Pharmacokinetics of Differently Designed Immunoliposome Formulations in Rats with or without Hepatic Colon Cancer Metastases

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Purpose. Compare pharmacokinetics of tumor-directed immunoliposomes in healthy and tumor-bearing rats (hepatic colon cancer metastases).

Methods. A tumor cell-specific monoclonal antibody was attached to polyethyleneglycol-stabilized liposomes, either in a random orientation via a lipid anchor (MPB-PEG-liposomes) or uniformly oriented at the distal end of the PEG chains (Hz-PEG-liposomes). Pharmacokinetics and tissue distribution were determined using [^{3}H]-cholesteryloleylether or bilayer-anchored 5-fluoro[^{3}H]deoxyuridine-dipalmitate ([^{3}H]FUdR-dP) as a marker.

Results. In healthy animals clearance of PEG-(immuno)liposomes was almost log-linear and only slightly affected by antibody attachment; in tumor-bearing animals all liposomes displayed biphasic clearance. In normal and tumor animals blood elimination increased with increasing antibody density; particularly for the Hz-PEG-liposomes, and was accompanied by increased hepatic uptake, probably due to increased numbers of macrophages induced by tumor growth. The presence of antibodies on the liposomes enhanced tumor accumulation: uptake per gram tumor tissue (2–4% of dose) was similar to that of liver. Remarkably, this applied to tumor-specific and irrelevant antibody. Increased immunoliposome uptake by trypsin-treated Kupffer cells implicated involvement of high-affinity Fc-receptors on activated macrophages.

Conclusions. Tumor growth and immunoliposome characteristics (antibody density and orientation) determine immunoliposome pharmacokinetics. Although with a long-circulating immunoliposome formulation, efficiently retaining the prodrug FUdR-dP, we achieved enhanced uptake by hepatic metastases, this was probably not mediated by specific interaction with the tumor cells, but rather by tumor-associated macrophages.

KEY WORDS: immunoliposomes; pharmacokinetics; colon cancer; liver metastases; tumor targeting; 5-fluorodeoxyuridine.

INTRODUCTION

Specific targeting of liposomes to tumor cells may be achieved by attaching tumor cell specific antibodies to long-circulating liposomes (immunoliposomes) (1–4). It has been shown that antibody-mediated target recognition by liposomes can be combined with long-circulating properties, provided that density, position, and orientation of the antibody molecules on the liposomes are chosen properly (2). Even then, however, increase in clearance occurs, not only for immunoliposomes (5,6) but also for liposomes targeted via peptides as cell-specific ligands (7). Clearance appeared to increase with increasing antibody densities, due to higher uptake in liver and spleen (5,6). This uptake is likely to be mediated by macrophages residing in these organs as we recently demonstrated for liver macrophages (Kupffer cells) exposed to PEG-immunoliposomes (8,9).

To establish optimal targeting conditions, *i.e.,* long circulation time combined with good target recognition, the antibody density needs to be below approximately 40 μg antibody per μmol total lipid (2) with the antibodies attached at the terminal end of the PEG-chains and in a correct orientation, *i.e.,* with the antigen-binding moieties exposed.

Fc-receptors on macrophages were shown to play an important role in uptake of nonpegylated immunoliposomes (10) as well as of PEG-immunoliposomes (8,9). By attaching the antibodies to the liposomes via their Fc-portion, Fc-mediated Kupffer cell uptake was reduced (8). However, with these liposomes, containing partially oxidized antibody at the PEG-terminus, scavenger receptors were implicated in Kupffer cell uptake (G.A. Koning et al., unpublished observations), indicating that the coupling chemistry may also be an important factor influencing immunoliposome pharmacokinetics and biodistribution.

