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Lymphatic uptake and biodistribution of liposomes after subcutaneous injection
IV. Fate of liposomes in regional lymph nodes

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Abstract

The ability of clodronate-containing liposomes to deplete lymph nodes of macrophages was used as a tool to investigate the fate of liposomes in regional lymph nodes after subcutaneous (s.c.) administration. Reduced lymph node localization of liposomes in macrophage-depleted lymph nodes confirmed that phagocytosis by macrophages plays an important role in lymph node retention of liposomes. Depletion of macrophages had less effect on lymph node localization of small liposomes than on the lymph node localization of large liposomes. Inclusion of distearoylphosphatidylethanolamine (DSPE)–poly(ethyleneglycol) (PEG–PE) into the liposomes, which is known to oppose macrophage uptake, did not affect lymph node localization in macrophage-depleted or control lymph nodes. We conclude that PEG-liposomes retained by lymph nodes are also taken up by lymph node macrophages. Morphological observations visualizing the uptake of PEG-liposomes by lymph node macrophages support this conclusion. © 1998 Elsevier Science B.V.

Keywords: Liposome; Subcutaneous; Macrophage; Depletion; Clodronate; Colloidal gold

1. Introduction

Following interstitial administration, large molecules and colloids which are too large to enter blood capillaries can be taken up by the lymphatic system. The ability of the lymphatics to take up colloids from interstitial spaces has been exploited for the targeting of drug carriers to the lymphatic system. In particular, liposomes, being versatile, well tolerated, biodegradable lipid vesicles [1], have re-

Abbreviations: Chol, cholesterol; DPPC, dipalmitoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; DSPE, distearoylphosphatidylethanolamine; EPC, egg-phosphatidylcholine; EPG, egg-phosphatidylglycerol; ID, injected dose, i.d., intradermal; i.m., Intramuscular; i.p. Intraperitoneal; PEG–PE, poly(ethyleneglycol)–distearoylphosphatidylethanolamine; PS, phosphatidylserine; s.c., Subcutaneous; TL, total lipid

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ceived considerable interest as vehicles for drug targeting to the lymphatic system. Subcutaneous (s.c.) injection has been the route of administration most extensively investigated for this purpose.

Following lymphatic uptake, liposomes pass through a system of lymphatic vessels and will encounter one or more lymph nodes where a fraction will be retained. It has been suggested that phagocytosis by macrophages is one of the major mechanisms of uptake of colloidal particles in lymph nodes [2,3]. Recently, it was observed that an increase in liposome size and the presence of phosphatidylserine (PS) in the liposomal bilayers substantially enhanced lymph node localization, supporting the hypothesis that macrophage uptake is the major mechanism involved in lymph node localization of liposomes [4]. However, on the other hand, coating of small (0.1 μm) liposomes with poly(ethyleneglycol) (PEG), which has proven to oppose macrophage uptake [5,6], hardly affected lymph node localization, suggesting that phagocytosis by macrophages is not the only mechanism involved in lymph node retention of liposomes [7].

The aim of the present study was to investigate the fate of liposomes in regional lymph nodes after s.c. administration in more detail. The ability of clodronate-containing liposomes to deplete macrophages in lymph nodes was used as a tool to investigate the localization of intranodal liposomes. Macrophages in regional lymph nodes were depleted by s.c. administration of clodronate-containing liposomes [8,9]. Localization of s.c. administered large (nonsized) and small (0.1 μm) liposomes was studied in lymph nodes depleted of macrophages and compared with localization in control (nondepleted) lymph nodes. Sterically stabilized liposomes containing the lipid derivative distearoylphosphatidylethanolamine (DSPE)–PEG (PEG–PE) (PEG-liposomes) were also included in this study. The study of PEG-liposomes was of interest as they are known to be able to resist phagocytosis by cells of the mononuclear phagocyte system (MPS) [10]. Therefore, less effect of lymph node macrophage depletion was expected in the case of PEG-liposomes. In order to visualize liposomes in regional lymph nodes, liposomes containing colloidal gold were prepared and their localization in regional lymph nodes was examined by light microscopy and transmission electron microscopy.

2. Material and methods

2.1. Chemicals

Egg-phosphatidylcholine (EPC) and egg-phosphatidylglycerol (EPG) were donated by Lipoid (Ludwigshafen). PEG–PE was obtained from Avanti Polar Lipids (Alabaster, AL, USA). 1α, 2α (n)-[^{3}H]-Cholesteryloleylether (spec. act. 1.71 TBq/mmol) was supplied by Amersham (Buckinghamshire, UK). Tetrachloroauric(III)acid ($HAuCl_4$), cholesterol (Chol), 4-(2-hydroxyethyl)-1-piperazine-ethane-sulphonic acid (HEPES) and diaminobenzidine-tetrahydrochloride (DAB) were obtained from Sigma Chemical (St. Louis, MO, USA). Dichloromethylene-bisphosphonate (clodronate) was a gift of Boehringer Mannheim. Hionic Fluor, Soluene-350 and Plasmasol were purchased from Packard Instruments (Downers Grove, IL, USA). All other reagents were of analytical grade.

2.2. Preparation of clodronate-containing liposomes

Clodronate-liposomes were prepared as described by Van Rooijen and Sanders [8]. Briefly, a mixture of EPC and Chol (molar ratio 6:1) in chloroform was evaporated to dryness by rotation under reduced pressure. The lipid film was dispersed by gentle rotation at room temperature in an aqueous solution containing clodronate (0.25 g/ml) and kept at room temperature for about 2 h under nitrogen. The resulting liposome dispersion (about 14 mM total lipid (TL)) was gently shaken and sonicated for 3 min. Nonencapsulated clodronate was removed by centrifugation of the liposomes (10,000 $\times$ g, 15 min). Liposomes were washed 2–3 times by centrifugation (25,000 $\times$ g, 30 min) using sterile phosphate buffered saline pH 7.4 (PBS; 3.6 mM KH_2PO_4, 6.4 mM Na_2HPO_4, 145 mM NaCl) and finally resuspended in sterile PBS and stored under nitrogen at 4°C until further use. The final liposome suspension typically contained about 27 mM TL and about 5 mg/ml clodronate.

