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Enhanced lymph node retention of subcutaneously injected IgG1-PEG$_{2000}$-liposomes through pentameric IgM antibody-mediated vesicular aggregation

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Abstract

An efficient strategy for enhancing the lymph node deposition of rapidly drained liposomes from the interstitial injection site is described. Subcutaneously injected small-sized immuno-poly(ethyleneglycol)-liposomes (immuno-PEG-liposomes), containing 10 mol% mPEG$_{350}$–phospholipid and 1 mol% PEG$_{2000}$–phospholipid in their bilayer and where IgG1 is coupled to the distal end of PEG$_{2000}$, not only drain rapidly from the interstitial spaces into the initial lymphatic system, but also accumulate efficiently among the lymph nodes draining the region when compared with non-PEG-bearing immunoliposomes where IgG is directly coupled to the phospholipid. Liposome deposition among the draining lymph nodes, however, was further enhanced dramatically following an adjacent subcutaneous injection of a pentameric IgM against the surface attached IgG molecules (IgM:IgG, 10:1) without compromising vesicle drainage from the interstitium. This is suggested to arise either as a result of formation of large immuno-aggregates within the lymphatic vessels with subsequent transport to and trapping among the regional lymph nodes and/or following IgM binding to Fc receptors of the lymph node sinus macrophages forming a platform for subsequent trapping of drained IgG-coupled liposomes. This lymph node targeting approach may be amenable for the design and surface engineering of any rapidly drained nanoparticulate system bearing peptides and proteins that can be aggregated with a desired monoclonal pentameric IgM.

Keywords: IgM; Immunoliposome; Lymph node; Lymphatic system; Macrophage; Targeting

1. Introduction

Under normal physiological conditions interstitially injected liposomes are drained into the initial lymphatic system through patent junctions in the lymphatic capillaries and are then conveyed to the regional lymph nodes via the afferent lymph [1]. Within the lymph nodes the drained vesicles are susceptible to extraction by macrophages of the medullary sinuses and paracortex; however, littoral cells and polymorphonuclear granulocytes also play some role in liposome clearance [2]. Such means of vesicle transportation from interstitial sites and clearance by lymph node scavenger cells has numerous medical applications to include lymphoscintigraphic tracing, lymph node mapping, antimicrobial and antigen delivery, and immune modulation [1,3,4]. Among the key physicochemical factors controlling the kinetics of liposome drainage through the ground substance of the interstitium into the initial lymphatic system and subsequent macrophage capture are vesicular size, morphology, and surface characteristics (e.g., electric charge, hydrophilicity/hydrophobicity, and ligand expression and density) [1,5–8]. For example, in rats although up to 50% of the injected dose (footpad injection) of anionic unilamellar liposomes (size range 90–120 nm) is usually drained into the lymphatic system within 6–10 h, liposome capture by resident phagocytic cells of the primary draining lymph node rarely exceeds 2–3% of this fraction [6]. Liposome clearance by macrophages of the secondary and tertiary nodes is even less efficient. Noncaptured vesicles subsequently gain access into the systemic circulation via thoracic duct and are cleared by hepatic and splenic macrophages.

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Rational strategies are therefore required to dramatically increase the retention and localization of interstitially injected liposomes among the regional draining nodes. Recently, work from this laboratory demonstrated that the rate of drainage and lymphatic distribution (macrophage capture) of interstitially injected liposomes in rats can be improved dramatically by simultaneous attachment of a targeting ligand, immunoglobulin-G (IgG), and inclusion of appropriate methoxypoly(ethyleneglycol)–phospholipid (mPEG-PL) conjugates into the liposomal bilayer [9]. However, the extent of both liposome drainage and macrophage targeting was dependent on the mode of IgG coupling and surface poly(ethyleneglycol) configuration. For instance, the lymph node retention (both primary and secondary nodes) of rapidly drained liposomes of 100–120 nm in size was not only increased following conjugation of a non-specific IgG to the distal end of a functionalized PEG_{2000}-PL, but adjusting the molecular architecture of surface exposed PEG_{2000} chains to a "nearly overlapped mushroom/mushroom–brush transition" regime, yielded vesicles with optimal target-binding capability [9]. The latter was achieved by inclusion of 10 mol% of $mPEG_{350}$–phospholipid conjugates into the bilayer of IgG-PEG_{2000}-liposomes. This paper demonstrates a further step towards improving the lymph node retention of IgG-PEG_{2000}-liposomes through *in vivo* vesicular aggregation in the lymphatic vessels with a pentameric IgM (*in vivo* conversion of "small" to "big"; Fig. 1) thus enhancing liposome clearance by resident macrophages via Fc receptors. The principle of this approach was inspired by a recent observation of Phillips et al. [10] where it was demonstrated that the retention of interstitially injected biotin-coated liposomes in draining lymph nodes could be increased dramatically following an adjacent avidin injection.