Prevention of recognition of the Fc moiety may also be achieved by coupling Fab′ or F(ab′)$_2$ fragments to liposomes (1,11). Coupling of Fab′ to the distal end of liposomal PEG did not seem to affect liposome pharmacokinetics, although still considerable amounts of liposomes were retrieved in the liver (1,11). Moreover, immunoliposomes with antibody fragments attached were reported to be more immunogenic than liposomes carrying intact antibodies; this effect was even further enhanced by the presence of PEG on the liposome surface (12).

Tumor development may also affect immunoliposome pharmacokinetics (2,13), either by increasing the uptake at the tumor site or by causing changes in the immune status, such as increasing the numbers of macrophages. We address the pharmacokinetic behavior of immunoliposomes in a rat model of metastatic tumor growth in the liver using syngeneic CC531 colon carcinoma cells (14,15). This model was shown to increase both the number and the activity of liver macrophages (15). We characterized the *in vivo* behavior of two

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ABBREVIATIONS: AUC, area under the % of injected dose vs. time curve; CC52, rat colon carcinoma CC531-specific antibody; CC531, rat colon carcinoma cell; chol, cholesterol; FCS, fetal calf serum; FUdR-dP, 5-fluorodeoxyuridine-dipalmitate; ^{3}H-COE, [^{3}H-]-cholesteryloleylether; Hz-PEG-DSPE, hydrazide-PEG-distearoyl-phosphatidylethanolamine; PEG, poly(ethyleneglycol); MPB-PE, maleimido-4-(*p*-phenylbutyryl)phosphatidylethanolamine; PC, phosphatidylcholine; t½, circulation half life; TL, total lipid (phospholipids and cholesterol).

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types of immunoliposomes in rats with and without CC531 liver metastases. The CC531-specific monoclonal antibody CC52 (16) or a control irrelevant antibody was attached to PEG-liposomes. Attachment was either after random thiolation of the antibody, to MPB-PE incorporated in the liposomal bilayer (3,17), or after oxidation of its Fc-moiety, to the PEG-terminus via Hz-PEG-DSPE (5,17,18). We also assessed the *in vivo* fate of 5-fluorodeoxyuridine-dipalmitate (FUdR-dP), a lipophilic derivative of the anti-cancer drug FUdR (3,17), following its incorporation into these immunoliposomes. Immunoliposomes containing this prodrug proved to be much more potent toward CC531 colon cancer cells *in vitro* than FUdR-dP in control liposomes without the specific antibody (3). We demonstrated previously that this drug is efficiently delivered intracellularly into CC531 cells, most likely via a selective transfer of the amphiphilic prodrug from the liposomes to the tumor cell membrane (17). FUdR-dP incorporated in nontargeted, non-long-circulating (conventional) liposomes showed rather high toxicity (19). By contrast, incorporation of this prodrug in targeted long-circulating liposomes resulted in improved therapeutic activity and less toxicity in an animal model of lung cancer compared to conventional liposomes (20).

Our ultimate goal is to develop a tumor targeted liposomal carrier with long-circulating properties for specific delivery of FUdR-dP to liver metastases of colon tumors.

MATERIALS AND METHODS

Materials

N-succinimidyl-S-acetylthioacetate (SATA) sodium periodate, *N*-acetylmethionine, and cholesterol (chol) were obtained from Sigma (St. Louis, MO). Egg yolk phosphatidylcholine (PC), maleimido-4-(*p*-phenylbutyryl)phosphatidylethanolamine (MPB-PE), and poly(ethyleneglycol)$_{2000}$-distearoylphosphatidylethanolamine (PEG$_{2000}$-DSPE) were purchased from Avanti Polar Lipids (Alabaster, AL). Hydrazide-PEG-DSPE (Hz-PEG-DSPE) was synthesized as described previously (18). [^{3}H-]cholesteryloleylether was obtained from Amersham (Buckinghamshire, UK). Sephadex G-50 and Sepharose CL-4B were from Pharmacia (Uppsala, Sweden). All other chemicals were analytical grade or the best grade available. Polycarbonate filters for liposome extrusion were from Costar (Cambridge, MA).

