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ORGAN DISTRIBUTIONS OF LIPOSOME-LOADED RAT PLATELETS

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Past *in vitro* functional assays have demonstrated that platelet function is not inhibited by liposome uptake. In the present study, the organ distributions of control and liposome-loaded Sprague-Dawley rat platelets were examined to determine whether liposome uptake enhances RES uptake. Platelets were isolated using STRactan density gradient centrifugation, incubated with small unilamellar liposomes *in vitro* for 1 hour, labeled with ^{51}Cr and injected into a cohort group of rats. One hour post-injection the spleen, liver, lungs, blood, kidneys and bladder contents were removed and the percentages of the recovered dose localized per total organ (%RD) were determined. The RES index, defined as $\%RD_{liver} + \%RD_{spleen}$, were 24.8 ± 4.5 and 20.5 ± 5.0 for the control platelets and liposome-loaded platelets, respectively. These results indicate that liposome uptake does not enhance RES uptake.

Liposomes have been used to deliver diagnostic and therapeutic drugs with moderate success. RES uptake and lack of targeting specificity of the liposomes have been the major problems encountered. Several groups have increased the circulation times by attaching either PEG or the ganglioside GM_1 to the liposome surfaces (1,2); the circulation half-lives for DSPC:cholesterol:PEG and GM_1 liposomes are 20.0 ± 3.5 and 16.4 ± 3.1 hours, respectively, as compared to 6.7 ± 4.5 hours for DSPC:cholesterol (2:1 mole ratio) liposomes (1). There have been some attempts to target specific tissue sites by the addition of ligands, such as human gamma globulin or aminomannose to the liposome surface; however this targeting has been largely limited to increased Kupffer cell and RES uptake (3,4) Addition of

Abbreviations: PEG: polyethylene glycol, RES: reticuloendothelial system, DSPC: distearoylphosphatidylcholine, SUV: small unilamellar vesicle, PRP: platelet rich plasma, RD: recovered dose.

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antibodies to the liposomes increases targeting specificity, but success has been limited since the liposomes are still prone to RES uptake (5).

Recently we studied the *in vitro* interactions of SUV with platelets with the objective of developing an effective drug delivery system. Specifically, we have examined the kinetics and mechanisms of uptake of SUV, with and without covalent attachment of ligands. Liposomes that have been studied include: DSPC:cholesterol (2:1 mole ratio) control liposomes (6) and aminomannose-, human gamma globulin- and transferrin-labeled control liposomes (7). From our data we have concluded that attachment of these surface ligands increases the targeting specificity and efficiency of *in vitro* platelet uptake. In addition the mechanisms of uptake and subsequent specific localization of the liposomes and their contents within the cells are dependent on the type of liposome used.

Platelets have the unique ability to target specific sites *in vivo* including areas of infection and inflammation, tumors and clots. Therefore we expect to combine this *in vivo* targeting capability with the platelets' propensity to take up liposomes *in vitro* in order to create an effective drug delivery system for diagnostic and therapeutic agents. Having a variety of trial liposome systems should be advantageous in the optimization of the parameters for drug delivery.

In vitro functional assays, including microaggregation, serotonin release and membrane integrity, suggest that platelet function is not inhibited by liposome uptake (6, 7). The present study was designed to determine whether uptake of liposomes enhances platelet RES clearance.

MATERIALS AND METHODS

Control Liposome Preparation. A chloroform solution of a 2:1 mole ratio of DSPC (Avanti Polar-Lipids, Inc.) and cholesterol (Sigma Chemical Co., cell culture tested) was made such that the total lipid content was approximately 20 mg. The solution was taken to dryness in a 100 ml round bottom flask with a Büchler "Rotovap" apparatus and was dried in a dessicator under vacuum overnight. The resulting phospholipid mixture was resuspended in 5 ml of PBS (0.90% NaCl: 0.12% $Na_2HPO_4{\cdot}7H_2O$: 0.013% $NaH_2PO_4{\cdot}H_2O$ w/w in water, pH 7.3), vortexed and 2.5 ml portions were sonicated for 15 minutes using a Heat Systems-Ultrasonics, Inc. sonicator with a microprobe. In studies examining liposome uptake versus incubation time and liposome concentration, five μCi of [^{3}H]cholesterylhexadecyl ether (New England Nuclear) were also added to the lipid prior to drying.