2. Materials and methods

2.1. Liposome preparation and characterization

All lipids were from Sigma (Poole, UK) with the exception of -(4′-(4″-maleimidophenyl)butyroyl)-phosphatidylethanolamine (MPB-PE), $mPEG_{350}$-distearoylphosphatidylethanolamine ($mPEG_{350}$-DSPE) and MPB-PEG_{2000}-DSPE, which were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Immunoliposomes were composed of egg phosphatidylcholine (egg PC), cholesterol (Chol), dicetylphosphate (DCP) and MPB-PE in a molar ratio of 6.925:6.925:1:0.15, respectively. Immuno-PEG-liposomes were composed of

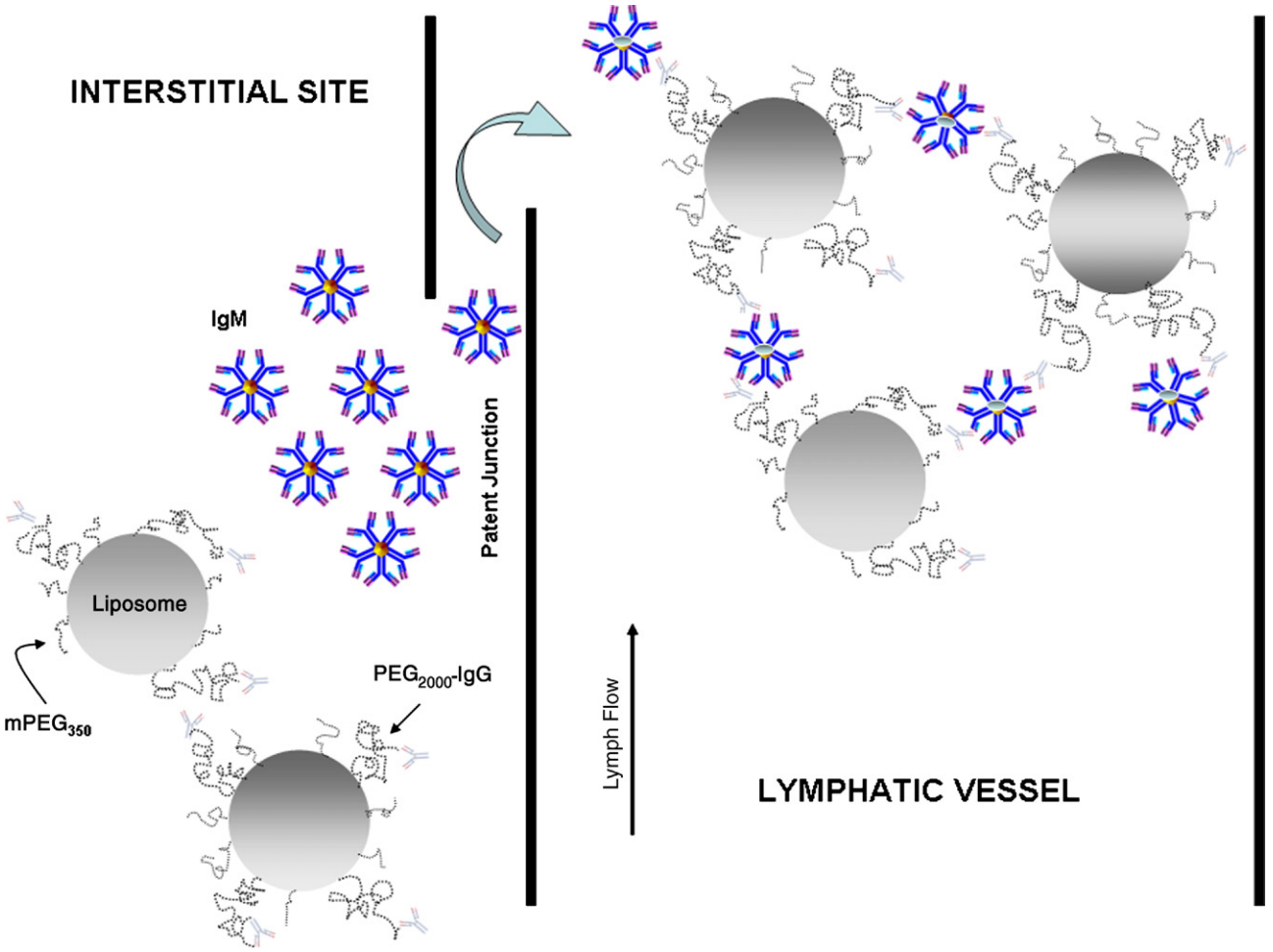

Fig. 1. Schematic representation of immuno-PEG_{2000}-liposome drainage from a subcutaneous injection site followed by IgM-mediated vesicular aggregation in the lymphatic vessel. Liposomes are injected first into the dorsal surface of rat footpad. Next, pentameric IgM molecules are injected subcutaneously proximal to the site of liposome injection. Both IgM and immuno-PEG_{2000}-liposome drain rapidly into the initial lymphatic vessels. Within the lymphatic vessels IgM molecules and liposomes encounter each other resulting in the formation of large immuno-aggregates. These entities drain into the regional lymph nodes where they become trapped.

egg PC, Chol, DCP, mPEG$_{350}$-DSPE, MPB-PEG$_{2000}$-DSPE in a molar ratio of 6.175:6.175:1:1.5:0.15, respectively. Liposomes were prepared by hydrating the dried lipid film with a buffer (25 mM HEPES, 25 mM Mes, 135 mM NaCl), pH 6.7, containing [^{125}I]-poly(vinylpyrrolidone) ([^{125}I]-PVP; Amersham International, Amersham, UK) as an established aqueous space label for *in vivo* studies [5,9]. Liposomes were extruded through polycarbonate Nuclopore filters of 100 nm in pore diameter using a high-pressure extruder. Liposome size distribution was determined by laser light scattering using a Malvern Zetasizer 3000 (Malvern Instruments, Malvern, UK) at 25 °C as described