Animals

Male Wag/Rij rats (200–220 g) were kept under clean conventional conditions and had access to food and water *ad libitum.*

Monoclonal Antibody

The monoclonal antibody CC52 (murine IgG$_1$), recognizing a surface antigen on CC531 colon adenocarcinoma cells (16), was purified from culture supernatant by protein A-Sepharose (Pharmacia, Woerden, The Netherlands) chromatography, according to the manufacturer's instructions. A murine IgG$_1$ monoclonal antibody against human B-cells, used as an irrelevant control antibody, was a gift from Dr. C. Thomas of our department.

Liposomes

Liposomes were composed of PC/Chol/PEG$_{2000}$-DSPE (23:16:1.6 molar ratio). Liposomes for antibody coupling contained in addition 0.025 mol MPB-PE or Hz-PEG-DSPE per mol total lipid (TL) (cholesterol and phospholipid). For preparation of liposomes containing Hz-PEG-DSPE an additional 0.015 mol PEG$_{2000}$-DSPE was added per mol of lipid to obtain the same total amount of PEG-DSPE (4 mol%). When required, liposomes were labeled with trace amounts of [^{3}H]-cholesteryloleylether (^{3}H-COE) (0.25 Ci/mol TL). Lipids dissolved in chloroform/methanol (9:1), were mixed and dried under reduced nitrogen pressure, dissolved in cyclohexane, and lyophilized. The lipids were hydrated in HN-buffer (10 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid [Hepes]), 135 mM NaCl), pH 7.4, for coupling of antibodies to MPB-PE containing liposomes in HN-buffer, pH 6.7. For coupling of antibodies to Hz-PEG-DSPE liposomes NaAc-buffer, pH 5.5 (100 mM sodiumacetate, 70 mM NaCl) was used. Liposomes were sized by repeated extrusion through filters with a pore size of 50 nm using a high-pressure extruder (Lipex, Vancouver, Canada). Phospholipid phosphorus of each liposome preparation was determined by a phosphate assay after perchloric acid destruction (21). Total liposomal lipid (TL) concentrations were calculated, taking into account the amount of cholesterol in the liposome preparations. Particle size and size distribution were determined by dynamic laser light scattering with a Nicomp model 370 submicron particle analyzer (Nicomp, Santa Barbara, CA). The diameter of the liposomes was obtained from the volume distribution curves produced by the particle analyzer. Liposome diameter after extrusion was 74 ± 8 nm for MPB-PEG-liposomes and 77 ± 10 nm for Hz-PEG-liposomes.

Coupling of Antibodies to Liposomes

The monoclonal antibody CC52 and the irrelevant mouse antibody mIgG1 were coupled to liposomes containing MPB-PE by a sulfhydryl-maleimide coupling method as described previously (3,17). The monoclonal antibody CC52 was coupled to liposomes containing Hz-PEG-DSPE via a hydrazone-linkage between a hydrazide moiety at the distal end of the PEG-chains and oxidized carbohydrates in the Fc-region of the antibody as described previously (5,17). Immunoliposomes were characterized by determining protein content (22), phospholipid phosphorus, and particle size. To MPB-PEG-liposomes 41.4 ± 7.3 μg CC52 and 69.0 ± 14.8 μg mIgG$_1$ per μmol total lipid (TL) were coupled, equaling an average of 35 and 58 antibody molecules per liposome, respectively. To Hz-PEG-liposomes 14.4 ± 0.6 (low density) or 44 μg (high density) of CC52 and 5.3 ± 0.3 μg mIgG$_1$ per μmol TL were coupled, equaling 12, 37, and 4 antibody molecules per liposome. Numbers of antibody molecules per liposome were calculated assuming unilamellar liposomes with a diameter of 100 nm with an average surface of the lipid headgroups (phospholipid and cholesterol) of 50 Å^2 to which IgG molecules are attached with a molecular weight of 150,000.

