Lymphatic Uptake and Biodistribution of Liposomes After Subcutaneous Injection: III. Influence of Surface Modification with Poly(ethyleneglycol)

Christien Oussoren[1,2] and Gert Storm[1]

Received March 18, 1997; accepted July 17, 1997

Purpose. The aim of the present paper was to assess the effect of inclusion of distearoylphosphatidylethanolamine-poly(ethyleneglycol) (DSPE-PEG) into liposomal bilayers on the lymphatic uptake and lymph node localization of liposomes after subcutaneous administration.

Methods. [^{3}H]-Cholesteryloleylether labeled liposomes of various composition and sizes were injected s.c. into the dorsal side of the foot of rats. At several time-points after injection, blood levels of liposomes were determined. Lymphatic uptake from the s.c. site of injection and lymph node localization in regional lymph nodes were determined at the end of the 52 h observation period.

Results. The results demonstrate that inclusion of DSPE-PEG into several types of liposomes has only a modest effect on lymphatic uptake. Also lymph node localization is only slightly affected by PEG-mediated steric stabilization.

Conclusions. Factors other than the presence of a steric barrier are more important in determining lymphatic uptake from the s.c. injection site. The observation that lymph node localization was only slightly affected by PEG-coating strongly suggests that macrophage uptake is not the only important mechanism of lymph node localization of s.c. administered liposomes.

KEY WORDS: liposomes; subcutaneous; lymphatic disposition; steric stabilization.

INTRODUCTION

The ability of the lymphatic system to take up colloidal particles from interstitial spaces has raised interest to use colloids, such as liposomes, for the delivery of diagnostic and therapeutic agents to regional lymph nodes following local administration. The subcutaneous (s.c.) route of administration has been most extensively investigated for delivery of particulates to the lymphatic system (1–3). Particles up to about 0.15 μm in diameter are transported from the s.c. injection site into the lymphatic capillaries and may localize in regional lymph nodes, whereas larger particles are trapped within the s.c. interstitial space for a long period of time (4,5). However, even lymphatic uptake and lymph node localization of small-sized particles is incomplete. Previous studies have demonstrated that lymphatic uptake and lymph node localization of small (0.07 μm) liposomes were typically about 60% and 1% of the injected dose, respectively (1).

The incomplete lymphatic uptake of s.c. administered particles from the injection site may be the result of interactions between the particle surface and components of the s.c. interstitium inducing formation of larger particles that are not taken up by the lymphatic capillaries but will remain at the s.c. site of injection. It has been suggested that it might be possible to increase lymphatic drainage of colloid particles by applying the concept of steric stabilization (2). Coating the liposomal surface with a sterically stabilizing, hydrophilic layer, may reduce nonspecific interactions of particles with components of the interstitium and inhibit the formation of particle structures too large for lymphatic uptake. Moreover, the increased hydrophilicity of particles with a sterically stabilized surface may allow improved migration through the aqueous channels of the interstitium possibly resulting in increased lymphatic uptake.

In the liposome field the most popular means to obtain sterically stabilized surfaces is to incorporate poly(ethyleneglycol) (PEG) conjugated to distearoyl-phosphatidylethanolamine (DSPE) into the liposomes. Most reports which have appeared in recent years used PEG-polymers with molecular weights of 1900 and 5000 Da. Upon reaching a lymph node, sequestration of liposomes may occur by either phagocytosis by cells belonging to the mononuclear phagocyte system (MPS) or by simple mechanical filtration. As steric stabilization of the liposomal surface with DSPE-PEG reduces adsorption of proteins to the liposomal surface, such liposomes would tend to avoid uptake by cells of the MPS (stealth effect). Therefore, PEG-coating of the liposomal surface may have a negative effect on lymph node localization. However, as hypothesized above, lymphatic uptake from the s.c. injection site may have increased as a result of the steric stabilization effect and, therefore, the net amount of liposomes reaching the lymph node may be higher in spite of the stealth effect.