previously [9]. Rat IgG1 (Serotec, UK) was thiolated, using *N*-succinimidyl-3-(2-pyridyldithio) proprionate followed by reduction with dithiotheritol (DTT) and characterized as before [9]. The thiolated antibody was coupled to liposomes via the linker lipids (MBP-PE or MBP-PEG$_{2000}$-DSPE). Non-reacted maleimide was blocked with DTT; free DTT and unbound antibody molecules were removed by passing the liposome suspension over a Sepharose CL-4B column in HEPES buffer. Antibody-conjugated liposomes were assayed for their protein content and phospholipid phosphorus and conjugation results are expressed as microgram bound antibody per micromoles phospholipid.

2.2. Lymphatic distribution studies

Male Wistar rats (n=3 for each determination), body weight 180±10 g, were injected subcutaneously into the dorsal surface of the left footpad with [^{125}I] PVP-encapsulated immunoliposomes or immuno-PEG-liposomes (2.8 μmol phospholipid) as described previously [9], followed by an adjacent subcutaneous injection (50 μl) of either a pentameric IgM (Sigma-Aldrich, UK; produced in mouse, ascites fluid) against rat IgG1, an irrelevant IgM, or saline proximal to the site of liposome injection. IgM–IgG interaction was first monitored by immunodiffusion. The ability of IgM to induce aggregation of IgG1-conjugated liposomes was confirmed by turbidity measurements.

Animals were sacrificed at various time points post liposome injection and associated radioactivity was measured in the footpad (whole foot), regional lymph nodes (the whole popliteal or primary node, iliac or secondary node, inguinal, and renal nodes), blood (samples taken from tail vein), and whole liver and spleen, using a gamma counter. Determination of the amount of liposomes in the blood was made on the assumption of total blood volume per rat of 7.5% of body weight. Biodistribution data in regional lymph nodes are presented as percent of injected dose/node±S.E.