Coupling of the antibodies resulted in a size increase of 10–20 nm for MPB-PEG-liposomes and 10–40 nm for Hz-PEG-immunoliposomes. No aggregate formation was observed. Liposomes were stored at 4°C under argon and used within 2 weeks after preparation.

Cell Culture

CC531 colon adenocarcinoma is a 1,2-dimethylhydrazine-induced carcinoma of the colon of WAG/Rij-rats (14). Cells were maintained at 37°C in a humidified atmosphere consisting of 5% CO_2 in air in 75 cm^2 culture flasks (Costar, Cambridge, MA). The medium used was RPMI 1640 medium with 25 mM Hepes (Gibco BRL, Breda, The Netherlands) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Gibco), fresh L-glutamine (2 mM), and penicillin/streptomycin (100 U/ml and 100 μg/ml, respectively) (Gibco). Cells were subcultured at 80% confluency.

Induction of Liver Metastases

Liver metastases were induced by inoculation of 5×10^5 CC531 colon adenocarcinoma cells into the portal vein of male WAG/Rij rats. CC531 cells were harvested by trypsinization (0.05% trypsin, 0.5 mM EDTA, and 200 μg DNase), washed three times with phosphate buffered saline (PBS) and resuspended in Hank's balanced salt solution at the desired concentration. Viability, as determined with the trypan-blue exclusion method, was >90%. Male WAG/Rij rats were anesthetized, underwent surgery, and tumor cells were inoculated into the portal vein as described before (15). Twenty-two days after inoculation of CC531 cells, liver metastases were macroscopically visible under the liver capsule as pale nodules. At that time all livers had metastases, although the number of visible nodules varied from one to 20 in the different livers. Metastases were never observed at the site of injection or in other organs.

Pharmacokinetics and Tissue Distribution Studies

For determination of tissue distribution, 10 μmol TL/kg of ^{3}H-COE-labeled liposomes was injected into male Wag/Rij rats (200–250 g) via the penile vein under light diethylether anesthesia. At the indicated times, blood samples were taken from the tail vein. The rat was anaesthetized by intraperitoneal injection of 20–25 mg sodium pentobarbital 24 h after injection of the liposomes and a blood sample was taken from the inferior vena cava. After a 5-min perfusion of the liver with PBS via the portal vein, liver and spleen were removed, the tumor nodules were carefully excised from the liver tissue and all tissues were processed for measurement of radioactivity as described before (23). Liver, spleen, and tumor tissue were weighed and subsequently homogenized using a Potter Elvehjem tube. Radioactivity was determined after solubilization of 0.4 ml of the homogenate in 100 μl 10% SDS and 4 ml scintillation liquid. Blood samples were allowed to clot for at least 1 h at 4°C; samples were then centrifuged and serum samples were taken for radioactivity measurement. The total amount of radioactivity in the serum was calculated using the equation: serum volume (ml) = [0.0219 × body weight (g)] + 2.66 (23). Results of serum clearance and organ uptake of the liposomes were calculated as a percentage of the injected dose. The pharmacokinetic parameters, area under percentage of injected dose vs. time curve (AUC_{0-24h}), and the circulation half lives ($t_{½}$), were calculated by fitting the percentage of injected dose vs. time data to a polyexponential curve using Kinfit from the MW/Pharm 3.02 software package from Medi/Ware developed in the department of Pharmacy, University of Groningen, The Netherlands.

