Mixed Micelles as a Proliposomal, Lymphotropic Drug Carrier

Andreas Supersaxo,[1,3] Wayne R. Hein,[2] and Hans Steffen[1]

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Four lipophilic, low molecular weight drugs solubilized in phosphatidylcholine–bile salt mixed micelles were injected s.c. into the hind legs of sheep and their cumulative recoveries in lymph draining from the site of application were determined. Surprisingly, the cumulative recoveries (percentage of dose) varied between less than 1 and 60%. We found that there is a correlation between the lipophilicity of the drug (log *P* octanol/water ~ $R_m°$ value) and the proportion of the dose absorbed by the lymphatic route. Drugs with $R_m°$ values >10 are absorbed preferentially by the lymphatics (>50% of dose), whereas compounds with $R_m°$ values <4 are hardly absorbed at all by the lymphatics (<10% of dose). By applying the prodrug principle we demonstrated that it is also possible to target drugs with $R_m°$ values <4 to the lymphatics. Furthermore, the analysis of the collected lymph samples by gel filtration, quasi-elastic light scattering, and electron microscopy revealed that, following s.c. administration, mixed micelles are converted into homogeneous, unilamellar vesicles. In conclusion, these results suggest that mixed micelles may represent a suitable delivery system for low molecular weight drugs whose targets are lymphoid cells. In addition, for drugs where liposomal application leads to a therapeutic advantage, the thermodynamically stable mixed micelle could be a good alternative to the liposome. However, for both applications a high drug lipophilicity is a prerequisite.

KEY WORDS: phosphatidylcholine–bile salt mixed micelles; subcutaneous administration; lymphotropic drug carrier; proliposomal formulation; sheep.

INTRODUCTION

Following s.c. administration a drug can be transported to the general cardiovascular pool either by the blood capillaries or by the lymphatics. For small molecules, up to 1 kDa, the blood capillary wall diffusivity is very high and represents a minor barrier to drug transport. However, for macromolecules the permeability through the blood capillary is low, and therefore, direct movement into the blood is restricted (1,2). Several authors showed that soluble macromolecules do not enter the bloodstream directly through the blood capillaries, but indirectly by way of lymphatic vessels (3–5). Based on these findings many approaches to deliver drugs selectively from the s.c. space into the lymphatics have been proposed such as macromolecular drug conjugates (6), activated carbon particles (7), emulsions (8), and liposomes (1,9).

Phosphatidylcholine–bile salt mixed micelles, a colloidal drug carrier, might provide another possibility to target drugs from the s.c. space into the lymphatics. The formation, size, and structure of bile salt–phosphatidylcholine mixed micelles have been studied systematically using various techniques such as quasi-elastic light scattering (10–12), small-angle X-ray scattering (13), calorimetry (14,15), and nuclear magnetic resonance (NMR) (16). The most widely accepted molecular model for the structure of bile salt–phosphatidylcholine mixed micelles is the "mixed disk" model proposed by Mazer *et al.* (10). In this model, phosphatidylcholine forms a cylindrical piece of bilayer surrounded by a ring of bile salt molecules oriented so that their hydrophilic parts interact with aqueous solvent. In addition, bile salt molecules are inserted into the bilayer in pair (see Ref. 10, Fig. 11).

To our knowledge no studies have assessed the feasibility of employing mixed micelles as a lymphotropic drug carrier. The present study evaluates the feasibility of using mixed micelles to target low molecular weight drugs from the s.c. space into the lymphatics.