3. Results and discussion

Immunoliposomes (IgG attached directly to the phospholipid) were of 126±30 nm (Z-average mean size) with a polydispersity index of 0.06, bearing 69±16 μg rat IgG1 bound per μmol phospholipid. Engineered IgG1-PEG$_{2000}$-liposomes (IgG is attached to the distal end of PEG$_{2000}$), containing 10 mol % mPEG$_{350}$–phospholipid conjugates in their bilayer, were of 134±31 nm (polydispersity index=0.12) with 62±14 μg IgG1 per μmol phospholipid. In accordance with the earlier report [9] IgG1-PEG$_{2000}$-liposomes drained at a faster rate from the footpad interstitium into the initial lymphatics when compared with that of immunoliposomes (Fig. 2a and b). This difference was previously suggested to arise from increased liposome surface hydrophilicity (due to close association of water molecules with PEG chains), which minimizes vesicle interaction with the ground substance of the interstitium [9]. Popliteal node (1° node) drains the footpad through lymph vessels; efferent popliteal trunks follow to a retroperitoneal lymphatic plexus dorsal to the iliac vessels and the main trunk continues to the iliac nodes (2° node). Therefore, we next examined liposome deposition in both popliteal and iliac nodes. Both popliteal and iliac node localization of IgG1-PEG$_{2000}$-liposomes was superior to that of immunoliposomes at all time points (Fig. 3), which

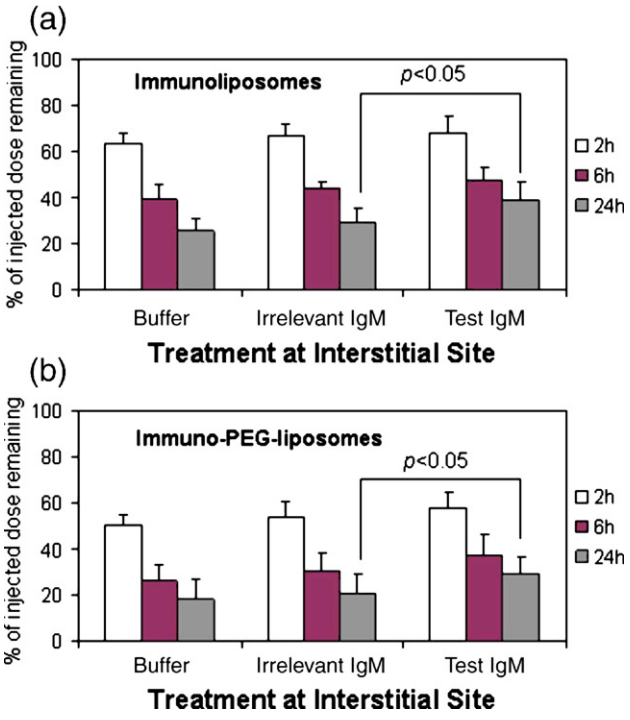

Fig. 2. Kinetics of liposome drainage from interstitial spaces of the footpad into the initial lymphatic system. Radiolabelled liposomes were injected subcutaneously into the dorsal surface of the left footpad followed by an adjacent subcutaneous injection of either buffer (saline) or a pentameric IgM against rat IgG1 (test IgM) or an irrelevant IgM.

may be attributed to their faster drainage from the interstitium, particularly within the first few hours of injection, and hence less exposure time to Fc receptor-bearing interstitial phagocytes. For example, at 24-h popliteal and iliac nodes retained 6.3±0.9% and 5.7±0.2% of injected dose of IgG1-PEG$_{2000}$-liposomes, respectively, whereas the corresponding values for immunoliposomes were 1.9±0.2% and 1.4±0.3% of injected dose, respectively.