Kupffer Cell Isolation, Culture, and Incubation with Liposomes

Kupffer cells were isolated after pronase digestion of the liver and purified by centrifugal elutriation as described before (9,10). Isolated Kupffer cells were resuspended in RPMI 1640 medium with 25 mM Hepes supplemented with 20% FCS, fresh L-glutamine (2 mM), and penicillin/streptomycin (100 U/ml and 100 μg/ml, respectively). In a humidified atmosphere containing 5% CO_2 in air, 5×10^5 cells/well were plated in 24-wells plates and maintained at 37°C. After 1 day the medium was replaced with the same culture medium containing 10% FCS. Cells were used in experiments 48 h after isolation. One hour before incubating the cells with liposomes, serum-containing medium was removed and replaced by serum-free medium. Incubations in the presence of proteolytic enzymes, which have been described to induce high affinity Fc-receptors on macrophages and monocytes (24,25) were performed to assess the effect of these receptors on uptake of immunoliposomes. Kupffer cells, except in control incubations, were treated with the proteolytic enzymes trypsin (0.5 mg/ml) or pronase (0.1 mg/ml) for 30 min at 37°C. Subsequently, cells were washed with PBS and incubated with 200 nmol ^{3}H-COE-labeled CC52-liposomes for 3 h in the absence of FCS. Incubations were stopped by removing the incubation medium followed by thorough washing of the cells with ice-cold PBS. Cells were lysed using 0.4 M NaOH. Cell-associated radioactivity was measured by liquid scintillation counting of aliquots of the lysed cell suspension and was normalized for the amount of cellular protein as determined by protein assay (26) using bovine serum albumin as a standard. Association of liposomes was calculated as nmol TL per mg cell protein and expressed as a percentage of control incubations without proteolytic enzymes.

Statistics

Statistical significance of differences was evaluated by a two-tailed unpaired Student's *t*-test.

RESULTS

Pharmacokinetic Behavior of PEG-Immunoliposomes in Healthy Rats

The pharmacokinetics of PEG-immunoliposomes and PEG-liposomes (without antibody) were examined in healthy rats (Fig. 1 and Table I). Liposomes were labeled either with ^{3}H-COE (Fig. 1a), a nondegradable bilayer marker (17), or with ^{3}H-FUdR-dP (Fig. 1b), a lipophilic prodrug of the anticancer drug FUdR. FUdR-dP remains strongly associated with the liposomal bilayer during circulation, but after uptake by cells it is degraded into ^{3}H-FUdR, released from the cells and rapidly excreted by liver and kidney (19,27). ^{3}H-FUdR-dP therefore represents a good marker for determining the concentration of liposomes in circulation, but not for studying tissue distribution. By using ^{3}H-COE both clearance and tissue distribution can be studied. Comparison of both labels renders information about the stability of FUdR-dP in the bilayer of PEG-immunoliposomes.

PEG-liposomes and PEG-immunoliposomes were both cleared from circulation with an approximately log-linear profile. PEG-liposomes had a $t_{½}$ of approximately 18 h, whereas that for PEG-immunoliposomes was only slightly lower. Attachment of antibodies to MPB-PEG-liposomes up to a den-

