The influence of repeated injections on pharmacokinetics and biodistribution of different types of sterically stabilized immunoliposomes

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Abstract

Sterically stabilized immunoliposomes (IL) with diameters of about 135 nm carrying mouse IgG, either coupled directly to the liposome surface, or linked to the terminal ends of grafted poly(ethylene glycol) (PEG) chains by a recently described conjugation procedure (Cyanur-PEG-PE), were intravenously injected into rats and the elimination kinetics and biodistribution were determined and compared with control liposomes. The amounts of conjugated antibodies were about 30 μg/μmol total lipid for all IL. In naive rats, plain pegylated liposomes displayed the longest blood circulation time, whereas the terminal-coupled IL exhibited the fastest elimination. Liposomes containing the underivatized anchor molecules circulate nearly as long as plain pegylated liposomes, indicating that the fast elimination of the IL can be attributed to the presence of antibodies.

A second injection of identical liposomes 14 days after the first injection had a considerable influence on the pharmacokinetic parameters of the liposomes. The circulation time of plain pegylated liposomes drastically dropped by half and their uptake by the liver increased concomitantly, indicating that the PEG, upon repeated injection, ceases to function as an efficient barrier reducing opsonization and/or immune reactions. The circulation time of conventional IL was moderately reduced upon a second injection, whereas that of the terminally coupled IL was nearly unaffected. These differences among the IL demonstrate that the pharmacokinetic behavior of IL is strongly dependent on the antibody conjugation site on the liposome. The observed effects of repeated injections were similar for liposomes of 90-nm diameter. The phenomena described may have important implications for the repeated application of IL as drug carriers.
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1. Introduction

Antibody-targeted liposomes, also referred to as immunoliposomes (IL), are regarded as promising targeting vehicles for systemic drug delivery in the treatment of several diseases such as cancer [1–3]. The basis of efficient liposomal drug targeting is a sufficiently long circulation lifetime to allow escape from the vascular bed. This can be achieved by sterically stabilizing unilamellar liposomes with surface-grafted poly(ethylene glycol) (PEG) chains [4,5]. Pegylated liposomes exhibit a dose-independent prolonged circulation in blood due to a reduced uptake by cells of the mononuclear phagocyte system. Thus, pegylated liposomes are able to accumulate in tumors or at sites of inflammation as a result of passive targeting [6,7].

For specific targeting purposes, the steric stabilization of liposomes by PEG can be combined effectively with antibody coupling [8,9]. Basically, two types of pegylated IL can be distinguished: (i) conventional IL with antibodies directly coupled onto the surface of PEG-containing liposomes [10,11] and, (ii) IL with antibodies coupled onto the terminal ends of surface-grafted PEG chains [12–14]. The latter formulations expose the antibodies in a better accessible

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Abbreviations: Chol, cholesterol; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; [3H]-COE, [3H]-cholesteryloleyl ether; IL, immunoliposomes; mAb, monoclonal antibody; N-Glut-PE, N-glutaryl-phosphatidylethanolamine; PBS, phosphate-buffered saline; PEG, poly(ethylene glycol); SPC, soy phosphatidyl choline; TL, total lipid

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and more flexible position, which was demonstrated to be advantageous for targeting purposes. For the preparation of PEG-terminally coupled IL, a variety of techniques are available, which differ in the chemistry of the coupling [12–16]. We recently introduced a conjugation technique, by which antibodies can be linked directly to a PEG-terminal cyanuric chloride (Cyanur-PEG-PE) without the need for prior antibody activation or derivatization [17].

The PEG-terminal IL have been optimized with respect to circulation half-life and targetability by varying liposome size, antibody coupling density as well as coupling technique [16]. However, even the most powerful liposomal drug formulation in many cases will require repeated administration. Consequently, it will not suffice to consider only the elimination kinetics of IL upon first injection. Pharmacokinetics of subsequently administered doses are at least equally important. For example, the use of species-incompatible antibodies or immunoconjugates might provoke an immune response of the organism, which in turn may strongly affect the pharmacokinetic profiles of subsequent doses.