2.3. Preparation of radiolabeled liposomes

Lymph node localization of liposomes was studied utilizing the following four liposome types: (1) large, nonsized EPC:EPG:Chol (molar ratio 10:1:4) (large liposomes), (2) small (0.1 μm) EPC:EPG:Chol (molar

ratio 10:1:4) (small liposomes), (3) large, nonsized EPC:EPG:Chol:PEG–PE (molar ratio 10:1:4:1) (large PEG-liposomes), and (4) small (0.1 μm) EPC:EPG:Chol:PEG–PE (molar ratio 10:1:4:1) (small PEG-liposomes). Liposomes were radiolabeled with a tracer amount of [^{3}H]-labeled cholesteryloleylether which has proven to be a reliable label to monitor the fate of liposomes in vivo [11,12]. Radiolabeled liposomes were prepared by the thin-film method [13]. A mixture of the appropriate amounts of lipids, including the [^{3}H]-label, was dissolved in a mixture of chloroform/methanol (4:1 v/v) and evaporated to dryness by rotation under reduced pressure at 40°C. After flushing the lipid film with nitrogen for at least 20 min, the film was hydrated in a sterile HEPES/glucose-buffer pH 7.4 (10 mM HEPES, 1 mM EDTA, 270 mM glucose). Small liposomes (0.1 μm) were prepared by extruding the liposome dispersion sequentially through two stacked 0.1-μm and two stacked 0.05-μm polycarbonate membrane filters (Nuclepore; Costar, Cambridge, MA, USA) under nitrogen pressure. Radioactivity of the radiolabeled liposomes was assayed in Hionic Fluor as a scintillation mixture with a Philips PW4700 liquid scintillation counter. The lipid concentration in the final liposome dispersion was about 30 mM TL.

2.4. Preparation of colloidal gold containing liposomes

To study lymph node localization of liposomes by light microscopy and transmission electron microscopy, colloidal gold-containing liposomes (gold-liposomes) were prepared. The following four liposome types were used: (1) large EPC:EPG:Chol (molar ratio 10:1:4) (large gold-liposomes), (2) small (0.1 μm) EPC:EPG:Chol (molar ratio 10:1:4) (small gold-liposomes), (3) large EPC:EPG:Chol:PEG–PE (molar ratio 10:1:4:1) (large PEG–gold-liposomes) and (4) small (0.1 μm) EPC:EPG:Chol:PEG–PE (molar ratio 10:1:4:1) (small PEG–gold-liposomes). Gold-liposomes were prepared as described by Hong et al. [14], with some modifications. Briefly, the appropriate amounts of lipids, dissolved in a mixture of chloroform/methanol (4:1 v/v), were evaporated to dryness by rotation under reduced pressure at 40°C. The lipid film was dissolved in cyclohexane and freeze dried overnight. The freeze-dried lipids were hydrated in a freshly prepared gold chloride/citrate solution (7.3 mM $HAuCl_4$, 27 mM trisodium citrate and 2.5 mM dipotassium carbonate) at 4°C. The resulting liposome dispersion (30 mM TL) was sized by extrusion at 4°C under nitrogen pressure. Large gold-liposomes were prepared by extruding twice through two stacked 1μm polycarbonate membrane filters. Small gold-liposomes (0.1 μm) were prepared by extruding sequentially through two stacked 0.1-μm and two stacked 0.05-μm polycarbonate membrane filters. Immediately after extrusion, the formation of colloidal gold was induced by incubating the liposome suspension at 37°C for 30 min. Free gold particles were removed by applying the gold-liposome dispersion onto a Sephacryl S-1000 SF column (Pharmacia, Woerden, The Netherlands) and subsequent elution with HEPES/NaCl buffer pH 7.4 (10 mM HEPES, 135 mM NaCl). The lipid concentration in the final liposome dispersion ranged between 5 and 15 mM TL. Encapsulation of colloidal gold into liposomes was examined by staining the liposome dispersion with a 2% phosphotungstic acid solution or a 1% uranylacetate solution for 30 s on a freshly glow-discharged carbon grid. Electron microscopic evaluation confirmed the absence of 'free' colloidal gold particles in the colloidal gold-containing liposome dispersions. Moreover, more than half of the visualized liposomes contained one or more colloidal gold particles.

2.5. Characterization of liposomes

Mean particle size was determined by dynamic light scattering at 25°C with a Malvern 4700 system using a 25-mW He–Ne laser (NEC, Tokyo, Japan) and the automeasure version 3.2 software (Malvern, Malvern, UK). For viscosity and refractive index, values of the appropriate aqueous medium were used. As a measure of particle size distribution of the dispersion, the system reports a polydispersity index. This index ranges from 0.0 for an entirely monodisperse up to 1.0 for a polydisperse dispersion. Lipid phosphate concentration was determined by the colorimetric method of Rouser et al. [15].

2.6. Animal experiments

Male Wistar rats (U:WU, CPB) from the animal facility of Utrecht University with an average weight

of 200–250 g were used. Animals received standard laboratory chow and water ad libitum. To eliminate macrophages in the popliteal lymph nodes [16], rats were injected with clodronate-liposomes (0.6 mg clodronate, 3 μmol TL) into the footpad and the dorsal side of the right foot. The extent of macrophage depletion was assessed by immunoperoxidase staining and acid phosphatase staining as described below. The influence of depletion of lymph node macrophages on the extent of localization in the popliteal lymph node of s.c. injected radiolabeled liposomes was investigated 6 days after treatment with clodronate-liposomes. Rats were injected s.c. with a single dose of [^{3}H]-labeled liposomes (3 μmol TL) into the dorsal side of the right foot (pretreatment with clodronate-liposomes) and left foot (control, i.e., no pretreatment with clodronate-liposomes). A blood sample was drawn from the tail vein 7 h post-injection. At this time-point peak blood levels are to be expected, as determined previously [4]. At the end of the observation period (i.e., 24 h post-injection), rats were killed by cervical dislocation and the site of s.c. injection (i.e., the whole foot), the popliteal lymph nodes and blood were collected.

To visualize the localization of liposomes in popliteal lymph nodes by light and transmission electron microscopy, rats were s.c. injected into the dorsal side of the foot with gold-liposomes. Rats received large gold-liposomes, large PEG–gold-liposomes, small gold-liposomes or small PEG–gold-liposomes (about 3 μmol TL). About 6 h post-injection, rats were killed by cervical dislocation and popliteal lymph nodes were collected.

2.7. Assessment of degree of macrophage depletion

The effect of macrophage depletion treatment was assessed by subjecting sections of popliteal lymph nodes to the following staining procedures: (A) immunoperoxidase staining using an monoclonal antibody directed against rat lymph node macrophages, and (B) acid phosphatase staining. (A) Immunoperoxidase staining: cryostat sections (8–10 μm) of popliteal lymph nodes were fixed on slides with acetone and air-dried. Slides were incubated for 60 min with monoclonal antibody ED3, which binds specifically to medullary and subsinusoidal macrophages in the outer cortex [17]. After washing three times with PBS the slides were incubated for 30 min with peroxidase-conjugated rabbit anti-rat immunoglobulin in PBS/BSA 0.5% (Daka, Copenhagen, Denmark) containing 1% normal rat serum. Slides were then rinsed three times with PBS and peroxidase-activity was determined with 0.5 mg DAB/ml in 0.05 M Tris buffer pH 7.6 containing 0.01% hydrogen peroxide for 10–15 min. After washing in 0.9% NaCl, staining was intensified by incubation with a $CuSO_4$ solution (5 mg/ml) in 0.9% NaCl for 15 min. After washing, sections were counter stained with haematoxylin for 15 s, dehydrated and mounted in malinol. Control slides were incubated with PBS/BSA 0.5% in the first step, instead of the monoclonal antibody solution. (B) Acid phosphatase staining: cryostat sections (8–10 μm) of popliteal lymph nodes were fixed with acetone and air dried. Acid phosphatase was determined, using naphtol-AS-MX-phosphate as a substrate [18] with hexazotized pararosaniline as diazonium salt [19] and counter stained with haematoxylin for 15 s, dehydrated and mounted in malinol.