MATERIALS AND METHODS

Materials

Epikuron 200 (lecithin from soyabeans: 92% phosphatidylcholine, 3% lysophosphatidylcholine, 0.23% DL-α-tocopherol, 0.23% residual oil) was purchased from Lucas Meyer, Hamburg, Germany. Egg phosphatidylcholine (99% phosphatidylcholine from egg yolk, grade 1) was supplied by Lipid Products, South Nutfield, UK. Sodium glycocholate (NaGC), sodium cholate (NaC), DL-α-tocopherol, very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and vitamin B12 were obtained from Sigma Chemical Co., St. Louis, MO. 5-Fluoro-2′-deoxyuridine (FUdR; Ro 5-0360), diclofensine (Ro 8-4650), diazepam (Ro 5-2807), and the retinoid Ro 23-6457 [(all-E)-3,7-dimethyl-9-[2-(trifluoromethyl)-6-(nonyloxy)phenyl]-2,4,6,8-nonatetraenoic acid] were from F. Hoffmann–La Roche, Basel, Switzerland. 5-[6-^{3}H]Fluoro-2′-deoxyuridine was purchased from NEN Research Products, Boston, MA. [^{14}C]Diclofensine, [^{14}C]diazepam, and [^{14}C]Ro 23-6457 were from F. Hoffmann–La Roche, Basel, Switzerland. 3′,5′-*O*-Dipalm-FUdR [3′,5′-bis(*O*-palmitoyl)-5-fluoro-2′-deoxyuridine] as well as the corresponding tritum-labeled compound was synthesized as described by Schwendener *et al.* (17). All other chemicals were of analytical grade and used without further purification.

Preparation of Mixed Micelles

The mixed micelles (phosphatidylcholine concentration: 30.8 mg/ml) were prepared according to the method described by Steffen and Schmidt (18). Soya phosphatidylcholine and sodium glycocholate at a molar ratio of 1:1 were dissolved in a round flask in chloroform/methanol (1:1, v/v%) and then appropriate amounts of the corresponding

[1] Pharmaceutical Research and Development, F. Hoffmann-La Roche Ltd., CH-4002 Basel, Switzerland.
[2] Basel Institute for Immunology, CH-4002 Basel, Switzerland.
[3] To whom correspondence should be addressed c/o Syntex Research, R1-266, 3401 Hillview Avenue, Palo Alto, California 94303.

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drugs together with traces of labeled compounds were added. The resulting solutions were evaporated to dryness under reduced pressure at 40°C and the formed lipid films were dispersed in 3.8% mannitol solution. The spontaneously formed mixed micelles were adjusted to pH 7, purged with N_2, and filtered through 0.22-μm sterile filters (Millex-GS, Millipore AG, Kloten, Switzerland). The concentrations of the drugs in the mixed micellar solutions were determined after filtration by radioactivity counting.

Preparation of 3′,5′-*O*-Dipalm-FUdR Liposomes

The liposomes (phosphatidylcholine concentration: 30 mg/ml) were prepared according to the detergent dialysis method described by Milsmann *et al.* (19).

Egg phosphatidylcholine, DL-α-tocopherol, and sodium cholate at a molar ratio of 1:0.005:1.75 were dissolved in a round flask in chloroform/methanol (1:1, v/v%) and then 1.4 mg of 3′,5′-*O*-dipalm-FUdR together with traces of the labeled compound was added. The resulting solution was evaporated to dryness under reduced pressure at 40°C and the formed lipid film was dispersed in 1 ml 10 m*M* phosphate buffer containing 0.9% NaCl, pH 7.4 (PBS). The obtained micellar solution was dialyzed against 1 liter PBS for 20 hr with a Lipoprep-GD-1 instrument (Diachema Ltd, Langnau, Zurich, Switzerland). Cellulose membranes with a molecular weight cut off of 10,000 were used (Diachema Ltd.). After completed dialysis the liposomes were purged with N_2 and filtered through 0.22-μm sterile filters (Millex-GS, Millipore AG, Kloten, Switzerland). The concentration of 3′,5′-*O*-dipalm-FUdR incorporated into the liposomes was determined after filtration by radioactivity counting and adjusted with PBS to 1 mg/ml. The prepared liposomes were essentially monodisperse (polydispersity: 2.3 ± 0.5), mean hydrodynamic radii of 45 ± 2 nm were measured (mean ± SD, $N = 3$).

Determination of the $R_m°$ Value

The $R_m°$ values were determined by the thin-layer chromatography (TCL) method as described by Butte *et al.* (20). Two microliters of a 0.5% sample solution in methanol was applied to HPTLC-RP18 plates (Merck, Darmstadt, Germany) and eluted with 10 different methanol/0.01 *M* phosphate buffer solutions, pH 7.4 (100–70% methanol, v/v%). After elution the dried chromatograms were visualized (UV 254 nm) and from the determined R_f values the R_m values were calculated [$R_m = \log (1/R_f - 1)$]. For all compounds the plots of the methanol concentrations of the mobile phase versus the R_m values showed a linear relationship. By linear regression analysis the intercepts (0% methanol) were calculated. These values are defined as $R_m°$ values. There is a good correlation between the $R_m°$ value determined by TLC and the log *P* octanol/water for compounds where log *P* was reported [e.g., diazepam: $R_m°$ value, 2.5; log *P*, 2.82 (21)].