As both lymphatic uptake and lymph node localization might be influenced by the presence of a hydrophilic PEG-coating on the liposomal surface, the ultimate effect of modification of the liposomal surface with DSPE-PEG on lymph node localization is difficult to predict. Indeed, there are indications in the literature that steric stabilization of colloidal particles would facilitate lymphatic uptake and reduce lymph node localization after s.c. administration (2,5–7). However, literature on this issue is very limited and the actual lymphatic disposition of PEG-containing liposomes from the s.c. injection site has not been reported yet. In this paper the influence of surface modification with DSPE-PEG on the lymphatic uptake and lymph node localization of s.c. administered liposomes was assessed.

MATERIALS AND METHODS

Chemicals

Egg-phosphatidylcholine (EPC) and dipalmitoylphosphatidylcholine (DPPC) were donated by Lipoid GmbH

[1] Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, participant in Groningen Utrecht Institute for Drug Exploration (GUIDE), Faculty of Pharmacy, Utrecht, The Netherlands.

[2] To whom correspondence should be addressed at the Department of Pharmaceutics, PO Box 80 082, 3508 TB Utrecht, The Netherlands.

ABBREVIATIONS: chol, cholesterol; DPPC, dipalmitoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; DSPE, distearoylphosphatidylethanolamine; EPC, egg-phosphatidylcholine; EPG, egg-phosphatidylglycerol; i.m., intramuscular; MPS, mononuclear phagocyte system; PEG, poly(ethyleneglycol); s.c., subcutaneous.

0724-8741/97/1000-1479$12.50/0

(Ludwigshafen, FRG). Distearoylphosphatidylethanolamine-poly(ethyleneglycol)2000 (DSPE-PEG2000) and distearoylphosphatidylethanolamine-poly(ethyleneglycol)5000 (DSPE-PEG5000) were obtained from Avanti Polar Lipids Inc. (Alabaster, Al, USA). 1α,2α(n)-[^{3}H]-Cholesteryloleylether (spec. act. 1.71 TBq/mmol) was supplied by Amersham (Buckinghamshire, UK). Cholesterol (Chol) and 4-(2-hydroxyethyl)-1-piperazine-ethane-sulphonic acid (Hepes) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Hionic Fluor, Soluene-350 and Plasmasol were purchased from Packard Instruments (Downers Grove, IL, USA). All other reagents were of analytical grade.

Preparation of Radiolabeled Liposomes

Liposomes were prepared by the thin film-extrusion method (8). [^{3}H]-Cholesteryloleylether was added as a marker of the lipid phase. 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 minutes, the film was hydrated in a sterile Hepes/glucose-buffer (10 mM Hepes, 1 mM EDTA, 270 mM glucose, pH 7.4). Intermediate (0.15 μm) liposomes were prepared by extruding the resulting liposome dispersion through two stacked 0.2 μm polycarbonate membrane filters (Nuclepore; Costar Co., Cambridge, MA, USA) under nitrogen pressure. Small (0.07 μm) liposomes were prepared by extruding sequentially through two stacked 0.1 μm and 0.05 μm polycarbonate membrane filters under nitrogen pressure.

Liposome Characterization

Radioactivity of the liposomal dispersions was assayed in Hionic Fluor as scintillation mixture and counted in a Philips PW 4700 liquid scintillation counter. 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 Ltd., Malvern, UK). For viscosity and refractive index, values of the Hepes/glucose buffer 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. The polydispersity index of the liposome dispersions used were in all cases <0.15.

Animals

Male Wistar rats (U:WU, CPB) from the animal facility of Utrecht University with an approximate body weight of 220 g were used. Animals received standard laboratory chow and water *ad libitum*.