2.8. Radioactivity measurements

Radioactivity in blood samples (120 μl) was determined by adding 120 μl of Plasmasol, 500 μl of

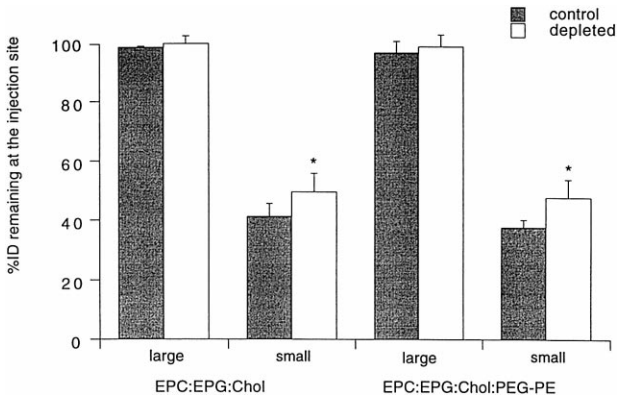


Fig. 1. Effect of pretreatment with liposomal clodronate on lymphatic uptake of s.c. administered liposomes. Macrophages in regional popliteal lymph nodes were eliminated by s.c. injection of clodronate-liposomes. Some 6 days after pretreatment with liposomal clodronate, rats were s.c. injected with large (nonsized) or small (0.1 μm) radiolabeled liposomes into the right foot (pretreated with clodronate) and left foot (control). Recovery of liposomal label from the s.c. injection site was determined 24 h post-injection. Values represent the mean percentage of injected dose remaining at the s.c. injection site (±sd of six animals). * $p < 0.05$.

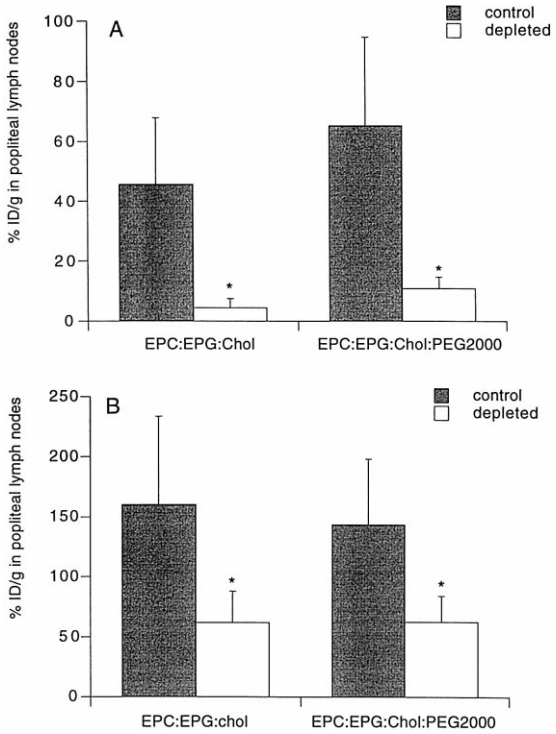


Fig. 2. Effect of depletion of lymph node macrophages on lymph node localization of s.c. administered liposomes. Macrophages in regional popliteal lymph nodes were eliminated by s.c. injection of clodronate-liposomes. Some 6 days after pretreatment with liposomal clodronate, rats were s.c. injected with radiolabeled liposomes into the right foot (pretreated with clodronate) and left foot (control). Localization of liposomal label was determined 24 h post-injection. (A) Lymph node localization of large (nonsized) liposomes. (B) Lymph node localization of small (0.1 μm) liposomes. Values represent the mean percentage of injected dose per gram tissue in popliteal lymph nodes ($\pm$sd of six animals). Note the differences in scale of the y-axes. $^{*}p < 0.05$.

water and 500 μl of 35% hydrogen peroxide. The samples were decolorized overnight at 40°C. Radioactivity was assayed in Plasmasol as a scintillation fluid. A total blood volume per rat of 75 ml/kg body weight was used for calculation of the percentage injected dose in the blood circulation [20,21]. Lymph nodes and feet were solubilized completely in an appropriate amount of Soluene-350 at 40°C. Solubilized lymph nodes and samples (500 μl) of solubilized foot were decolorized with 200 μl of 35% hydrogen peroxide overnight at 40°C. Radioactivity of the decolorized samples was assayed in Hionic Fluor as scintillation fluid.

Lymphatic uptake is defined as the percentage injected dose (%ID) radioactivity (i.e., 100%) minus the percentage of dose radioactivity recovered from the injection site. Lymph node localization is expressed as the percentage of injected dose of radioactivity per gram lymph node (popliteal) tissue (%ID/g). Results represent the mean of 6 rats $\pm$ standard deviation (sd).

2.9. Light and electron microscopy

Localization of gold-liposomes in regional lymph nodes was studied by light microscopy of silver-enhanced semi-thin sections and transmission electron microscopy. Popliteal lymph nodes of rats injected with gold-liposomes were fixed in a 2% solution of glutaraldehyde in PBS. The samples were then processed for resin histology, employing post-fixation in buffered osmium tetraoxide, alcohol dehydration and embedding in Epon 812 epoxy resin. For electron microscopy some tissue blocks were stained en bloc with 1% *p*-phenylenediamine in 70% alcohol to enhance lipid visualization. For light microscopy, 1-μm sections were cut on a LKB III Ultratome (LKB Producter, Stockholm, Sweden) and mounted on glass slides. The gold particles were enhanced using an Intense™, M silver enhancement kit RPN 491 (Amersham International, Buckinghamshire, UK) and the sections were stained with 1% toluidine blue and

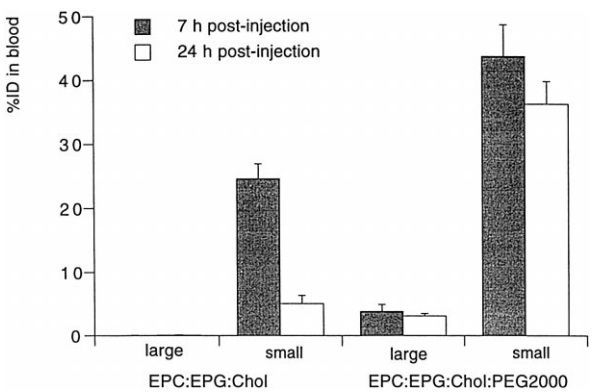


Fig. 3. Effect of surface modification with PEG on blood levels of s.c. administered liposomes. Macrophages in popliteal lymph nodes were eliminated by s.c. injection of clodronate-liposomes. Six days after pretreatment with liposomal clodronate, rats were s.c. injected with large (nonsized) or small (0.1 μm) radiolabeled liposomes into the right foot (pretreated with clodronate) and left foot (control). Recovery of liposomal label in blood was determined 7 h and 24 h post-injection. Values represent the mean percentage of injected dose circulating in the blood ($\pm$sd of six animals).