Animals and Surgical Procedures

White Alpine and Black Jura sheep of both sexes, aged 2–4 years, were obtained from Versuchsbetrieb Sennweid, Olsberg, Switzerland. The cannulation of the efferent duct of the popliteal lymph node was done as described by Hall and Morris (22). After the operation sheep were maintained in metabolism cages and fed on commercial pellets, and lymph was collected as described by Miyasaka and Trnka (23).

Lymphatic Absorption Studies

The absorption studies were performed using the cannulated sheep model as described by Supersaxo *et al.* (4). This system allows the collection of lymph draining directly from the injection site. Experiments were started 2–3 days after surgery. After collecting a blank lymph sample, 1 ml of the following test formulations was injected s.c. into the lower part of the lymph cannulated leg: (I) 3′,5′-*O*-dipalm-FUdR mixed micelles and liposomes (1 mg/ml), (II) diclofensine mixed micelles (1 mg/ml), (III) diazepam mixed micelles (0.8 mg/ml), (IV) Ro 23-6457 mixed micelles (0.4 mg/ml), and (V) FUdR mixed micelles (0.3 mg/ml). Each drug formulation was tested in three separate sheep. For each experiment freshly prepared formulations were used. Lymph was collected continuously in heparinized tubes (5 U heparin/ml lymph). The collection tubes were changed at the indicated intervals. After centrifugation (200*g*, 10 min, 4°C) all lymph plasma samples were kept at 4°C until assayed. The concentration of the drugs in lymph were determined by radioactivity counting.

Determination of Radioactivity

To 50 μl test sample (mixed micelles, liposomes, or lymph plasma) 2 ml of liquid scintillation cocktail (Emulsifier 299, Packard Instruments International, Zurich, Switzerland) was added, and the radioactivity present determined by liquid scintillation spectroscopy (Liquid Scintillation Analyzer 2000 CA Tri-Carb, Packard). Efficiency of counting was determined by comparision with an external standard.

Determination of Vesicle Diameter

Hydrodynamic radii and polydispersity (0–2, homogeneous; 5–9, heterogeneous) of the 3′,5′-*O*-dipalm-FUdR liposomes and the vesicles recovered in lymph after administration of mixed micelles were measured by quasi-elastic light scattering (Nano-Sizer PSM 78, Coulter Electronics, Krefeld, Germany).

Electron Microscopy

Freeze-fracture electron micrographs of lymph plasma samples were taken with a Philips 301 electron microscope as described by Müller *et al.* (24).

Gel Filtration Chromatography

A column (53 × 0.9 cm) filled with Bio-Gel A-5m, 200–400 mesh (Bio-Rad Laboratories, Richmond, CA), and previously equilibrated with a 10 m*M* phosphate buffer containing 0.9% NaCl, pH 7.4 (PBS), was used. The probes (200 μl) were eluted with PBS at a flow rate of 6.9 ml/hr. Aliquots of 1 ml were collected (Fraction collector Frac-300, Pharmacia). The eluate was monitored at 280 nm using a variable-wavelength detector (Knauer) or by determining the radioactivity in the collected fractions.

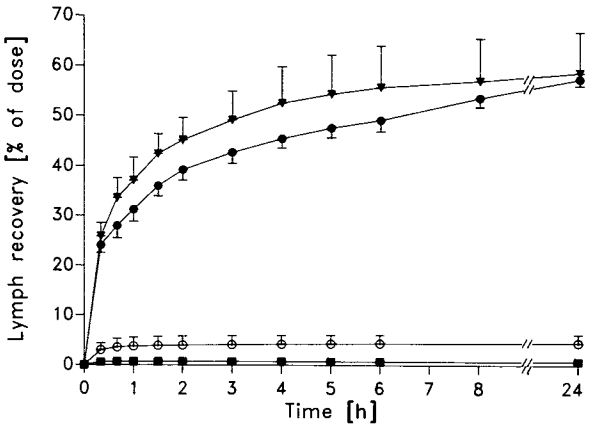

Fig. 1. Cumulative recovery of 3',5'-*O*-dipalm-FUdR (▼), Ro 23-6457 (●), diclofensine (○), and diazepam (■) in the efferent lymph from the right popliteal lymph node following s.c. administration into the lower part of the right hind leg. The four drugs were injected solubilized in mixed micelles. Each point and bar shows the mean and SD of three experiments performed in three separate sheep.