Although particulate systems and vesicles >150 nm are more susceptible to macrophage recognition and clearance than their smaller counterparts, they move very slowly from the interstitial injection site into the initial lymphatics; the drainage may often take periods of days depending on particle surface hydrophobicity [1,3,11,12]. However, this slow transit may induce local inflammation and renders particles and liposomes susceptible to macrophage clearance at the injection site thus limiting their direct transport to the lymphatic system [1,11]. Therefore, for lymphatic transport smaller vesicles are of preferred choice. In order to further enhance lymph node retention of the rapidly drained small-sized immunoliposomes and IgG1-PEG$_{2000}$-liposomes we induced vesicle aggregation within the lymphatic vessels and lymph nodes. This was achieved by an adjacent subcutaneous injection of a monoclonal pentameric IgM against the rat IgG1 (IgM:IgG, 10:1) proximal to the site of liposome injection. Firstly, IgM injection had no significant effect on liposome drainage at 2 and 6 h, irrespective of the antibody-conjugated liposome type and despite occurrence of some mild inflammatory reactions, when compared with the corresponding

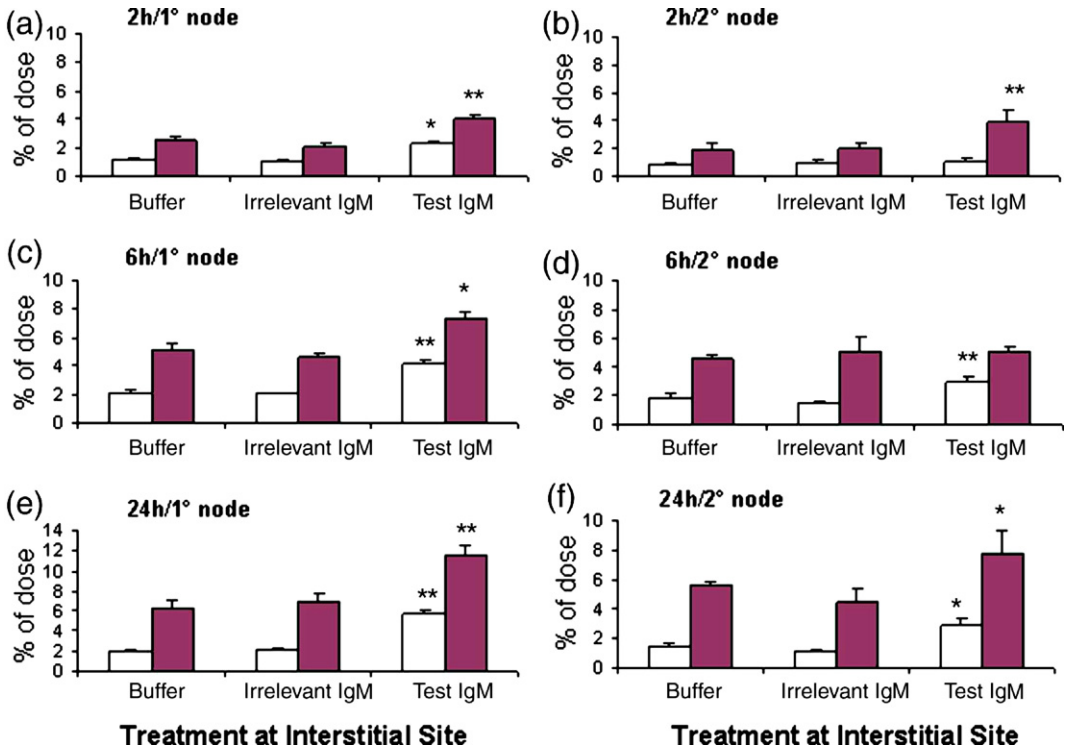

Fig. 3. The extent of liposome retention in 1° (popliteal) and 2° (iliac) lymph nodes. The open and closed columns represent immunoliposomes and IgG1-PEG$_{2000}$-liposomes, respectively. *$p<0.05$, **$p<0.01$ compared with the irrelevant IgM administration.