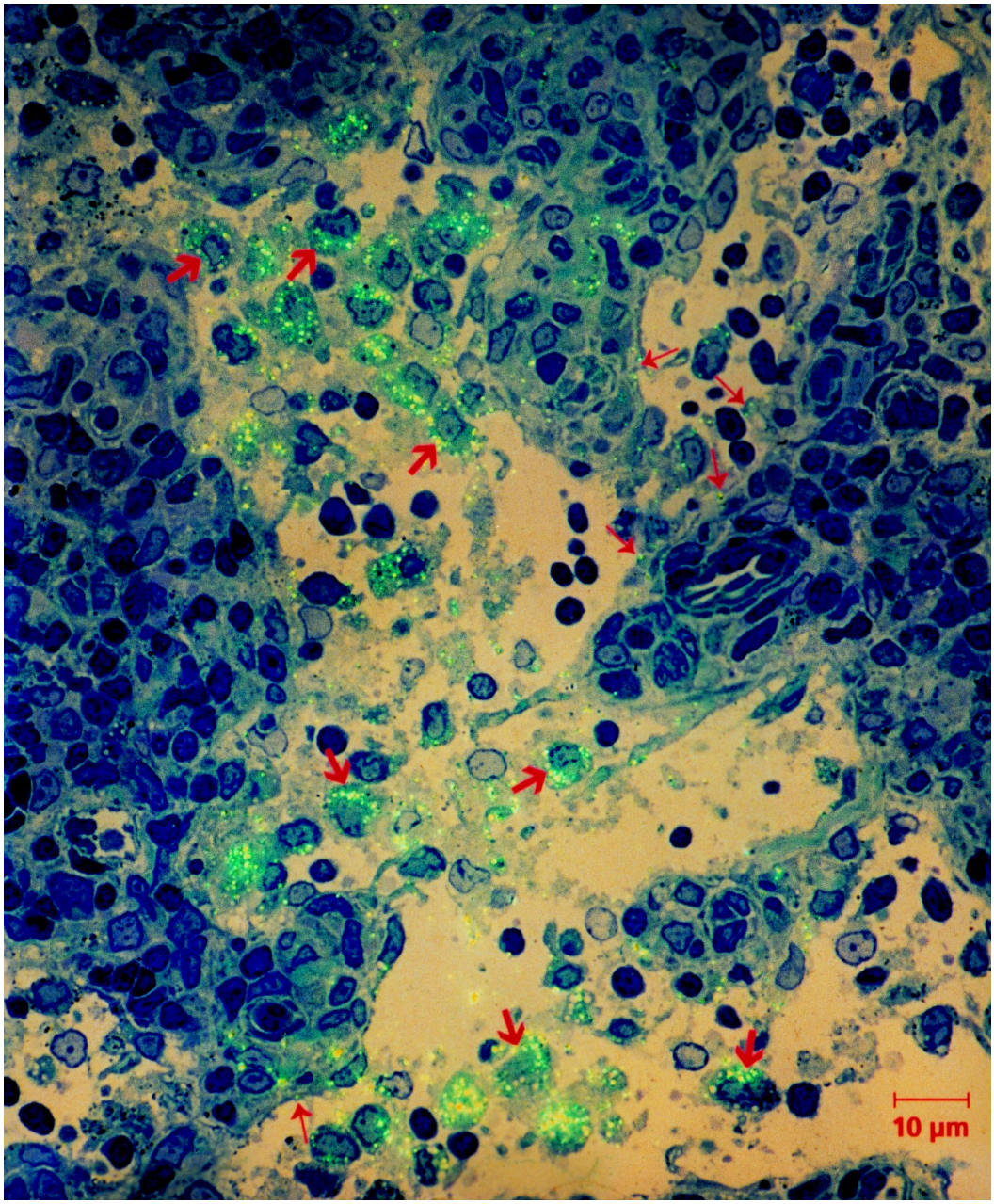
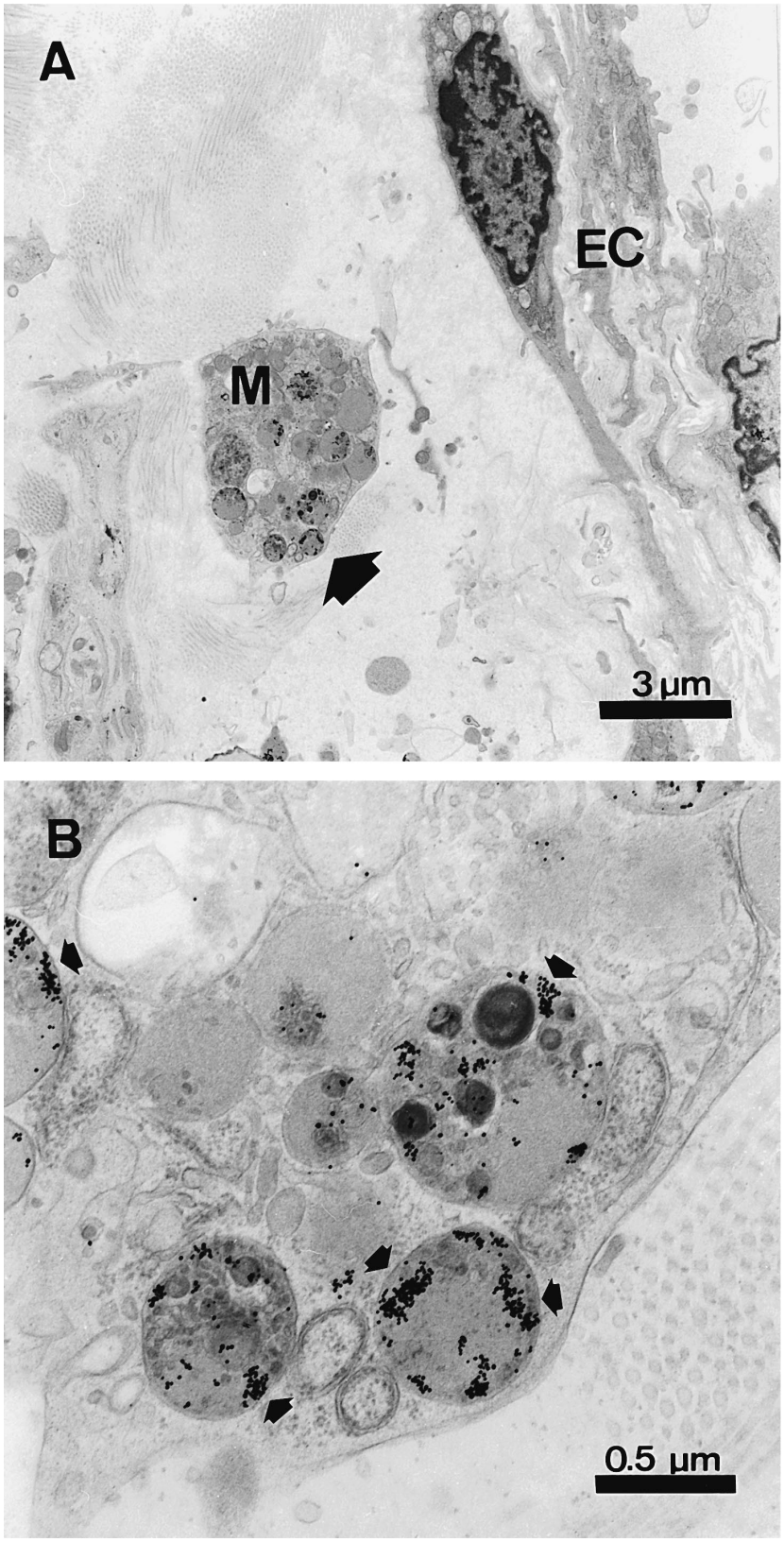