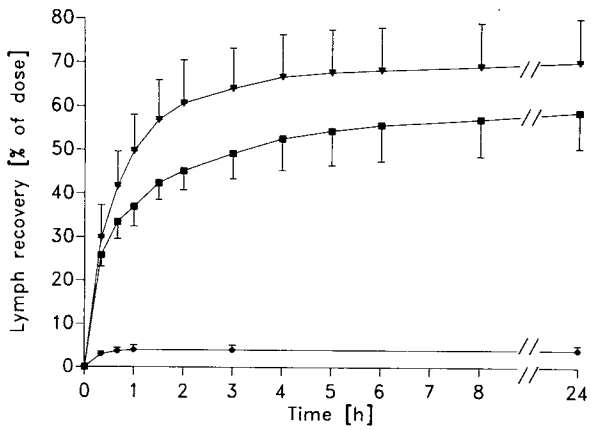

Fig. 3. Cumulative recoveries of FUdR and 3',5'-*O*-dipalm-FUdR in the efferent lymph from the right popliteal lymph node following s.c. administration of 3',5'-*O*-dipalm-FUdR liposomes (▼), 3',5'-*O*-dipalm-FUdR mixed micelles (■), and FUdR mixed micelles (●). All three preparations were injected into the lower part of the right hind leg. Each point and bar shows the mean and SD of three experiments performed in three separate sheep.

RESULTS

Influence of Drug Lipophilicity ($R_m°$ Value) on Lymphatic Absorption

Four lipophilic, low molecular weight drugs solubilized in mixed micelles were injected s.c. into the hind legs of sheep and their cummulative recoveries in lymph draining from the site of application were determined. The recovery in lymph was calculated as the product of the concentration in lymph and the volume of lymph collected for each time interval and was expressed as a percentage of the administered dose. The cumulative recoveries determined were 58.6 ± 9.9% (3',5'-*O*-dipalm-FUdR), 57.3 ± 1.8% (Ro 23-6457), 4.5 ± 1.9% (diclofensine), and 0.7 ± 0.4% (diazepam), respectively (mean ± SD; $N = 3$) (Fig. 1). No activity was found in the cell pellets recovered by centrifugation. Figure 2 shows that there is a relationship between the $R_m°$ value of the drug and the proportion of the dose absorbed by the lymphatics. Drugs with $R_m°$ values >10 (3',5'-*O*-dipalm-FUdR and Ro 23-6457) are absorbed preferentially by the lymphatics, whereas compounds with $R_m°$ values <4 (diclofensine and diazepam) are hardly absorbed at all by the lymphatics.

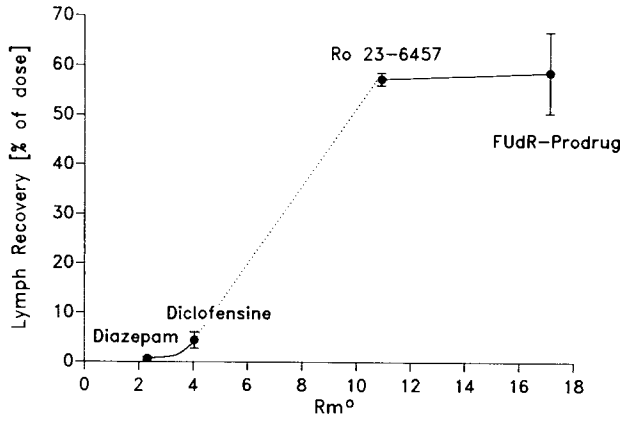

Fig. 2. Correlation between the $R_m°$ value and the cumulative recovery of 3',5'-*O*-dipalm-FUdR ($R_m°$: 17.5), Ro 23-6457 ($R_m°$: 10.6), diclofensine ($R_m°$: 4.0), and diazepam ($R_m°$: 2.5) in the efferent lymph from the right popliteal lymph node following s.c. administration into the lower part of the right hind leg. The four drugs were injected solubilized in mixed micelles. Each point and bar shows the mean and SD of three experiments performed in three separate sheep.