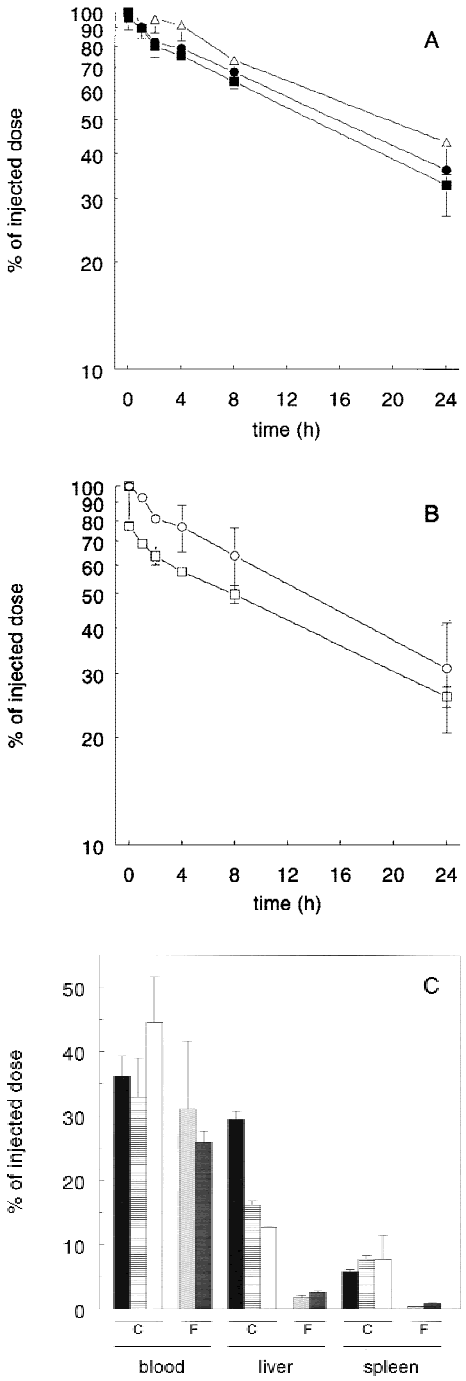

Fig. 1. Serum disappearance (A + B) and tissue distribution (C) of immunoliposomes labeled with ^{3}H-COE or ^{3}H-FUdR-dP in healthy rats. Rats were injected with 10 μmol lipid/kg of ^{3}H-COE-labeled (A) or ^{3}H-FUdR-dP-labeled (B) immunoliposomes (antibody density in μg antibody/μmol of total lipid in parentheses). (A) ^{3}H-COE-labeled (immuno)liposomes: (●) CC52-MPB-PEG (40 μg/μmol), (△) PEG, and (●) CC52-Hz-PEG (6 μg/μmol). (B) ^{3}H-FUdR-dP-labeled (immuno)liposomes: (○) CC52-MPB-PEG (30 μg/μmol) and (□) CC52-Hz-PEG (14 μg/μmol). (C) Tissue distribution 24 h after injection of immunoliposomes. The following liposome types were studied. ^{3}H-COE-labeled (bars indicated with C): CC52-MPB-PEG (40 μg/μmol) (filled bars), CC52-Hz-PEG (6 μg/μmol) (horizontally hatched bars), PEG (open bars); or ^{3}H-FUdR-dP-labeled (bars indicated with F): CC52-MPB-PEG (30 μg/μmol) (hatched bars) and CC52-Hz-PEG (14 μg/μmol) (gray bars). Values are presented as a percentage of injected dose (means ± SD, $n = 3$).

sity of 40 μg/μmol TL influenced pharmacokinetic behavior of the liposomes only slightly (Table I). Hz-PEG-liposomes with low antibody density (6 μg/μmol) showed pharmacokinetics identical to MPB-PEG-immunoliposomes. However, if the antibody-density on Hz-PEG-liposomes was increased up to 14 μg/μmol a faster clearance and a slightly but significantly lower AUC was observed compared with PEG-liposomes ($p = 0.016$). Uptake of the liposomes in liver and spleen is presented in Fig. 1c. Surprisingly, attachment of antibodies to MPB-PEG-liposomes at concentrations that only slightly increased clearance resulted in a doubling of the liver uptake compared with control PEG-liposomes. Hz-PEG-immunoliposomes (6 μg/μmol) were taken up by the liver to a comparable extent as control PEG-liposomes. No differences were seen in the spleen uptake of these three types of liposomes.

The pharmacokinetic behavior of both MPB and Hz-PEG-immunoliposomes labeled with ^{3}H-FUdR-dP (Fig. 1b) incorporated in their bilayers were virtually identical to that of PEG-immunoliposomes labeled with the metabolically inert ^{3}H-cholesteryloleylether (Fig. 1a), demonstrating a sustained association of the prodrug with the immunoliposomes while in circulation. Only low levels of ^{3}H-FUdR-dP were retrieved in liver and spleen, reflecting rapid intracellular degradation of the prodrug by macrophages and fast excretion of the label from the liver as was previously shown (19,27).