Fig. 4. Lightmicrograph of 1 μm resin silver-enhanced section of a popliteal lymph node isolated 6 h after s.c. injection of small (0.1 μm) gold-liposomes composed of EPC:EPG:Chol (molar ratio 10:1:4) into the foot. Paracortical sinus; macrophages (large arrows) and endothelial cells (small arrows) containing colloidal gold particles.

basic fuchsin. The sections were examined using a Zeiss Axioplane light microscope (Oberkochen, Hertfordshire, UK) using polarized light. Following light microscopy, the resin specimen blocks were trimmed and 70–90 nm sections of the selected area of the lymph nodes were cut. The sections were collected

Fig. 5. Transmission electron micrographs of 70–90 nm resin section of a popliteal lymph node isolated 6 h after s.c. injection of small (0.1 μm) gold-liposomes composed of EPC:EPG:Chol (molar ratio 10:1:4) into the foot. (A) Macrophage (M) containing colloidal gold near endothelial cell (EC). (B) Enlargement of A; intracellular vesicles containing colloidal gold particles (arrows).


A
EC
M
3 µm
B
0.5 µm

on G200 copper grids and stained with aqueous uranyl acetate and lead citrate (Reichert Ultrastainer; Leica, Rijswijk, The Netherlands) before examination at an accelerating voltage of 60 kV in a Philips EM201 transmission electron microscope (Philips Analytical, Electron Optics, Eindhoven, The Netherlands). The lightmicrographs and the transmission electron micrographs shown should be considered as typical examples of the evaluated sectioned material.

2.10. Statistics

The effect of different treatments was compared by a two-tail Student's *t*-test assuming equal variances with 95% confidence interval. Differences were considered significant when the *p*-value was less than 0.05.

3. Results

3.1. Effect of macrophage depletion on degree of lymph node localization of liposomes

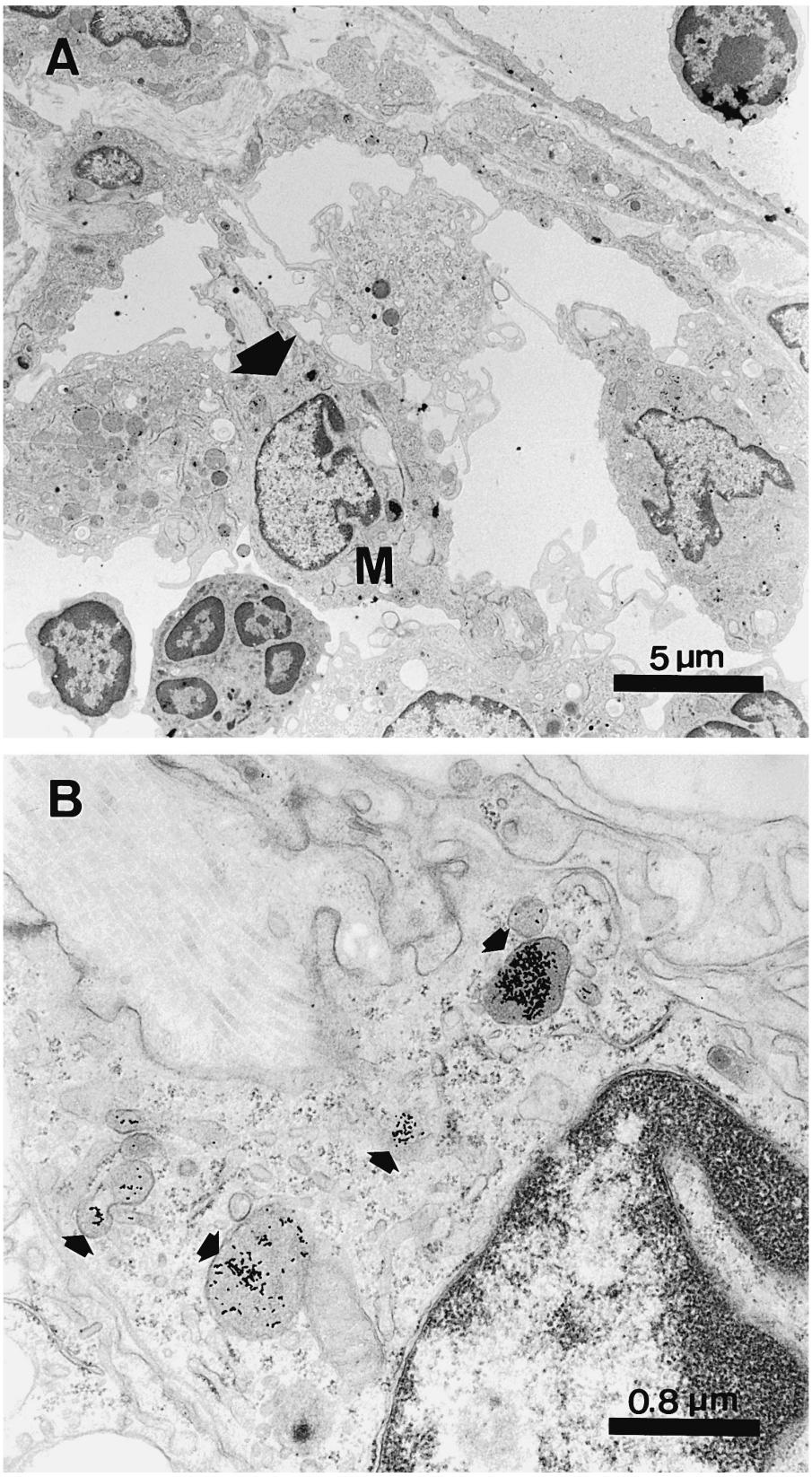
Lymph node localization of four types of liposomes, composed of EPC:EPG:Chol (molar ratio 10:1:4) differing in size (large and small (0.1 μm)) with or without PEG–PE (6 mol%), was compared. Liposomes were radiolabeled with a trace amount of [^{3}H]-cholesteryloleylether. Lymph node localization of radiolabeled liposomes was determined in popliteal lymph nodes depleted of macrophages and in control (nondepleted) lymph nodes derived from the contralateral side of the body. The so-called macrophage 'suicide technique' was used to deplete popliteal lymph nodes of macrophages [9]. S.c. injection of clodronate-liposomes results in depletion of macrophages lining the subcapsular sinus and those in the medulla of regional popliteal lymph nodes [22]. To determine the effectiveness of the liposomal clodronate-treatment, the degree of macrophage depletion was assessed. The extent of macrophage depletion was studied 6 days after s.c. injection of clodronate-containing liposomes. This time-point was considered appropriate for performing the lymph node localization experiments as elimination of macrophages in popliteal lymph nodes after s.c. administration of clodronate-liposomes in the footpad of mice was found to be completed 5 days post-injection; repopulation was not observed within 1 month after treatment [22]. Immunoperoxidase staining revealed that in popliteal lymph nodes draining the s.c. injection site of clodronate-liposomes, macrophages in the medulla and the subcapsular sinuses were completely eliminated. Few macrophages were still present in the paracortical area as revealed by acid phosphatase staining. Macrophages in the contralateral popliteal lymph nodes were not affected (results not shown).