Use of Prodrug Principle Enhances Lymphatic Absorption

By applying the prodrug principle we next tested whether we could also target drugs with $R_m°$ values <4 to the lymphatic system by means of mixed micelles. For this purpose 3',5'-*O*-dipalm-FUdR, a lipophilic prodrug of FUdR, was synthesized. By conversion of FUdR to its prodrug we could increase the $R_m°$ value from −1.4 for FUdR to 17.5 for the prodrug. Concomitantly, the lymph recovery increased from 4.0 ± 1.5% for FUdR to 58.6 ± 9.9% for the prodrug (mean ± SD, $N = 3$) (Fig. 3).

Mixed Micelles Versus Liposomes

The results presented in Fig. 3 also demonstrate that

Table I. Vesicle Diameters Measured in Efferent Lymph from the Right Popliteal Lymph Node Collected During the First 20 min After s.c. Administration of Mixed Micelles Containing the Indicated Drugs into the Lower Part of the Right Hind Leg

Drug	Diameter (nm)[a]	Polydispersity[a,b]
FUdR-prodrug	66.5 ± 0.7	2.5 ± 0.7
Ro 23-6457	63.3 ± 3.5	3.3 ± 0.6
Diclofensine	62.2 ± 4.6	3.0 ± 0.7
Diazepam	65.0 ± 6.2	3.0 ± 1.0

[a] Each value represents the mean and SD of three experiments performed in three separate sheep.

[b] 0–2, homogeneous: 5–9, heterogeneous (Coulter Nanosizer PSM78 manual).

mixed micelles are about as efficient a lymphotropic carrier as are liposomes. The cumulative recovery of 3′,5′-*O*-dipalm-FUdR in lymph following s.c. administration in mixed micelles or in small unilamellar liposomes was 58.6 ± 9.9 and 69.8 ± 13.0%, respectively (mean ± SD, $N = 3$).

Micelle-to-Vesicle Transitition *in Vivo*

During the experiments of using mixed micelles as a lymphotropic drug carrier, we observed that the collected lymph was opalescent, similar to that after s.c. administration of liposomes. Quasi-elastic light scattering measurements revealed that the opalescence was due to particles with a narrow size distribution around 65 nm (Table I). Electron micrographs showed that these particles were small unilamellar vesicles (Fig. 4).

Gel Filtration

The collected lymph samples were next fractionated on a Bio-Gel A-5m column. The results of typical experiments are illustrated in Fig. 5. The elution profile of lymph collected following s.c. administration of 3′,5′-*O*-dipalm-FUdR and Ro 23-6457 in mixed micelles indicates that the bulk of