A recent study demonstrated that after a single dose of plain pegylated liposomes, the pharmacokinetics of subsequent doses is substantially altered. The enhanced blood clearance of the second dose was attributed to immunogenic reactions evoked by the first injection, involving an unidentified soluble serum factor functioning as opsonin [18]. Several studies describe the adjuvant activity of liposomes in the immune response to incorporated or surface-attached protein antigens [19,20]. Despite this obvious immunogenic potency of IL, only a few studies recognize its significance. Phillips et al. [21] investigated the immunogenicity in mice of conventional pegylated IL with mouse IgG, surface attached via an avidin bridge. They detected a strong immunogenic reaction, which was even enhanced by the presence of PEG. In only one study PEG-terminal coupled IL were considered: Harding et al. [22] attached chimerized mouse IgG onto hydrazide-reactive PEG derivatives and administered these IL repeatedly to rats. The decrease in circulation half-life they observed correlated well with a significant increase in anti-IgG titers. Because the strong immune response toward the coupled IgG could be specified as Fc directed, the coupling of less immunogenic Fab fragments was suggested for future IL applications.

However, these studies neither allow to determine a correlation between structural parameters of IL and their immunogenicity nor do they provide a comparison between immunogenic potency of conventional versus terminally coupled IL.

The present study investigates the pharmacokinetic behavior of repeatedly injected conventional and terminally coupled IL in rats as an indicator for liposomal immunogenicity. Furthermore, we paid special attention to the biocompatibility of the recently described antibody coupling anchor Cyanur-PEG-PE.

We found that the presence of IgG is an important factor in the enhanced elimination of liposomes, even upon the first

administration. However, the extent by which circulation half-life at the second injection is reduced varies among the liposome preparations. Because the terminally coupled IL using Cyanur-PEG-PE retain a circulation time which is slightly higher than for conventional ones, they appear from this study as acceptable carriers for a liposomal drug therapy.

Obviously, IL cannot indiscriminately be considered as highly immunogenic; the liposomal structure and antibody coupling technology are important factors determining the extent to which liposomal pharmacokinetics are altered upon repeated administration.

2. Materials and methods

2.1. Materials

Soy phosphatidylcholine (SPC) was obtained from Lucas Meyer (Hamburg, Germany). Cholesterol (Chol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) were purchased from Sigma (Deisenhofen, Germany). Poly(ethylene glycol)-PE (PEG-DSPE 2000) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). [^{3}H]-cholesteryloleyl ether (^{3}H-COE) was obtained from Amersham (Buckinghamshire, UK). These substances were used without further purification. The membrane anchors for antibody coupling, Cyanur-PEG-PE and *N*-glutaryl-phosphatidylethanolamine (N-Glut-PE) as well as PEG-PE 3000, were synthesized as previously described [17]. All salts and buffers were of analytical grade.

The purified murine IgG (reagent grade) was purchased from Sigma.

2.2. Animals

About 10 weeks old specified pathogen-free male Wag/Rij rats (Harlan, Horst, The Netherlands) were kept under standard laboratory conditions with access to standard laboratory chow and water ad libitum.

2.3. Preparation of IL

Large unilamellar vesicles (LUV) were prepared by extruding multilamellar vesicles. For this purpose, a lipid film (10 μmol) was suspended in 1 ml aqueous solution (0.15 M HEPES or NaCl) at 60 °C. The resulting multilamellar vesicles were extruded (Extruder, Lipex Biomembrane, Vancouver, Canada) six times through a polycarbonate filter with a pore size of 100 nm, followed by extrusion through a filter with a pore size of 50 nm (Costar, Bodenheim, Germany). Vesicle size was determined by dynamic light scattering using a Malvern Autosizer II c (Malvern, UK) in mass distribution mode. The lipid composition of vesicles was adjusted according to the coupling procedure. The basic lipid composition was SPC/Chol 2:1 (molar ratio), to which either 5 mol% N-glut-PE or 5 mol% Cyanur-PEG-PE were added

to prepare IL. The addition of PEG-PE 2000 diminished the fraction of SPC. When required, liposomes were labeled with trace amounts of ^{3}H-COE (0.25 Ci/mol).