Determination of Liposome Diameters and Estimation of Liposome Numbers. Liposome diameters for different preparations were determined by photon correlation spectroscopy at 480 nm with a Malvern Instruments PCS 100 system. The numbers of [^{3}H]cholesterylhexadecyl ether-labeled liposomes were calculated by assuming that the counts per weight lipid were constant for all preparations, the average surface area for a phospholipid molecule is 0.7 nm^2 (8), and the average

molecular weight for the lipid is 656 g/mol. The numbers of non-radiolabeled liposomes were determined by performing phosphate assays (9) on the samples and assuming a 0.7 nm^2 phospholipid surface area. The light scattering data indicated that liposome diameters were 68-80 nm. The numbers of liposomes reported have all been normalized assuming that the diameters of the liposomes for all experiments were 74 nm.

Platelet Rich Plasma Isolation. Male Sprague-Dawley rats weighing approximately 250 g were anesthetized with ether. Blood was collected using a 10 ml syringe with an 18 gauge needle that contained 0.2 ml of a 19% citrate solution from the posterior vena cava at kidney level. The needle was removed and the blood was dripped slowly into a centrifuge tube containing 2 ml of BSG-citrate solution (6.83 g NaCl, 4 g sodium citrate, 2.0 g dextrose, 0.218 g H_2KPO_4 and 1.22 g HNa_2PO_4 adjusted to 1 liter using H_2O, pH 7.4). After slow inversion of the tube twice, an additional 2 ml of BSG-citrate was added. Each blood sample was kept separate throughout the centrifugations: 150 x g, 3 minutes to obtain the PRP. The remaining red blood cell fraction was resuspended in 9 ml BSG-citrate and centrifuged again under the same conditions. The PRP samples from each rat were pooled.

^{51}Chromium Labeling. 0.5 mCi of sodium ^{51}chromate was added to the PRP and incubated at room temperature for 30 minutes.

Platelet Isolation. STRactan gradients were prepared as follows. A stock solution of 30% STRactan (arabinogalactan, St. Regis, Tacoma, WA) was prepared in BSG-citrate and kept frozen until used; 10% and 20% solutions were prepared immediately before use by dilution with BSG-citrate. Three ml of the 10% STRactan solution were added to a 15 ml polypropylene centrifuge tubes. Four ml of the 20% STRactan solution were carefully layered on the bottom of the tubes. Three ml of the ^{51}Cr labeled PRP were layered on top of the other layered solutions and the tubes were centrifuged at 700 x g for 20 minutes. The cloudy layers of platelets were removed, pooled, resuspended in 9 ml of BSG-citrate and centrifuged at 500 x g for 10 minutes to wash off the remaining STRactan. The cells were resuspended in modified Tyrodes solution (1.0 g dextrose, 1.0 g $NaHCO_3$, 0.2 g KCl, 8.0 g NaCl, 0.05 g Na_2HPO_4 and 0.14 g $CaCl_2$ in 1 liter H_2O, pH 7.4).

Incubation with Liposomes. Platelets were incubated with the liposomes at 37°C for 1 hour. One hour allows significant uptake while minimizing platelet exposure to 37°C, which has been shown to induce platelet membrane lesions (6). Throughout the experiments we attempted to incubate a constant ratio of 2.1×10^{12} liposomes to 2.0 mg platelet protein (0.81×10^9 platelet/mg protein (10)). After incubation, 9 ml of BSG-citrate was added to each tube prior to centrifugation at 500 x g for 10 minutes to inhibit aggregation. Centrifugation was repeated and the cells were finally resuspended in BSG-citrate. The Peterson modification of the Lowry protein assay with a bovine serum albumin standard and without trichloroacetic acid precipitation was used to estimate the amounts of protein in the samples (11).

Organ Distribution. The rats were anesthetized with 30 mg nembutal/kg rat weight injected intraperitonially. Approximately 2 mg protein (0.5 ml) of control or liposome-loaded platelets was injected into each rat via the saphenous vein using a 1 ml syringe with a 23 gauge needle. Sufficient control and liposome-loaded platelets were retained to determine the total injected dose. Platelet localization in the spleen, liver, lungs and blood were determined one hour post-injection. Small amounts of tissue were repeatedly washed in cold isotonic saline, weighed and gamma counted. Preliminary studies have shown that repeated washings in 200-300 ml of saline is as effective as whole organ perfusion in removal of blood-borne radioactivity (12). The total volume of blood per rat was determined by assuming an 8% blood volume (ml) per kg rat body weight. Organ uptake of platelets is expressed as the mean percentage of the recovered dose localized per total organ (%RD) ± the standard deviation and was compared by the *t* test utilizing a 95% confidence level. Platelet organ localizations were evaluated in groups of six rats.

RESULTS

In vitro uptake of liposomes by rat platelets. 1.6 x 10^9 rat platelets were incubated with 7.1 x 10^{11}, 2.1 x 10^{12} and 3.5 x 10^{12} liposomes for 1 hour at 37°C. Uptake increased with increasing numbers of incubated liposomes (Figure 1).