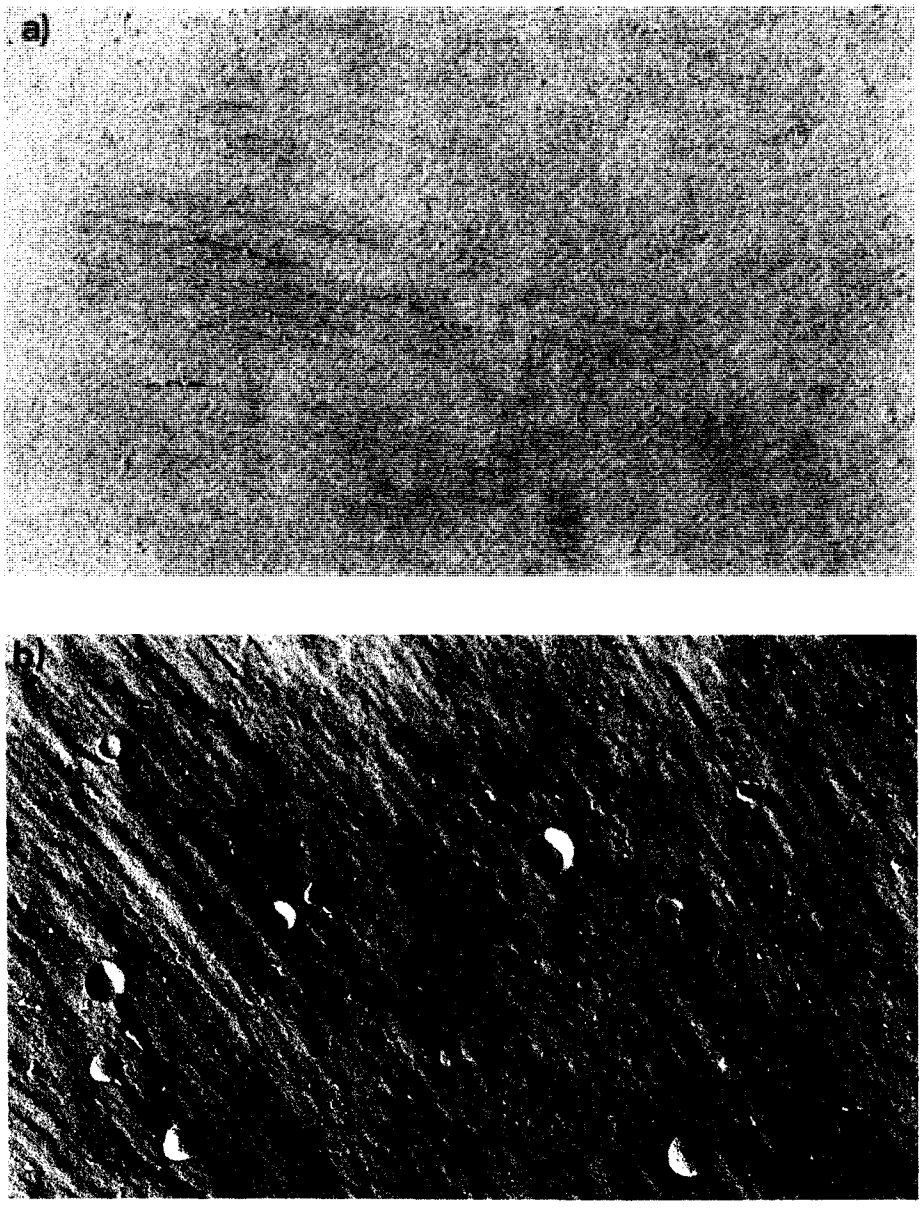

Fig. 4. Electron micrographs of efferent lymph plasma from the right popliteal lymph node collected before (a) and during the first 20 min after (b) i.d. administration of 3′,5′-*O*-dipalm-FUdR mixed micelles into the lower part of the right hind leg.

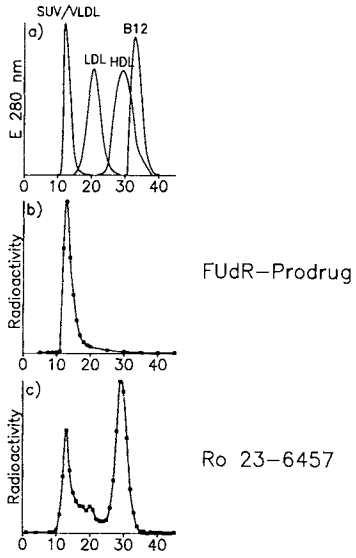

Fig. 5. Bio-Gel A-5m elution patterns of SUV (small unilamellar vesicles; mean diameter, 45 nm), VLDL, LDL, HDL, and B12 (a) and of efferent lymph from the right popliteal lymph node collected during the first 20 min after s.c. administration of 3′,5′-*O*-dipalm-FUdR mixed micelles (b) and Ro 23-6457 mixed micelles (c) into the lower part of the right hind leg.

3′,5′-*O*-dipalm-FUdR recovered in lymph was associated with the newly formed vesicles, whereas the retinoid Ro 23-6457 was associated with the vesicles (39.4 ± 11.9%) as well as bound to lymph proteins, mainly to HDL (31.5 ± 11.4%). No radioactivity was found in the elution volume available to small molecules.