For all coupling procedures, an initial phospholipid/Ab molar ratio of 1000:1 was chosen, as previously optimized by Hansen et al. [13]. The coupling yield of all reactions was quantified with a protein determination assay according to Peterson [23] and correlated to the actual liposome concentration determined by phosphate assay [24].

Liposomes containing Cyanur-PEG-PE were prepared in 0.15 M NaCl. The indicated amounts of antibodies, which were dissolved in borate buffer (pH 8.8), were added to this preparation and incubated at room temperature for about 16 h. Unbound antibodies were separated by passing the liposomes over a Sepharose 4B (Sigma) column and eluting them with phosphate-buffered saline (PBS) pH 7.4.

To form a protein linkage to liposomal N-Glut-PE, 6 mg EDC was added to 10 μmol liposomes in HEPES (pH 7.4) followed by a 6-h incubation period at room temperature and by gel permeation chromatography (Sephadex G 50, Pharmacia, Sweden) to remove excess EDC. Antibodies were added and the mixture was incubated overnight at room temperature. Unbound antibodies were separated from the IL by gel permeation chromatography on Sepharose 4B.

2.4. Pharmacokinetics and tissue distribution studies

For determination of tissue distribution, 20 μmol total lipid (TL)/kg of ^{3}H-COE-labeled liposomes was injected into male Wag/Rij rats (210–250 g) via the penile vein under light diethyl ether anesthesia. At the indicated times, blood samples were taken from the tail vein. Twenty-four hours after injection of the liposomes, rats were anesthetized by intraperitoneal injection of 20–25 mg sodium pentobarbital and a blood sample was taken from the inferior vena cava. After a 3-min perfusion of the liver with PBS (37 °C) via the portal vein, liver, spleen, heart, lungs and kidneys were removed. The organs were processed for measurement of radioactivity as described before [25]. Briefly, the organs were homogenized using a Potter Elvehjem tube. Radioactivity of the homogenate (0.1–0.4 ml) was determined after adding 100 μl 10% sodium dodecyl sulfate and 4 ml scintillation liquid. Blood samples were allowed to clot for at least 3 h at 4 °C followed by centrifugation. The radioactivity measurements of the serum samples were used to calculate the total amount of radioactivity in serum according to the equation: serum volume (ml) $= [0.0219 \times$ body weight (g)] $+ 2.66$ [25]. Results of serum clearance and organ uptake of the liposomes were calculated as percentage of the injected dose.

The pharmacokinetic parameters were calculated by fitting the percentage of injected dose versus time for each rat taking a compartment free analysis using Topfit version 2.0 [26]. Because the elimination curves do not follow clear first-order kinetics, we took the time point at which 50% of the liposomes were eliminated from the blood from the elimination graphs to allow comparison between the different types of liposomes, instead of a calculated half-life.

2.5. Determination of anti-PEG-PE antibody titers in serum

The search for anti-PEG-PE antibodies in the 24-h serum samples was performed by a sandwich ELISA technique. The ELISA plates (Microlon, Greiner Labortechnik, Germany) were coated with PEG-PE 2000 as described before [27]. Coating was established by determining phospholipid phosphorus [24] after solubilization of the coating using Triton X-100.

Initial serum dilution was 1:5 in PBS. Coated plates were incubated with serum samples for 90 min at 37 °C. After washing, peroxidase-labeled rabbit (anti-rat IgG) was used to assay for bound rat antibodies.

3. Results and discussion

3.1. Preparation and characterization of IL

Conventional IL with antibodies directly coupled onto the liposomal surface were constructed using N-Glut-PE as an anchor, to which antibodies can be attached after carbodiimide activation via an amide bond, as previously described [17,28]. Five mole percent of PEG-PE 2000 was included in the lipid mixture to obtain liposomes with long circulation characteristics.

