}-containing liposomes, strong RES blockade would be expected.

In addition to the ability of G_{M1}-containing LUVs with entrapped doxorubicin to blockade liver uptake, a further point of interest concerns the different uptake behavior of the liver and spleen. An ability to dramatically block liver uptake by a small pre-dose of liposomally entrapped doxorubicin is consistent with specific uptake of liposomes by Kupffer cells [11,12], and argues against a non-specific mechanism. Similarly, the increases in spleen uptake seen as a result of liver blockade imply that liposome uptake in the spleen by fixed macrophages plays a relatively minor role in liposome clearance. Rather, these results suggest a non-specific filter model [13,14], where the spleen accumulates liposomes not cleared by the liver.

In summary, the results presented here indicate that G_{M1}-containing liposomes do not avoid uptake by the RES, particularly the Kupffer cells of the liver. Arguments suggesting that liposomes containing G_{M1} exhibit extended lifetimes by avoiding the fixed and free macrophages of the RES, and that, therefore, such formulations containing cytotoxic drugs will not impair or blockade the normal functioning of the RES would therefore appear invalid. In addition, our results suggest that the roles of liver and spleen in liposome clearance are different and should be considered independently of each other. Finally, in our view, it is likely that the presence of lipids such as G_{M1} which lead to extended circulation lifetimes reduces the rates of uptake by the RES but does not influence the basic mechanism of clearance.

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