Some 6 days after s.c. administration of clodronate-liposomes, radiolabeled liposomes were injected s.c. into the dorsal side of the right (pretreated) and left (control) foot. Lymphatic uptake from the s.c. injection site and lymph node localization were determined 24 h post-injection.

Fig. 1 presents the %ID remaining at the s.c. injection site. Lymphatic uptake of the larger liposome types was low compared to uptake of the small liposome types (about 98 and 40%ID remaining at the injection site, respectively). Pretreatment with clodronate-liposomes had practically no effect on lymphatic uptake of the various liposome types investigated; a slight negative effect was observed only in case of the two small liposome types. The effect of inclusion of PEG-PE on the lymphatic uptake of small and large liposomes was negligible regardless of prior treatment with clodronate-liposomes (Fig. 1).

Lymph node localization of large, nonsized liposomes in nondepleted, control lymph nodes and lymph nodes depleted of macrophages is presented in Fig. 2A. About 46%ID/g (i.e. 0.2%ID) was recovered from the control lymph nodes 24 h after injection of nonsized, large liposomes. Depletion of macrophages resulted in a drastically decreased lymph node localization of large liposomes (87% reduction). Inclusion of PEG–PE did not influence lymph node localization of large liposomes in macrophage-depleted and control (nondepleted) lymph nodes. The effect of