Terminally PEG-coupled IL were prepared by using Cyanur-PEG-PE as a membrane anchor [17,29]. To obtain comparable steric stabilization of all IL, either 5 mol% of Cyanur-PEG-PE anchor ("Cyanur 5") or 3 mol% Cyanur-PEG-PE plus 2 mol% PEG-PE 2000 ("Cyanur 3") was incorporated.

To avoid immune-specific biodistribution of the IL, we used an irrelevant, highly purified mouse IgG for coupling to the liposomes. The coupling reactions did not lead to a significant increase in particle size (Table 1).

Table 1
Structural parameters of the administered immunoliposomes

Sample	Lipid composition (molar ratio)	Particle size (nm)	Coupled antibodies [μg/μmol TL]	Coupled antibodies per liposome
N-Glut-PE	SPC/Chol/N-Glut-PE/PEG-PE 2000 (6:3:0.5:0.5)	142 ± 6	24 ± 2.8	30
Cyanur 5	SPC/Chol/Cyanur-PEG-PE (6.5:3:0.5)	138 ± 8	30 ± 4.1	38
Cyanur 3	SPC/Chol/Cyanur-PEG-PE/PEG-PE 2000 (6.5:3:0.3:0.2)	136 ± 8	26 ± 4.0	32
Control	SPC/Chol/PEG-PE 2000 (6.5:3:0.5)	131 ± 7	–	–

Data are means ($n > 5$) ± S.D.

The amounts of coupled antibodies of about 30 μg/μmol TL used in this study have been shown to be an optimal compromise between targetability and long circulation of IL [30,31].

3.2. Single-dose pharmacokinetics of IL

The blood clearance profiles in naive rats in Fig. 1 demonstrate that all preparations display prolonged blood retention with some minor differences between the preparations. Control liposomes, without coupled antibodies, display the longest circulation time. In contrast to previous reports, the preparations do not show ideal first-order elimination kinetics. They tend to show biphasic kinetics with a fast initial elimination during the first 4 h followed by slower second phase. We tentatively attribute these differences to the lipid composition used in this study and to the size of our liposome preparations. We used unsaturated soy PC as the main phospholipid, which tends to produce less stable liposomes upon contact with serum (data not shown) than more rigid saturated phospholipid used by others [18,22]. When ignoring the biphasic profile and approximating it as log-linear, we can calculate a half-life for the control of about 13 h, which is in agreement with several studies on elimination kinetics of pegylated liposomes [4,16]. The slightly shorter circulation time than that reported by Harding et al. [22] or Koning et al. [32] might also be attributed to the larger particle size in our study. It is evident that the surface-bound antibodies enhance the blood clearance, because all these IL types display shorter half-lifes than the control. The pharmacokinetic parameters obtained from a log-linear approximation for all preparations are summarized in Table 2.

From Table 2, it is also apparent that there is a difference between conventional and terminally coupled IL. The terminally coupled IL Cyanur 3 and 5 were cleared faster than the N-Glut-PE IL, which may be attributed to a better accessibility of the antibody positioned at the PEG terminus. Liposomes containing Cyanur-PEG-PE without coupled antibodies circulate slightly longer than the antibody-coupled liposomes. The accumulation of Cyanur 3 and 5 IL in the liver is higher than that of the other liposomes (Table 2). The uptake by spleen does not correlate with the circulation time. Uptake in lungs, kidney and heart was always below 1% of injected dose (not listed).

3.3. Pharmacokinetics after repeated administration

Most therapeutic applications of liposomes will require repeated injections. This may lead to immunological reactions, especially when species-incompatible antibody targeting is applied, resulting in altered pharmacokinetics. To evaluate such complications, we analyzed the pharmacokinetic behavior of the liposomes upon repeated injection. Following a first injection with non-labeled liposomes, rats were reinjected after 14 days with liposomes of the same composition, containing a trace amount of ^{3}H-COE as a marker (Fig. 2 and Table 3). It was expected, based on previous studies, that any change in pharmacokinetic profile may require 1–3 weeks to develop [18].