Animal Experiments

To study the biodistribution of radiolabeled liposomes, rats were injected s.c. with a single dose of [^{3}H]-labeled liposomes (about 2.5 μmol total lipid (TL)) into the dorsal side (i.e. the upper side) of the right foot. At various time-points post-injection a blood sample was drawn from the tail vein under light ether anesthesia. At the end of the observation period, rats were anesthetized with ether and about 10 ml of blood was taken by heart puncture. Rats were then killed by cervical dislocation. The site of s.c. injection and regional lymph nodes (popliteal and iliac) (9) were collected and assayed for radioactivity. The animal experiments adhered to the "Principles of Laboratory Animal Health Care".

Radioactivity Measurements

Radioactivity in blood samples (120 μl) was determined by adding 120 μl of Plasmasol, 500 μl of water and 500 μl of 35% hydrogenperoxide. The samples were decolorized overnight at 40°C. Radioactivity was assayed in Plasmasol as scintillation fluid. A total blood volume per rat of 75 ml/kg body weight was used for calculation of the percentage of dose in the blood circulation (10). Lymph nodes, and the right foot 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% hydrogenperoxide overnight at 40°C. Decolorization of samples was repeated until the samples were only slightly colored. Radioactivity of the decolorized samples was assayed in Hionic Fluor as scintillation fluid. Lymphatic uptake is defined as the percentage injected dose radioactivity (i.e. 100%) minus the percentage of dose radioactivity recovered from the injection site. Lymph node localization is expressed as the percentage injected dose radioactivity per gram lymph node tissue (popliteal and iliac lymph nodes). Relative lymph node localization was calculated by dividing the percentage of dose per gram lymph node tissue by the lymphatically absorbed fraction (i.e. the fraction injected dose taken up from the injection site). Results represent the mean of 4 rats ± standard deviation (sd).

Pharmacokinetics

All pharmacokinetic parameters were calculated using the curve fitting program KINFIT 3.0 (MEDI\WARE BV, Groningen, The Netherlands) for each animal individually. Mean and sd were calculated for each treatment group.

Statistics

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

RESULTS AND DISCUSSION

A s.c. administered carrier system for the delivery of diagnostic, therapeutic and immunomodulatory agents to regional lymph nodes should combine two major characteristics: efficient uptake by the lymphatic system and high lymph node localization. The concept of steric stabilization of colloidal particles has been proposed to optimize both these characteristics by manipulating surface properties (2,6). For liposomes, surface modification with DSPE-PEG has been suggested to result in high blood levels and lower accumulation in regional lymph nodes than liposomes lacking the PEG-coating (5,7). This paper reports on a detailed study aimed to investigate the influence of DSPE-PEG inclusion on the lymphatic uptake and lymph node localization of liposomes after s.c. administration.

DSPE-PEG2000 and DSPE-PEG5000 were incorporated into the bilayer of the several liposome types given in Table 1. Liposomes were radiolabeled with a tracer amount of [^{3}H]-cholesteryloleylether, which has proven to be a reliable label to monitor the fate of liposomes in vivo (11,12). A single injection of liposomes (containing about 2.5 μmol total lipid) was given s.c. into the dorsal side of the foot of rats. Lymphatic uptake and lymph node localization were determined 52 h after injection. To check whether inclusion of DSPE-PEG into the liposomal bilayer indeed resulted in effective steric stabilization of the liposomes, blood levels of liposomes were also determined to confirm prolonged circulation characteristics.