Fig. 6. Transmission electron micrographs of 70–90 nm resin section of a popliteal lymph node isolated 6 h after s.c. injection of large PEG–gold-liposomes composed of EPC:EPG:Chol:PEG–PE (molar ratio 10:1:4:1) into the foot. (A) Macrophage (M) in lymph node sinus. (B) Enlargement of A; intracellular vesicles containing colloidal gold particles (arrows).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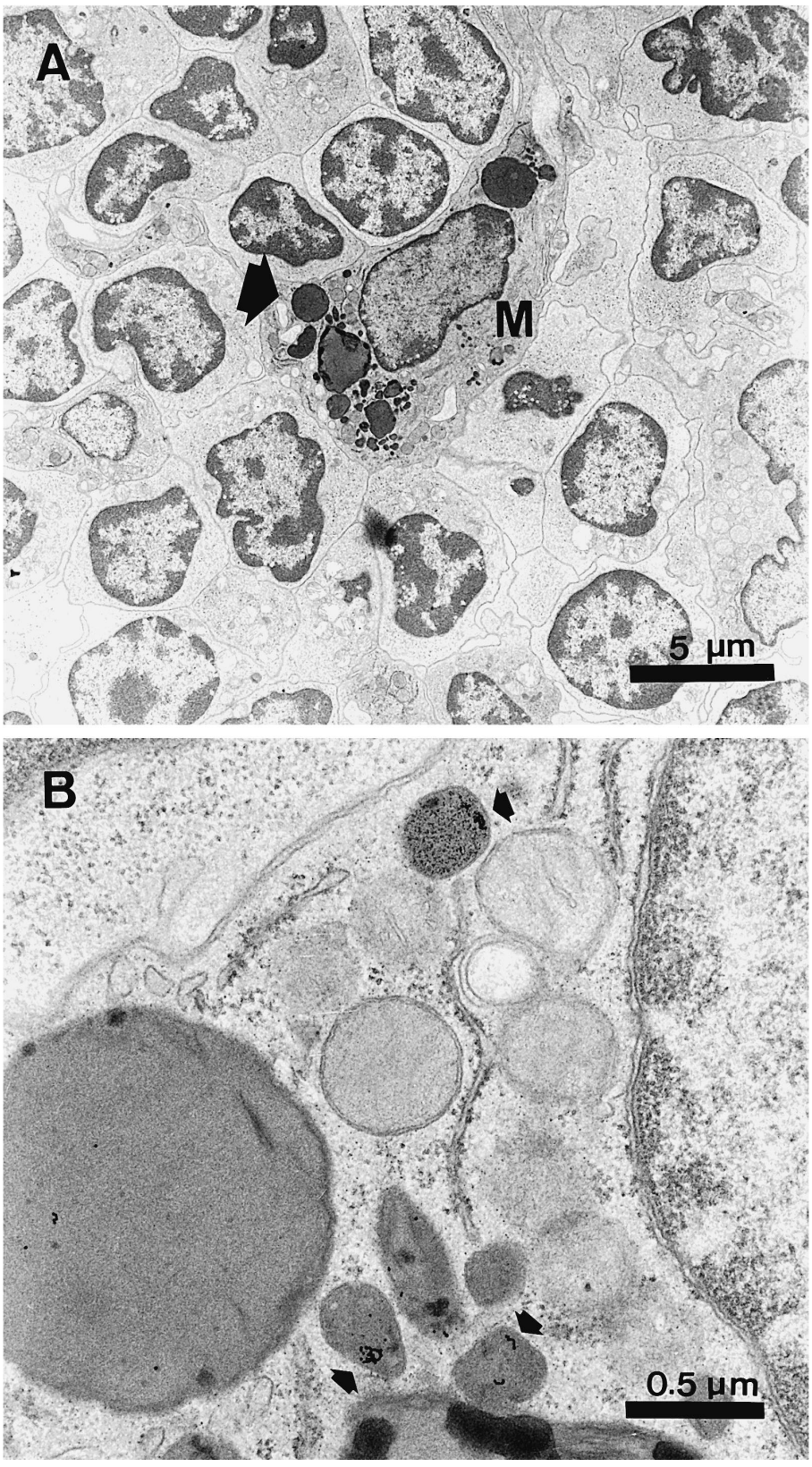
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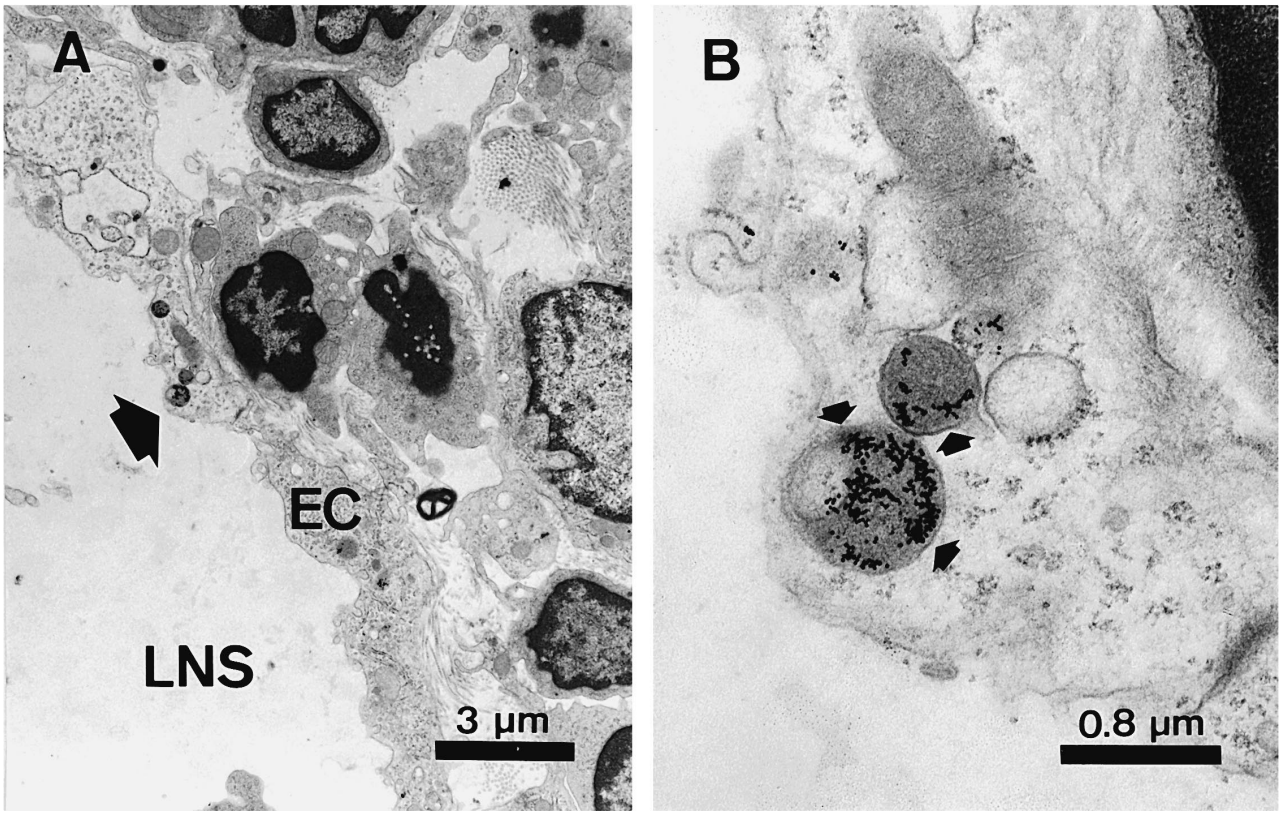

Fig. 8. Transmission electron micrographs of 70–90 nm resin section of a popliteal lymph node isolated 6 h after s.c. injection of large PEG–gold-liposomes composed of EPC:EPG:Chol:PEG–PE (molar ratio 10:1:4:1) into the foot. (A) Endothelial cell (EC) lining lymph node sinus (LNS). (B) Enlargement of A; Intracellular vesicles containing colloidal gold particles (arrows).

macrophage depletion on lymph node localization of small (0.1 μm) liposomes is shown in Fig. 2B. About 160%ID/g (i.e. 0.6%ID) was recovered from control the lymph nodes 24 h after injection of small liposomes. Localization of small liposomes in lymph nodes depleted of macrophages was substantially less than in control lymph nodes. However, the effect of depletion was less as compared to the effect on localization of large liposomes (59% vs. 87% reduction, respectively). No significant effect of the presence of PEG–PE on liposome localization in macrophage-depleted and control lymph nodes was observed. As a control, lymphatic uptake from the s.c. injection site and lymph node localization of large, nonsized radiolabeled liposomes was studied 6 days after pretreatment with empty liposomes (i.e., devoid of clodronate) prepared in a similar way as clodronate liposomes. Pretreatment with empty liposomes did not affect lymphatic uptake or lymph node localization of the large liposomes (results not shown).

Fig. 3 shows blood levels of liposomes at 7 and 24 h after injection. Subcutaneous administration of large liposomes yielded much lower blood levels than s.c. administration of small liposomes at both time-points. Inclusion of PEG–PE resulted in considerably higher blood levels, both for large and small liposomes. These results are in line with our earlier observations on the pharmacokinetics of conventional liposomes and PEG-liposomes in rats not pretreated with liposomal clodronate [4,7].

Fig. 7. Transmission electron micrographs of 70–90 nm resin section of a popliteal lymph node isolated 6 h after s.c. injection of small (0.1 μm) PEG–gold-liposomes composed of EPC:EPG:Chol:PEG–PE (molar ratio 10:1:4:1) into the foot. (A) Macrophage (M) between lymphocytes in paracortex. (B Enlargement of A; intracellular vesicles containing colloidal gold particles (arrows).