Compared to the elimination kinetics of the first dose, the circulation time of the second dose of plain control lip-
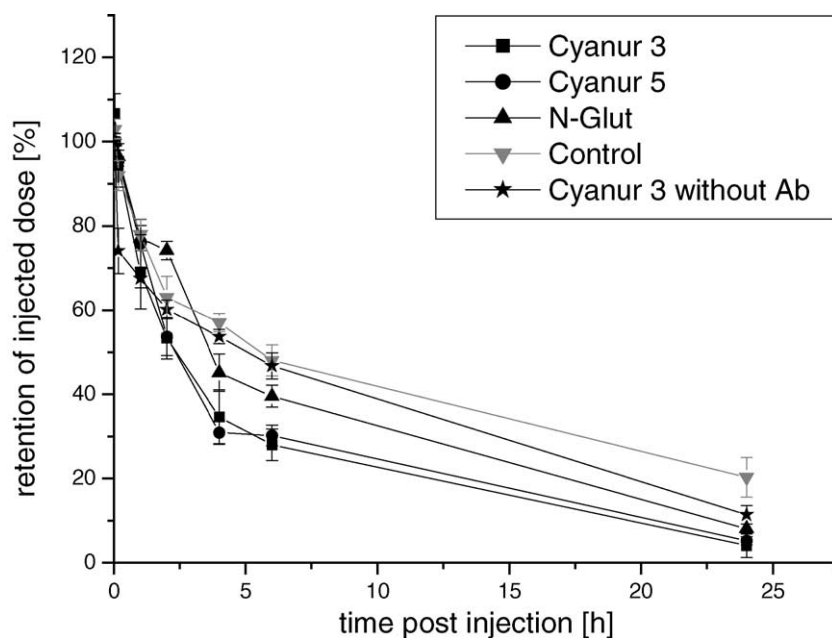

Fig. 1. Pharmacokinetic profiles of plain and antibody-coupled liposomes containing a ^{3}H-COE label in naive rats. Data represent the mean of three experiments ± S.D.

Table 2
Pharmacokinetic parameters and organ distribution of different liposomal preparations labeled with ^{3}H-COE 24 h after single injection into rats

Sample	50% Value [h][a]	AUC [%h/%] ± S.D.	Liver uptake [%] ± S.D.	Spleen uptake [%] ± S.D.
Cyanur 3	2.5	605 ± 96	49.6 ± 3.0	3.5 ± 0.03
Cyanur 5	2.5	658 ± 84	52.3 ± 4.1	8.1 ± 1.4
N-Glut	3.7	920 ± 73	30.8 ± 1.8	5.9 ± 0.9
Control	5.9	1364 ± 163	39.0 ± 2.8	6.4 ± 1.8
Cyanur 3 without Ab	5.2	917 ± 89	33.8 ± 3.0	6.0 ± 1.1

Data represent the means of three independent experiments.

[a] Time at which 50% of the liposomes have been removed from the blood; data obtained from the elimination curves as described in the Materials and methods section.

Table 3
Pharmacokinetic parameters and organ distribution of different liposomal preparations labeled with ^{3}H-COE 24 h after a second administration into rats 14 days following a first injection with the same preparation

Sample	50% Value [h][a]	AUC [%h/%] ± S.D.	Liver uptake [%] ± S.D.	Spleen uptake [%] ± S.D.
Cyanur 3	3.0	715 ± 62	53.0 ± 2.1	6.5 ± 0.5
Cyanur 5	1.3	378 ± 112	63.3 ± 4.2	10.9 ± 1.6
N-Glut	1.4	455 ± 63	60.7 ± 3.8	7.4 ± 1.2
Control	0.8	242 ± 39	60.6 ± 4.4	6.4 ± 1.7
Cyanur 3 without Ab	3.6	828 ± 89	35.1 ± 2.1	5.8 ± 0.7
Control 3000	2.5	315 ± 35	62.3 ± 1.8	6.0 ± 0.9

Data represent the means of three independent experiments.