control experiments (adjacent saline injection or an irrelevant IgM injection), whereas at 24 h significantly more liposomes were retained at the injection site (Fig. 2). The latter may correspond to the formation of some local immuno-aggregates and initiation of further inflammatory reactions. Liposome retention in the popliteal node (Fig. 3a, c and e) was increased dramatically at all time points in animals that received monoclonal IgM injection when compared with that of the corresponding controls (saline and irrelevant IgM injections). The effect, however, was more profound with IgG1-PEG$_{2000}$-liposomes than that of immunoliposomes, which may be ascribed to their faster drainage from the injection site thus encountering more IgM (which also drains rapidly into the lymphatic system) within the lymphatic vessels. Remarkably, the extent of IgG1-PEG$_{2000}$-liposomes in the iliac node (Fig. 3b, d and f) was also increased by the monoclonal IgM, particularly at 24 h; this presumably corresponds to trapping of smaller immuno-aggregates that escaped filtration at the popliteal node level. The described strategy therefore enhances liposome retention among a chain of draining lymph nodes (liposome retention in both inguinal and renal nodes was also increased; data not shown). The enhanced lymph node retention of liposomes following monoclonal IgM treatment was also associated with lower levels of vesicle capture by phagocytic elements of the liver and the spleen when compared with control (irrelevant antibody, Fig. 4).

When monoclonal IgM and antibody-conjugated liposomes were mixed prior to subcutaneous injection, liposomes drained poorly into the lymphatic system (more than 80% of vesicles remained at injection site at 24 h post injection) and total node retention (popliteal and iliac nodes) did not exceed 3.5% of the injected dose. On the basis of these observations we suggest that the enhanced lymph node retention of IgG1-PEG$_{2000}$-liposomes

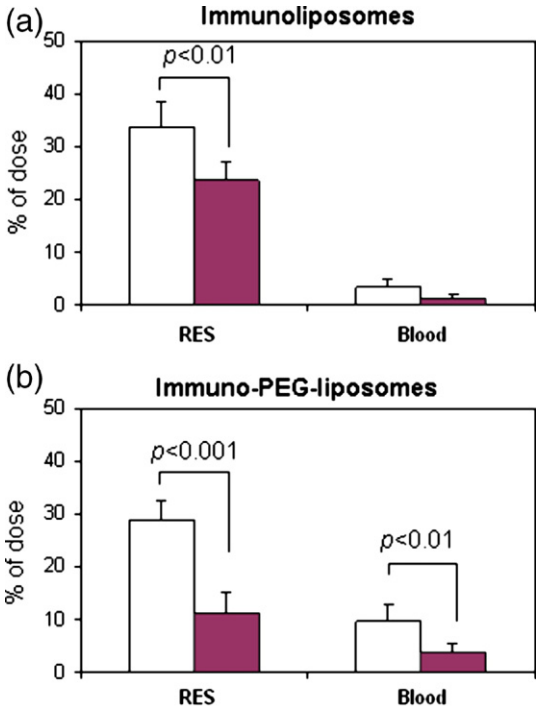

Fig. 4. Liposome levels among the organs of the reticuloendothelial system, RES (liver + spleen) and in the blood at 24 h post interstitial injection. The open and closed columns represent animals that received an adjacent subcutaneous injection of an irrelevant IgM (control) and test IgM, respectively.

following immediate monoclonal IgM injection is not only due to their fast drainage from the injection site but also as a result of formation of some liposome–IgM aggregates presumably within the lymphatic vessels, which become trapped in the next encountered lymph node and/or in lymph node sinuses. With regard to the latter speculation it is possible that the rapidly drained IgM molecules may bind to macrophage Fc receptors in the draining lymph node sinuses thus forming platforms for subsequent trapping of IgG-coupled liposomes. Although IgM is not cationic, this proposed mechanism is rather similar to the previously described avidin/biotin-liposome system, where on the basis of intracavitary injections it was concluded that the positively charged avidin may bind to the negatively charged surface of endothelial cells in the lymph nodes and subsequently act as a platform for trapping of drained biotin-tagged liposomes [13,14].

In the hindlimb and forelimb, gentle massage of the area drained by the lymphatic vessels results in an increased flow of lymph [15]. Thus local massaging may accelerate the formation of liposome–IgM aggregates thereby further enhancing their lymph node retention, but this strategy awaits further investigation. Nevertheless, the described lymph node targeting approach may be amenable for design and surface engineering of any rapidly drained nanoparticulate system bearing peptides and proteins that can be aggregated with a desired monoclonal pentameric IgM.

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