The highest lymphatic uptake (71% of the injected dose (%ID)) was observed after s.c. administration of 0.07 μm EPC:Chol liposomes containing 10 mol% DSPE-PEG5000 (Fig. 1). The PEG-induced increase in lymphatic uptake was about 1.2-fold as compared to similar liposomes lacking the PEG-coating (control liposomes). Remarkably, inclusion of 5 mol% DSPE-PEG2000 or 5 mol% DSPE-PEG5000 in the same liposome type did not result in increased lymphatic uptake. High lymphatic uptake (69 %ID) was also observed after administration of 0.07 μm DPPC:Chol liposomes containing 5 mol% DSPE-PEG5000. In this case, lymphatic uptake increased 1.4-fold as compared to similar control liposomes. Coating of the same DPPC:Chol liposomes with 5 mol% DSPE-PEG2000 did not result in a significant increase in lymphatic uptake which was about 50 %ID (Fig. 1). Lymphatic uptake of the larger 0.15 μm EPC:Chol liposomes was much less compared to 0.07 μm EPC:Chol liposomes, as only about 20 %ID was taken up from the injection site (Fig. 1). This much lower uptake is in accordance with previous studies (1,4,5), which showed that size is the most important factor influencing lymphatic uptake of liposomes after s.c. administration. Inclusion of 5 mol% DSPE-PEG2000 or 5 mol% DSPE-PEG5000 in the 0.15 μm EPC:Chol liposomes yielded a slight increase (1.7 and 1.5-fold, respectively) in lymphatic uptake. Steric stabilization conferred by the higher molecular weight DSPE-PEG5000 was not superior to that conferred by DSPE-PEG2000. The rate of lymphatic uptake (absorption rate (t1/2abs)) was not influenced by steric stabilization of the liposomal surface (Table 2). For all liposome types the t1/2abs was about 2–4 h. However, because of the limited number of observations in the absorption phase, the t1/2abs should be considered as approximate values and, therefore, should be used with care.

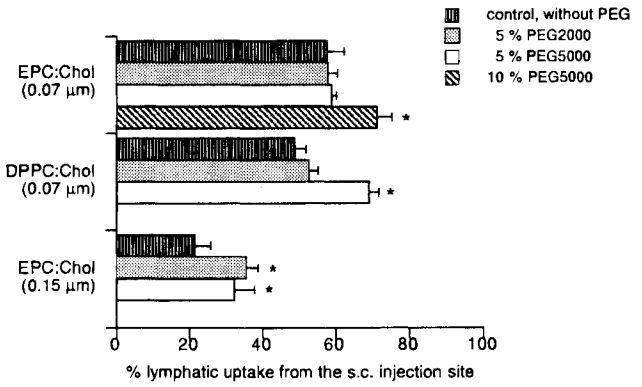

Fig. 1. Effect of PEG-coating on the lymphatic uptake of s.c. administered liposomes. A single injection of several types of liposomes (about 2.5 μmol TL) was given s.c. into the dorsal side of the foot of rats. Values represent the mean percentage of the injected dose remaining at the injection site ± sd of 4 animals 52 h post-injection.* $p < 0.05$.

Thus, the above observations demonstrate that inclusion of DSPE-PEG into several types of liposomes did not influence lymphatic uptake to the extent that was anticipated based on recently published results (2,5). The highest lymphatic uptake was achieved by inclusion of 5 mol% DSPE-PEG5000 into the bilayers of 0.07 μm DPPC:Chol liposomes and 10 mol% DSPE-PEG5000 in 0.07 μm EPC:Chol liposomes. However, the fraction remaining at the injection site is still a substantial proportion (about 30 %ID). Apparently, factors other than the hydrophilicity of the liposomal surface are more important in determining lymphatic uptake from the s.c. injection site. Speculating, incomplete lymphatic uptake may be related to the heterogeneous size distribution of the liposomal dispersion with liposomes larger than the mean size being retained at the site of injection. Another possible explanation is the alteration of the interstitial pressure during the initial period of uptake. When the pressure in the interstitial tissue exceeds that within the lymphatic capillaries, intercellular junctions in the lymphatic capillaries open up and lymph flow and thus lymphatic uptake