[a] Time at which 50% of the liposomes have been removed from the blood; data obtained from the elimination curves as described in the Materials and methods section.

osomes was strongly diminished. This confirms similar observations by Dams et al. [18], who interpreted this as the result of a reaction of the immune system toward the first dose, leading to opsonization of the second dose by a soluble serum factor. Consequently, despite their prolonged circulation time in naive organisms, normal pegylated liposomes should not be regarded as non-immunogenic.

In view of the behavior of the plain liposomes, we expected an even faster clearance of a second dose of IL as a result of the immunogenic effect of the coupled antibodies. Surprisingly, however, the second dose of IL was not eliminated faster than the second dose of plain control liposomes (Fig. 2). In fact, cyanur IL tend to display even longer circulation times.

The moderate change in elimination rate of the IL suggests that the coupled antibodies of murine origin are either not strongly immunogenic or they are not readily processed

by cells of the immune system due to the presence of the PEG chains. The shorter circulation time of a second dose of the N-Glut-PE liposomes, as compared to that of both terminally coupled IL with better antibody exposition, does not support the latter explanation.

The data for the liver uptake are generally in accordance with the serum elimination values. Whereas the liver uptake of the Cyanur 3 or 5 IL only moderately increased by about 10–20% compared to the first dose, the liver uptake of the N-Glut IL and of the controls was drastically increased (Table 3).

Because the coupled murine antibodies seem to contribute little to the altered liposome pharmacokinetics after repeated injection, we further investigated the role of the PEG. Our results showed that especially the clearance of the liposomes
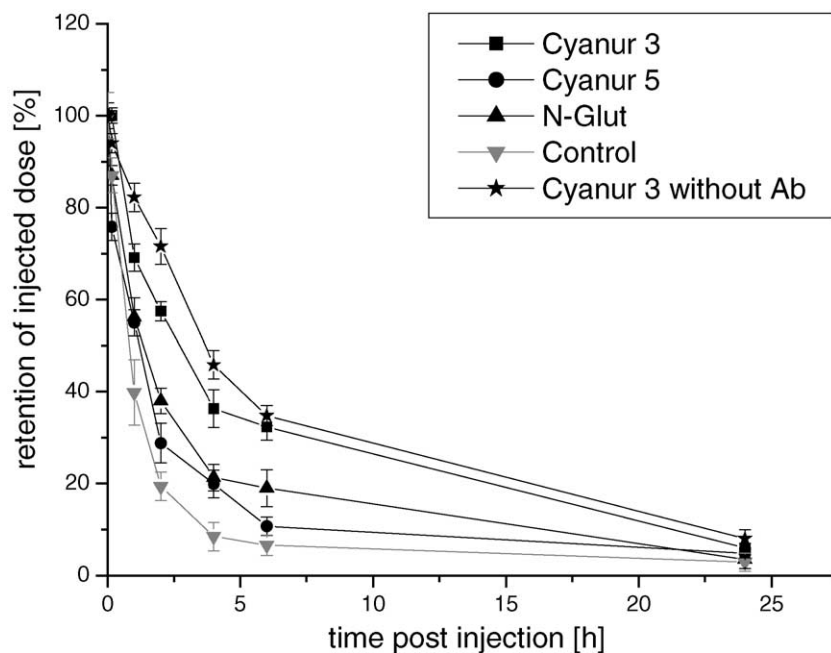

Fig. 2. Blood clearance curves obtained following a second injection of plain liposomes or IL 14 days after first injection in rats. Data are means of at least three experiments ± S.D.

containing PEG-PE 2000 (control and N-Glut-PE) is enhanced upon second injection, but not that of the Cyanur-PEG-PE IL or the Cyanur liposomes without antibodies. This might imply an immunogenic role of PEG-PE as already described by Phillips et al. [21]. Because liposome-induced anti-phospholipid antibodies are known to have a significant effect on liposome circulation as an intrinsic adjuvant factor [33], we searched for anti-PEG-PE antibodies in the 24-h serum samples. However, no evidence was found for the presence of anti-PEG-PE antibodies in the different serum samples.