3.2. Microscopic observations

The different liposome types under investigation were labeled with colloidal gold to visualize their localization in control (nondepleted) popliteal lymph nodes. Colloidal gold has proven to be a useful electron-dense marker for microscopic evaluation of localization of liposomes in vivo [14]. Typical examples of light and transmission electron microscopy are presented. Light microscopy of silver-enhanced semi-thin sections of popliteal lymph nodes isolated 6 h after s.c. administration of gold-liposomes revealed an intracellular presence of liposomes. Large liposomes concentrated predominantly in macrophages in the subcapsular sinus (results not shown). Small liposomes, on the other hand, tended to localize in sinus macrophages in the cortical and medullary sinuses rather than in the subcapsular sinus. Endothelial cells lining the sinuses were found to contain a high amount of gold particles only after administration of small liposomes (Fig. 4). Transmission electron microscopy confirmed macrophages as the predominant site of liposome localization. In all cases, colloidal gold was found in intracellular vesicles (Fig. 5). Remarkably, also PEG-liposomes were found to be localized primarily within the macrophages (Figs. 6 and 7). Also in endothelial cells lining the lymphatic sinuses, colloidal gold was observed in some cases (Fig. 8).

4. Discussion

The main objective of the present study was to gain more insight into the fate of liposomes in regional lymph nodes after s.c. administration of liposomes. Selective depletion of macrophages from various tissues in vivo has been exploited to investigate the role of these cells in defined biological processes [9]. Here, experiments were designed to study the effect of liposomal clodronate-mediated depletion of macrophages in lymph nodes on the intranodal localization of s.c. administered liposomes. Prior to that, it was evaluated whether treatment with clodronate-liposomes influences the degree of lymphatic uptake of s.c administered liposomes from the s.c. injection site. Lymphatic uptake was only slightly affected by pretreatment with clodronate-liposomes (Fig. 1). Therefore, it is concluded that the fraction of liposomes reaching the regional lymph nodes after pretreatment with clodronate-liposomes is about the same to that reaching control lymph nodes. Hence, the interpretation of the present lymph node localization findings is not complicated by liposomal clodronate-induced differences in degree of lymphatic uptake from the injection site.

Pretreatment with liposomal clodronate resulted in drastic reduction of lymph node localization of large as well as small liposomes (Fig. 2). However, the reduction in lymph node localization was substantially higher (about 1.5-fold) in the case of the large liposomes compared to the small liposomes. The difference in degree of lymph node localization in macrophage-depleted lymph nodes between small and large liposomes may be related to the ability of small liposomes to penetrate more efficiently into the nodal tissue than large liposomes. As macrophages in the subcapsular sinus and medulla were completely depleted, clodronate-liposomes are probably phagocytosed predominantly by macrophages present in these lymph node areas. The large liposomes will likely follow the routing of the large, clodronate-liposomes through the lymph node and, therefore, tend to pass through lymph node regions which are depleted from macrophages. Consequently, phagocytosis of these large liposomes will be strongly reduced. Considering the finding that lymph node retention is also strongly reduced (Fig. 2A), the involvement of macrophages for lymph node localization of large liposomes is clearly indicated. Small liposomes, as a result of their size, might be able to penetrate more efficiently into deeper areas of the lymph node and may, therefore, reach lymph node regions not encountered by the large clodronate-liposomes. Thus, phagocytosis of small liposomes may still occur by macrophages present in nondepleted areas of the lymph node. A second explanation for the difference in effect of liposomal clodronate-treatment on lymph node localization between large and small liposomes relates to the possibility of uptake of small liposomes by cells other than macrophages. Small liposomes may be taken up by cells, such as endothelial cells, capable of pinocytosis of macromolecules and very small particles [23,24]. This explanation seems in line with the light microscopy results. Small gold-liposomes localized to a high extent in sinus macrophages, but

colloidal gold was also observed in endothelial cells lining the sinuses (Fig. 5). After administration of large gold-liposomes, uptake in endothelial cells was negligible. It is known that when macrophages are eliminated, other cells may partly take over the function of endocytosis [25–27]. As these cells will take up smaller particles only, the depletion treatment exerts less effect on the localization of small liposomes.

As surface modification with PEG is known to resist phagocytosis of liposomes by macrophages, it was anticipated that lymph node localization of PEG-liposomes would be substantially lower as compared to liposomes lacking the PEG-coating. Moreover, one would expect that the depletion of macrophages has only a minor effect on lymph node localization of PEG-liposomes. Surprisingly, however, we observed that inclusion of PEG–PE did not significantly influence lymph node localization in control and depleted lymph nodes. The presence of PEG–PE did not affect lymph node localization in control lymph nodes. In addition, macrophage depletion also had a strong effect on the degree of localization of PEG-liposomes. Moreover, large numbers of colloidal gold particles were observed in macrophages after administration of PEG–gold-liposomes by transmission electron microscopy (Figs. 7 and 8). These results can not be attributed to a failure to achieve effective steric stabilization, as the PEG-liposomes proved to be long-circulating after reaching the blood circulation (Fig. 3). We hypothesize that the initial mechanism of lymph node localization may be the result of mechanical depth filtration in the meshwork of reticular cells in the lymph node. Because of the slow progression of the liposomes through the lymph node, enough time is available for an effective interaction of the PEG-liposomal surface with phagocytic cell membranes and, given enough time, even PEG-liposomes will be taken up by macrophages. In this context, one should realize that it is the rate, rather than the extent of phagocytosis, that is affected by the hydrophilic PEG-coating; macrophages of liver and spleen still ingest a substantial part of an i.v. dose of PEG-liposomes. Partial removal of the steric barrier from the particle surface has been suggested to be responsible for the observed uptake of poloxamine-908 coated polystyrene particles by spleen macrophages [5,10,28]. However, in the view of the relatively early observation time point (6 h post-injection) and the much stronger anchoring of PEG to the liposomal bilayers via the hydrophobic anchor DSPE as compared to the physically adsorbed poloxamine-908, this possibility seems unlikely. The reason for the apparent efficient uptake of PEG-liposomes by lymph node macrophages remains to be investigated. Observations on earlier time-points after injection might reveal differences between the localization of control and PEG-liposomes.

For treatment of diseases with lymphatic involvement (e.g., cancer, HIV), it is desirable to develop approaches for the delivery of diagnostic and therapeutic agents to the diseased lymph nodes. Also, lymphatic targeting of antigens to induce immune responses is of interest as lymph nodes play an important role in establishing humoral and cellular immunity by the processing of antigens. Results presented in this paper establish that phagocytosis by macrophages is the most important mechanism for lymph node localization of large liposomes. Depletion of macrophages had less effect on the lymph node localization of small liposomes. Small liposomes may reach macrophages in regions of lymph nodes not reached by large liposomes. In addition, small liposomes may be taken up by cells other than macrophages, such as endothelial cells lining the lymph node sinuses. Remarkably, inclusion of PEG–PE into the liposomes did not reduce lymph node localization in control lymph nodes. Macrophage depletion had also a strong effect on the lymph node localization of PEG-liposomes. Therefore, it is concluded that PEG-liposomes retained by lymph nodes are to a large extent taken up by lymph node macrophages. Morphological observations visualizing the uptake of PEG-liposomes by lymph node macrophages support this conclusion. The results presented in this paper are relevant for the design of liposomes for targeting of diagnostic agents, drugs and antigens to regional lymph nodes.

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