Pharmacokinetic Behavior of PEG-Immunoliposomes in Rats with Hepatic Colon Cancer Metastases

We also determined the pharmacokinetic behavior of PEG-liposomes and PEG-immunoliposomes in rats with liver metastases of CC531 colon adenocarcinoma. Liposomes were injected i.v., 21 days after tumor cell inoculation into the portal vein. Pharmacokinetics were monitored during 24 h and are presented in Fig. 2 and Table II.

A striking difference compared to the healthy animals was the initial rapid phase of elimination converting the clearance curve to biphasic. Approximately 30–40% of the PEG-(immuno)liposomes were cleared from circulation within the first 2–3 h. Liposomes remaining after the initial phase of clearance were removed more slowly with a $t_{1/2}$ of more than 10 h. In tumor-bearing rats, clearance of CC52-MPB-PEG-immunoliposomes (41 μg/μmol) showed similar pharmacokinetics to plain PEG-liposomes (Table II). In contrast, MPB-PEG-liposomes with an irrelevant murine IgG_1 attached were cleared more rapidly, especially in the initial clearance phase. As a consequence, these liposomes had a significantly lower AUC. This increased clearance was accounted for by a two-fold increased uptake of liposomes in the liver as can be seen in Fig. 2b. This increased clearance is probably due to the higher antibody density of these liposomes as compared with CC52-MPB-PEG-liposomes (Table II). CC52-MPB-PEG-liposomes showed a higher level of liver uptake (30 to 40% of injected dose) compared with PEG-liposomes (20% of injected dose), which is barely reflected in the plasma levels. PEG-liposomes on the other hand, displayed a higher level of uptake in the spleen, indicating that the increased liver uptake of CC52-MPB-PEG-liposomes affects the uptake by the spleen.

CC52-Hz-PEG-immunoliposomes (14 μg CC52/μmol

Table I. Pharmacokinetics of PEG-Immunoliposomes in Healthy Rats

Liposome type	Antibody density (μg/μmol TL)	Label	$AUC_{0\text{-}24h}$ (% I.D. × h/ml)	$t_{1/2}$ (h)	*n*	*p*-value[a]
CC52-MPB-PEG	40	^{3}H-COE	2328 (±209)	16.8 (±1.2)	3	0.202
CC52-MPB-PEG	30	^{3}H-FUdR-dP	2068 (±642)	15.0 (±4.2)	3	0.151
CC52-Hz-PEG	6	^{3}H-COE	2144 (±334)	16.3 (±3.0)	3	0.176
CC52-Hz-PEG	14	^{3}H-FUdR-dP	1668 (±112)	16.1 (±1.5)	3	0.016
PEG	—	^{3}H-COE	2809 (±580)	18.3 (±3.3)	3	

Rats were injected with PEG-(immuno)liposomes labeled either with ^{3}H-COE or ^{3}H-FUdR-dP at a dose of 10 μmol TL/kg body weight. Serum disappearance was determined during 24 h after injection (Figs. 1a and b). From these values the area under the % of injected dose vs. time curve and the circulation half-life were calculated as mentioned in the methods section. Values are means ± SD ($n = 3$).
[a] Statistical significance of difference of AUC compared to AUC of PEG-liposomes. *P*-values < 0.05 are considered significant.

TL) were removed from circulation more rapidly than PEG-liposomes or CC52-MPB-PEG-liposomes, especially in the initial clearance phase, resulting in a significantly lower AUC ($p = 0.005$ and $p = 0.018$, respectively). The increased clearance was accompanied by higher uptake in the liver. Hz-immunoliposomes with only 5 μg/μmol of irrelevant IgG_1 attached to their PEG-termini, showed a comparable circulation time and AUC as PEG-liposomes, indicating that the antibody density strongly influences the circulation times of these liposomes. The most rapid clearance profile was observed after injection of CC52-Hz-PEG-liposomes with high antibody-density (44 μg/μmol). These liposomes were removed from circulation extremely rapidly; within 1 min approximately half of the injected dose was already cleared and after 1 h liposome levels in circulation were virtually zero. The AUC of these liposomes dropped 20-fold compared with CC52-Hz-PEG-liposomes (14 μg Ab/μmol TL) and 40-fold as compared with PEG-liposomes without antibody. This rapid clearance was a consequence of high uptake in the liver.