Another difference between the IL is the molecular weight of the PEG derivatives. In contrast to the commonly used PEG-PE 2000, Cyanur-PEG-PE contains a PEG chain of a molecular weight of about 3000. Dams et al. [18] interpreted the strong increase in elimination of liposomes containing PEG-PE 2000 as the result of opsonization by a serum component. It is conceivable that the production of such a factor is modulated by the PEG chain length. Therefore, we prepared control liposomes containing 5 mol% PEG-PE 3000. In addition, the second dose of these PEG 3000 liposomes shows a reduced circulation time (Fig. 3) similar to the PEG 2000 control liposomes. Therefore, there is no indication that the higher molecular weight of PEG is responsible for the pharmacokinetic differences between Cyanur 3 or 5 and the other preparations. Furthermore, because the Cyanur liposomes without coupled antibodies circulate much longer (Fig. 2) than control PEG-3000 liposomes, which differ from the former only in the terminal cyanuric chloride moiety, this emphasizes a potential role of the PEG terminus in pharmacokinetic behavior.

These data indicate that, although elimination of pegylated liposomes from the circulation is enhanced upon repeated injection, possibly as a result of an immunogenic response, the pharmacokinetic behavior of IL is also strongly dependent on structural parameters, which cannot be generalized easily. A second dose of our terminally PEG-coupled IL was eliminated with a calculated half-life of more than 6 h, whereas the hydrazide-PEG-coupled IL used by Harding et al. [22] displayed a half-life of only 0.5 h after the second injection. They found a much stronger immunogenicity against coupled whole antibodies compared to coupled Fab fragments, leading them to recommend the latter for use as homing devices to minimize immune reactions. Because we used whole murine IgGs, versus antibodies of human origin in the study of Harding et al., and because we did not test for the presence of antibodies against IL in our study, we cannot directly compare the two studies.

However, considering the pharmacokinetics of plain and IL in our study, the antibodies display obviously a much lower influence on pharmacokinetics. Consequently, the strong immunogenic activity of the IL in the study of Harding et al. may be related to liposomal characteristics, such as the hydrazide coupling method they used. It seems less likely that animal differences (Sprague–Dawley rats versus Wag/Rij rats in our study) caused the difference with our observations. In any case, the differences between the results in these two studies underscore the influence of liposomal characteristics and antibody conjugation techniques on the pharmacokinetics of follow-up doses of plain as well as IL.

It is well recognized that liposomal size has strong implications on the pharmacokinetics. The liposomes used in the
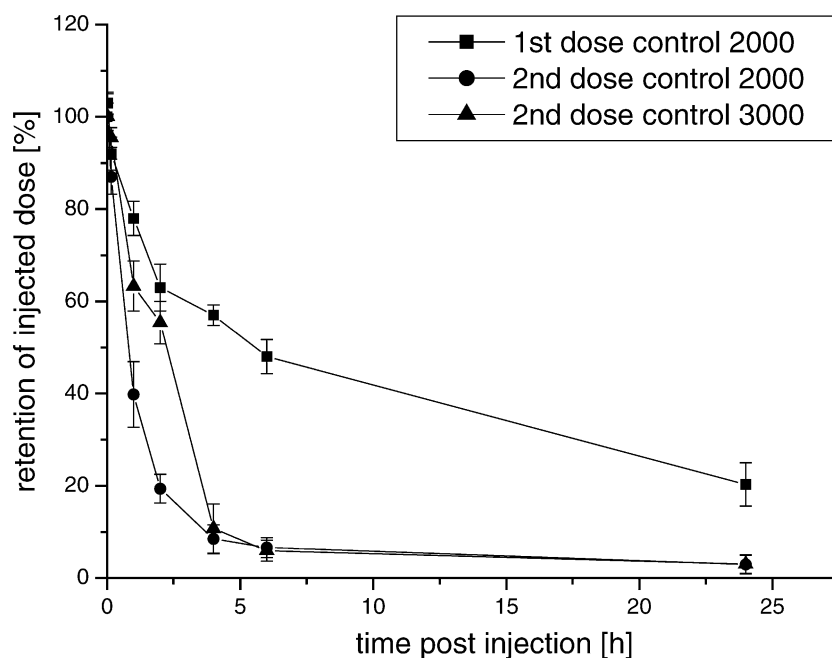

Fig. 3. Comparison of the pharmacokinetic profiles of plain liposomes containing either PEG-PE 2000 or PEG-PE 3000. Data are means of three experiments ± S.D.