Table 1. Liposome Types Investigated

Lipid composition[a]	Molar ratio	DSPE-PEG (mol%)	Mean size (μm)	Polydispersity index
EPC:Chol (control)	2:1	0	0.07	0.07
EPC:Chol:PEG2000	1.85:1:0.15	5	0.07	0.08
EPC:Chol:PEG5000	1.85:1:0.15	5	0.07	0.05
EPC:Chol:PEG5000	1.7:1:0.3	10	0.07	0.10
DPPC:Chol (control)	2:1	0	0.07	0.10
DPPC:Chol:PEG2000	1.85:1:0.15	5	0.07	0.07
DPPC:Chol:PEG5000	1.85:1:0.15	5	0.07	0.12
EPC:Chol (control)	2:1	0	0.15	0.14
EPC:Chol:PEG2000	1.85:1:0.15	5	0.15	0.08
EPC:Chol:PEG5000	1.85:1:0.15	5	0.15	0.07

[a] PEG was incorporated as DSPE-PEG.

Table 2. Influence of Inclusion of DSPE-PEG into Several Liposome Types on Blood Pharmacokinetic Parameters

Liposome type	Mean size (μm)	$t_{1/2abs}$ (h)	t_{max} (h)	C_{max} (%ID)	$t_{1/2elim}$ (h)	AUC (h nmol/ml) ($\times 10^2$)
EPC:Chol	0.07	2.3 ± 1.6	5 ± 1	26 ± 2	6 ± 2	6 ± 0
EPC:Chol:PEG2000 5 mol%	0.07	3.5 ± 0.4	11 ± 1	44 ± 3	22 ± 1	29 ± 2
EPC:Chol:PEG5000 5 mol%	0.07	3.3 ± 0.4	11 ± 1	44 ± 3	25 ± 2	33 ± 1
EPC:Chol:PEG5000 10 mol%	0.07	2.9 ± 0.3	9 ± 1	37 ± 2	20 ± 0	22 ± 1
DPPC:Chol	0.07	4.1 ± 1.5	11 ± 2	29 ± 3	17 ± 2	16 ± 2
DPPC:Chol:PEG2000 5 mol%	0.07	2.3 ± 0.4	8 ± 1	31 ± 3	23 ± 2	20 ± 2
DPPC:Chol:PEG5000 5 mol%	0.07	2.3 ± 1.1	7 ± 2	33 ± 5	15 ± 1	15 ± 1
EPC:Chol	0.15	3.9 ± 1.6	7 ± 1	12 ± 2	7 ± 3	4 ± 1
EPC:Chol:PEG2000 5 mol%	0.15	3.1 ± 0.6	9 ± 1	26 ± 2	17 ± 2	14 ± 1
EPC:Chol:PEG5000 5 mol%	0.15	3.5 ± 1.1	11 ± 2	23 ± 3	21 ± 2	15 ± 1

Note: A single dose (about 2.5 μmol TL per rat) of radiolabeled liposomes was injected s.c. into the dorsal side of the foot of rats. Values were calculated for each individual animal and represent the mean ± sd of 4 animals. ($t_{1/2abs}$; absorption half-life, t_{max}; time at which C_{max} was reached, C_{max}; maximum blood level, $t_{1/2elim}$; elimination half-life, AUC; area under the curve).

of interstitial fluid increases (13,14). As lymphatic uptake may be the result of an elevated interstitial pressure caused by the injection itself, lymphatic absorption may slow down dramatically when the interstitial pressure is normalized (15).

Lymph node localization of the various liposome types under investigation is presented in Fig. 2. DSPE-PEG-mediated steric stabilization had hardly any effect on lymph node localization when the results are expressed as the percentage of injected dose/gram lymph node tissue (%ID/g). Lymph node localization decreased slightly when 0.07 μm EPC:Chol liposomes were coated with 5 mol% DSPE-PEG2000 and 5 mol% DSPE-PEG5000 (Fig. 2A). It should be realized, however, that as discussed above, if lymphatic uptake increases, the fraction of injected liposomes reaching regional lymph nodes also increases. Consequently, lymph node localization should be corrected for the lymphatically absorbed fraction (i.e. the difference between the administered dose and the amount recovered at the injection site) to establish the net effect of the steric PEG-layer on lymph node localization. To this end, the 'relative lymph node localization' was calculated by correcting the percentage of dose recovered in the lymph nodes for the absorbed fraction (Fig. 2B). Clearly, the highest relative lymph node localization (370% of absorbed fraction/g) was seen for 0.15 μm EPC:Chol liposomes, which is very likely the result of their larger liposome size, as reported previously (1). In general, inclusion of DSPE-PEG in small liposomes induced a modest decrease in relative lymph node localization with the strongest effect observed in the case of EPC:Chol:DSPE-PEG5000 10 mol% and DPPC:Chol:DSPE-PEG5000 5 mol% (35% and 43% reduction, respectively).