DISCUSSION

The present study demonstrates that phosphatidylcholine–bile salt mixed micelles may be used to target low molecular weight drugs to the lymphatics draining the site of s.c. administration. However, efficient targeting of drugs to lymphoid tissue by means of mixed micelles is feasible only for highly lipophilic drugs. We found that when administered in mixed micelles, drugs with $R_m°$ values >10 are preferentially absorbed by the lymphatics (>50% of dose), whereas compounds with $R_m°$ values <4 were hardly absorbed at all by the lymphatics (<10% of dose). However, due to the lack of lymph recovery data for drugs with $R_m°$ values between 4 and 10 (we did not find an appropriate model drug), we cannot exclude the possibility that less lipophilic drugs ($R_m°$ values between 4 and 10) may also be preferentially delivered to the lymphatics by means of mixed micelles. By applying the prodrug principle, we also demonstrated that it is possible to target drugs with $R_m°$ values <4 to the lymphatics. Furthermore, our data show that mixed micelles are as efficient as liposomes in terms of their capacity to function as lymphotropic drug carriers. Compared with liposomes and other lymphotropic drug carriers such as emulsions, mixed micelles have the important advantage of being thermodynamically stable.

Another interesting result of the present study is the observation that following s.c. administration mixed micelles are converted into small unilamellar vesicles. This transition from mixed micelles to vesicles is a well known *in vitro* phenomenon. Several authors showed that vesicles can spontaneously form from phosphatidylcholine–bile salt mixed micelles upon dilution, dialysis, or gel filtration (25). To our knowledge the present investigation illustrates for the first time that this micelles-to-vesicle transition also occurs *in vivo* following s.c. administration of a mixed micellar drug solution, probably as a result of the combined effect of dilution and dialysis.

Our results suggest that mixed micelles may represent a suitable delivery system for drugs which stimulate, inhibit, or modulate the function of a distinct lymphoid-cell population. However, there are limitations to using mixed micelles or other lymphotropic drug carriers, in that targeting will be restricted to those lymphoid tissues which directly drain the site of application and high drug levels will not persist for long periods after a single administration. However, it may be possible to achieve more generalized effects. Lymphocytes recirculate continually from the blood to the lymph, and most of this migration occurs in lymph nodes (26,27). Therefore, by continuous local delivery of a drug to one or more lymph nodes, recirculating lymphocytes would be exposed periodically to high levels of the drug. By maintaining this special microenvironment over extended periods in areas of cell migration, it may become feasible to alter the immunological activity of more widely dispersed lymphoid tissues without having high levels of drug in the general circulation.

Another potential clinical application for lymphatic administration of drugs is the treatment of tumors which metastasize along the lymphatics (e.g., malignant melanoma, carcinomas of the lung, colon, breast, testis, prostate) (28). This could be done readily when the drainage site of the primary tumor can be reached by s.c. injection. Tumors localizing in internal organs would generally be inaccessible to therapy of this type. Endoscopic administration of a mixed micellar drug solution might overcome this problem in some cases, e.g., bronchoscopic application for access to lymph nodes draining the lung and mediastinum, the usual sites of initial metastasis from carcinoma of the lung (29).

It has been demonstrated that administration of antifungals (e.g., amphotericin B), antitumor drugs (e.g., doxorubicin, platinium complexes), and immunomodulators (e.g., muramyl dipeptide and lipophilic analogues, antigens) (for review see Ref. 30) in liposomes leads to a therapeutic advantage. Our results show that the thermodynamically stable mixed micelle might offer an alternative to liposomes. However, a prerequisite for a successful use of mixed micelles as proliposomal formulation is that the drug will remain associated with the *in vivo* formed vesicles, as in the case of 3′,5′-*O*-dipalm-FUdR.

In summary, the present study shows that phosphatidylcholine–bile salt mixed micelles might be used as a lymphotropic drug carrier or as a proliposomal formulation. For both applications a high drug lipophilicity is a prerequisite. Depending on the intended use of mixed micelles, the drug should be released from the *in vivo* formed vesicles within the lymphatic system or remain associated with them. It will be important to assess whether or not targeting drugs in this way has therapeutic advantages and studies aimed at evalu-

ating the biological activity of immunosupressive drugs delivered in mixed micelles are currently in progress.

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