Table 4
The influence of reducing the particle size to about 90 nm on the pharmacokinetic parameters for a second injection into rats, 14 days following a first injection with the similar probe

Sample	50% Value [h][a]	AUC [%h/%] ± S.D.	Liver uptake [%] ± S.D.	Spleen uptake [%] ± S.D.
Cyanur 3	10.9	1447 ± 89	15.3 ± 1.6	3.1 ± 0.8
Cyanur 5	15.2	1490 ± 103	16.3 ± 2.1	2.0 ± 0.6
N-Glut	3.6	763 ± 82	41.7 ± 4.6	3.8 ± 0.5
Control	3.1	690 ± 97	42.0 ± 4.0	1.5 ± 0.7

Organ distribution was determined 24 h after injection, serum disappearance was determined during this time. Data represent the means of three independent experiments.

[a] Time at which 50% of the liposomes have been removed from the blood; data obtained from the elimination curves as described in the Materials and methods section.

present study were around 135 nm in diameter and thus slightly larger than those in other pharmacokinetic studies on IL [18,31]. Therefore, we also performed experiments on the effect of repeated injection using liposomes of the same composition but with a size of around 90 nm. The antibody density on the smaller liposomes of about 18 IgG per liposome is identical to that on the liposomes described by Harding et al. [22]. The pharmacokinetic data in Table 4 demonstrate that a decrease in particle size leads to an increase in circulation time of the Cyanur IL. Furthermore, these data confirm and emphasize the differences between terminal-coupled and conventional IL, which are even more conspicuous for the 90-nm liposomes than for the larger liposomes in Table 3. Cyanur 3 and 5 display a very long circulation time, whereas N-Glut IL and control liposomes behave similar to the larger liposomes with relatively short half-lifes. Concomitantly, the liver uptake of the latter two preparations was higher than that of the Cyanur preparations.

4. Conclusions

We investigated the influence of repeated injections of liposomes on liposome pharmacokinetics, an aspect of the therapeutic use of (immuno)liposomes that has attracted little attention up to now.

Two types of IL were prepared which were identical with respect to antibody density and orientation; one in which the antibody was coupled directly to the bilayer lipids and another in which it was coupled to the terminal end of a bilayer-anchored PEG chain. This allowed us to correlate differences in pharmacokinetics with structural liposome parameters such as antibody accessibility or steric stabilization.

The elimination kinetics in naive rats demonstrates that coupled antibodies strongly increase the elimination rate, and because the Cyanur-PEG-PE IL display the shortest half-life, elimination may directly be correlated with better antibody exposure at the liposomal surface.

However, after a second injection, elimination kinetics no longer obeys this simple rule. A second dose of plain pegy-

lated liposomes was rapidly eliminated. Obviously, the steric barrier of the liposomes by PEG-PE seems to be less efficient in reducing opsonization and immune reactions. However, an active role of PEG-PE in eliciting specific antibodies could not be established. These data could have implications for the therapeutic application of pegylated liposomes as drug delivery systems.

The coupled antibodies seem to play a less important role in increasing the elimination than is often assumed, because IL were eliminated not faster than control liposomes. This study for the first time directly compares the behavior of different types of IL upon repeated injections and demonstrates a relatively low immunogenicity of our Cyanur-PEG-PE IL. Although we do not fully understand this phenomenon at this point, it is obvious that the applied antibody coupling method is an important parameter in the repeated-injection pharmacokinetics. These experiments confirm the biocompatibility of the Cyanur-PEG-PE IL, whereas their relatively long circulation time upon repeated injections demonstrates their potential as therapeutic drug delivery vehicles.

Further experiments, which should consider changes in the application intervals, modifications in the coupled antibodies or the use of Fab fragments could shed more light on this behavior.

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