The question arises why the PEG-sterically stabilized surface does not exert a stronger negative effect on lymph node localization than observed. It is generally assumed that liposomes are retained in lymph nodes via phagocytic capture by macrophages. The observation that the steric barrier imposed by the hydrophilic PEG-chains does not strongly suppress lymph node localization indicates that macrophage uptake is not the predominant mechanism of lymph node localization of liposomes. Rather, simple mechanical filtration of (PEG)-liposomes may be the major mode of lymph node localization. A supportive observation is that incubation of the 0.07 μm EPC:Chol liposomes with fresh rat serum (for 30 min at 37°C) prior to s.c. administration did not have a significant effect on

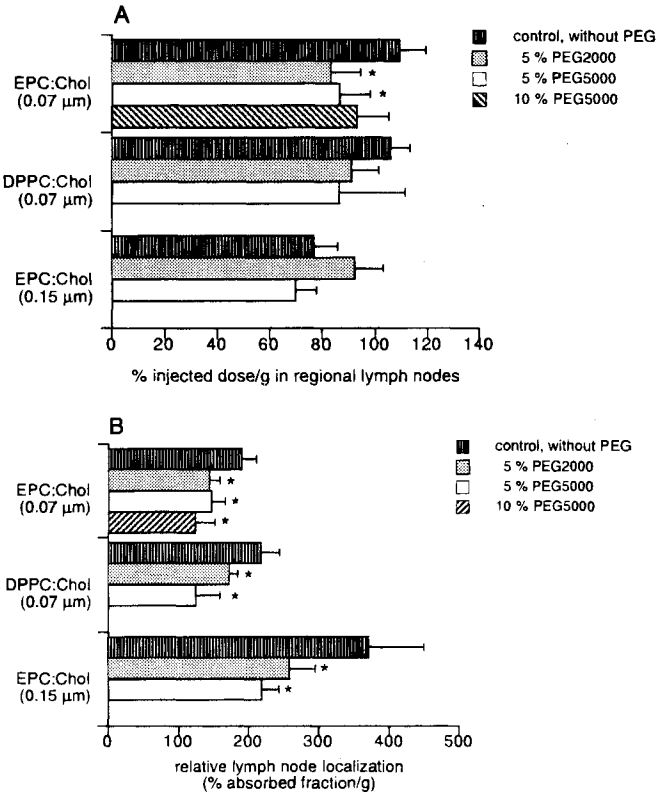

Fig. 2. Effect of PEG-coating on lymph node localization of s.c. administered liposomes. A single injection of several types of liposomes (about 2.5 μmol TL) was given s.c. into the dorsal side of the foot of rats. A: Lymph node localization; percentage of injected dose in the regional lymph nodes per gram tissue 52 h post-injection. B: Relative lymph node localization; percentage of the lymphatically absorbed fraction in the regional lymph nodes per gram tissue 52 h post-injection. Values represent the mean ± sd of 4 animals.* $p < 0.05$.