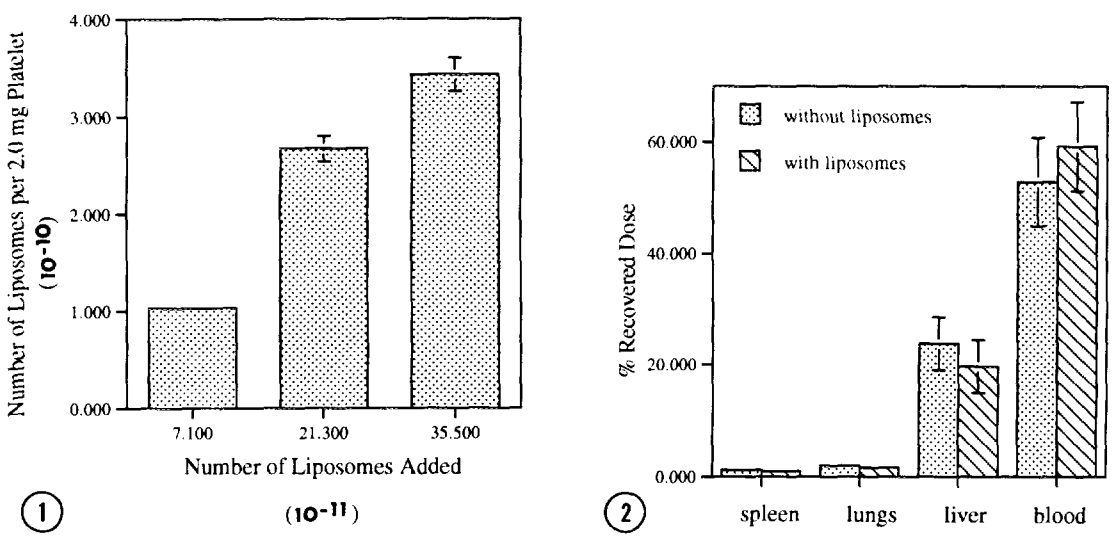

Figure 1. Liposome concentration dependence of Spraque-Dawley rat platelet uptake of [^{3}H]cholesterylhexadecyl ether labeled liposomes. 1.6 x 10^9 platelets were incubated with varying amounts of [^{3}H]cholesterylhexadecyl ether labeled liposomes at 37°C for 1 hour.

Figure 2. Organ distributions of control and liposome-labeled platelets one hour post-injection. The data are represented as the percentage of the recovered dose localized per total organ (%RD) ± SD.

Organ distributions. The %RD for the spleen, liver, lungs and blood were 1.10 ± 0.28, 23.73 ± 4.21, 1.87 ± 0.36, and 52.83 ± 6.98 for the control platelets and 0.93 ± 0.22, 19.62 ± 4.82, 1.55 ± 0.40, and 59.18 ± 7.96 for the liposome-loaded platelets (Figure 2). The RES indexes, defined as the combined %RD of the liver and spleen, were 24.8 ± 4.5 and 20.5 ± 5.0 for the control platelets and liposome-loaded platelets, respectively.

The data are reported as the percentage of the recovered dose rather than the injected dose. By combining the counts recovered from the bladder and kidneys plus those in the spleen, liver, lungs and blood (recovered dose), we account for greater than 75% of the injected dose.

DISCUSSION

The requirements for liposome-loaded platelets to be a successful drug delivery system are efficient loading without loss of platelet function and targeting capability. The results of all *in vitro* function assays with human platelet, including microaggregation, serotonin release and membrane integrity lead to the conclusion that platelet function is unimpaired by liposome uptake (6).

The current study has demonstrated that liposome uptake does not enhance RES uptake or decrease circulation of platelets. Initial uptake by the liver and spleen within the first 15 minutes is not uncommon. It is hypothesized that during this time platelets that are minimally damaged undergo repair and are rereleased (13). However, subsequent accumulation of platelets in the RES is often indicative of irreversible platelet damage (14, 15). Localization within the lungs can indicate platelet aggregation and formation of microemboli (12). No significant differences in the organ distributions or the RES indexes have been demonstrated in our studies. Therefore, we conclude that liposome uptake does not impair platelet function or circulation.

The ability to target might be dependent on factors other than those responsible for normal circulation. Therefore future studies will focus on experiments that assess the ability of the liposome-loaded platelets to target thrombi and tumors. Additionally, imaging thrombi and tumors using liposome-encapsulated imaging agents, including contrast dyes or radiolabels, will be examined and compared to conventional techniques. The efficacy of delivery of thrombolytic and anti-cancer drugs delivered using this method will also be addressed in detail.

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