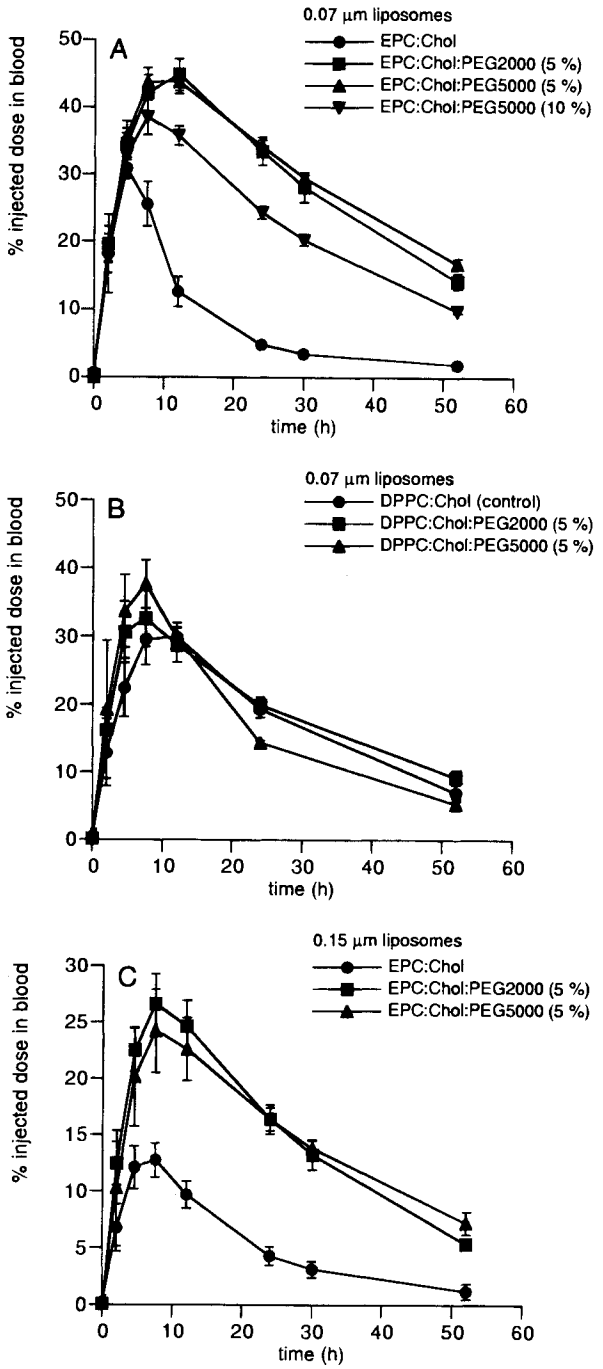

Fig. 3. Effect of PEG-coating on blood levels of s.c. administered liposomes. A single injection of several types of liposomes (about 2.5 μmol TL) was given s.c. into the dorsal side of the foot of rats. A: Effect of PEG-coating on blood levels of 0.07 μm EPC:Chol liposomes. B: Effect of PEG-coating on blood levels of 0.07 μm DPPC:Chol liposomes. C: Effect of PEG-coating on blood levels of 0.15 μm EPC:Chol liposomes. Values represent the mean ± sd of 4 animals. Note the differences in the y-axis scale.

lymph node localization of these liposomes (result not shown). The lack of effect of this pre-opsonization procedure also suggests that macrophage uptake is not the only important mechanism of liposomal lymph node localization.

To assess the effect of steric stabilization on blood circulation times of liposomes, blood levels of radioactivity were determined at several time-points post-injection (Fig. 3). Estimates for pharmacokinetic parameters in blood are given in Table 2. As compared to control (non-coated) EPC:Chol liposomes, elimination half-lives in blood increased 3 to 4-fold, peak blood levels 2-fold and the area under the curve (AUC) 4 to 6-fold. These findings confirmed that inclusion of DSPE-PEG into EPC:Chol liposomes indeed provided effective steric stabilization of the liposomal surfaces. Therefore, the lack of a strong effect of DSPE-PEG inclusion on lymphatic uptake and lymph node localization of these liposomes can not be attributed to an ineffective steric barrier. Inclusion of 5 mol% DSPE-PEG2000 and 5 mol% DSPE-PEG5000 into 0.07 μm DPPC:Chol liposomes did not result in major changes in blood pharmacokinetics. This is unlikely to be caused by ineffective steric stabilization, but rather by a failure of the steric barrier to improve the already long circulation times of the small, non-coated control DPPC:Chol liposomes. Small liposomes composed of saturated phospholipids and cholesterol such as the DPPC:Chol liposomes used in this study are known to circulate for prolonged periods without the requirement of a sterically stabilized surface (16).

In conclusion, in contrast to suggestions by others (5–7) the findings presented in this paper do not indicate that steric stabilization of liposomes has profound effects on lymphatic uptake from the s.c. injection site and lymph node localization. Apparently, the presence of an inert, steric barrier is not an important factor in determining lymphatic uptake of liposomes after s.c. administration. The observation that lymph node localization was only slightly affected by PEG-mediated steric stabilization strongly suggests that macrophage uptake is not the only important mechanism of lymph node localization of s.c. administered liposomes.

REFERENCES

1. C. Oussoren, J. Zuidema, D. J. A. Crommelin, and G. Storm. Lymphatic uptake and biodistribution of liposomes after subcutaneous injection. II. Influence of liposome size, composition and dose. *Biochem. Biophys. Acta* In press.
2. A. E. Hawley, S. S. Davis, and L. Illum. *Adv. Drug Deliver. Rev.* **17**:129–148 (1995).
3. H. M. Patel. Fate of liposomes in the lymphatics. In G. Gregoriadis (Ed), *Liposomes as drug carriers*, John Wiley and Sons Ltd, New York, 1988, pp. 51–62.
4. A. Tümer, C. Kirby, J. Senior, and G. Gregoriadis. *Biochim. Biophys. Acta* **760**:119–125 (1983).
5. T. M. Allen, C. B. Hansen, and L. S. S. Guo. *Biochim. Biophys. Acta* **1150**:9–16 (1993).
6. S. M. Moghimi, A. E. Hawley, N. M. Christy, T. Gray, L. Illum, and S. S. Davis. *FEBS Lett.* **344**:25–30 (1994).
7. V. S. Trubetskoy, J. A. Cannillo, A. Milshtein, G. L. Wolf, and V. P. Torchilin. *Magn. Reson. Imaging* **13**:31–37 (1995).
8. F. Olson, C. A. Hunt, F. Szoka, W. J. Vail, and D. Papahadjopoulos. *Biochim. Biophys. Acta* **551**:295–303 (1979).
9. N. Tilney. *J. Anat.* **109**:369–383 (1987).
10. W. A. Ritschel. *STP Pharma* **3**:125–141 (1987).
11. G. L. Pool, M. E. French, R. A. Edwards, L. Huang, and R. H. Lumb. *Lipids* **17**:448–452 (1982).
12. Y. Stein, G. Halperin, and O. Stein. *FEBS Lett.* **111**:104–106 (1980).
13. P. H. Cox. The kinetics of macromolecule transport in lymph and colloid accumulation in lymph nodes. In P. H. Cox (Ed), *Progress in Radiopharmacology*, Elsevier, Amsterdam, 1981, pp. 267–292.

14. C. M. O'Driscoll. Anatomy and physiology of the lymphatics. In W. N. Charman and V. J. Stella (Eds), *Lymphatic transport of drugs*, CRC Press, Boca Raton, 1992, pp. 1–35.
15. C. Oussoren, J. Zuidema, D. J. A. Crommelin, and G. Storm. Lymphatic uptake and biodistribution of liposomes after subcutaneous injection. I. Influence of the anatomical site of injection. *J. Liposome Res.* **7**:85–99 (1997).
16. J. Senior. *Ther. Drug Carrier Syst.* **3**:123–193 (1987).