Targeting of Immunoliposomes to Liver Metastases of CC531 Colon Cancer

Accumulation of different types of PEG-(immuno)liposomes in tumor nodules in the liver was assessed. From 2 to 11% of the injected dose were retrieved in the tumor nodules in the liver (Fig. 2b). These values are obviously influenced by the size of the tumor, and to allow better comparison between the different types of liposomes we calculated the percentage of injected dose per gram of tumor tissue and also compared this with the specific accumulation of liposomes in liver tissue in Fig. 3.

All liposome formulations carrying surface-attached antibodies, either randomly or uniformly oriented, and either tumor-specific or irrelevant, tended to accumulate in higher levels in the tumor nodules than PEG-liposomes without antibody (Figs. 2b and 3). Between the different immunoliposome types no significant differences in tumor accumulation were observed. The increased tumor uptake values of the immunoliposomes relative to PEG liposomes were only marginally significant for CC52-MPB-PEG liposomes (41 μg/μmol) and CC52-Hz-PEG liposomes (14 μg/μmol) (with $p = 0.076$ and $p = 0.060$, respectively). Differences were not significant for the nonspecific IgG_1-containing liposomes. Tumor to liver uptake ratios ranged from a high of 0.8 for CC52-MPB-PEG-liposomes and IgG_1-MPB-PEG-liposomes to a low of 0.5 for CC52-Hz-PEG-liposomes and PEG-liposomes.

The rapidly cleared CC52-Hz-PEG-liposomes (44 μg

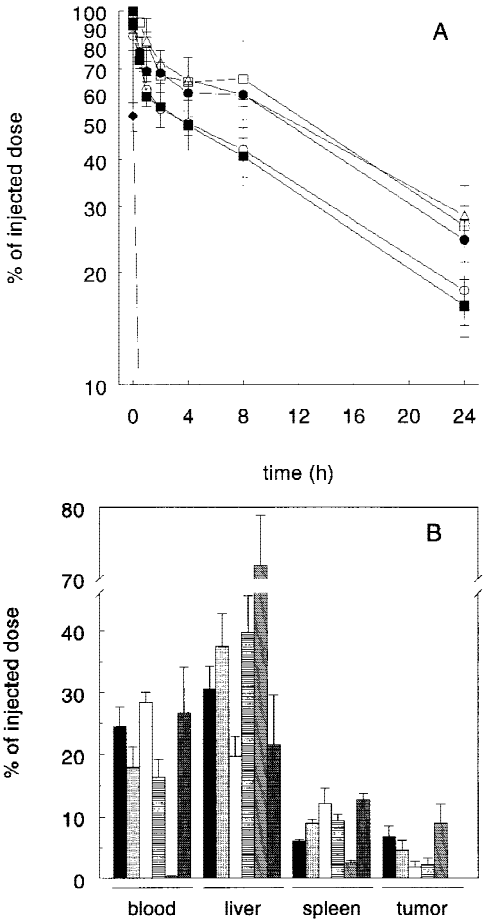

Fig. 2. (A) Serum disappearance of ^{3}H-COE-immunoliposomes in rats with liver metastatic growth of CC531 colon cancer 21 days after inoculation of the tumor cells. Rats were injected with 10 μmol lipid/kg of the following liposome types (antibody density in μg antibody/μmol of total lipid in parentheses): (●) CC52-MPB-PEG (41 μg/μmol), (○) IgG_1-MPB-PEG (69 μg/μmol), (△) PEG, (●) CC52-Hz-PEG (14 μg/μmol), (◆) CC52-Hz-PEG (44 μg/μmol) or (□) IgG_1-Hz-PEG (5 μg/μmol). (B) Tissue distribution 24 h after injection of immunoliposomes. The following liposome types were studied. CC52-MPB-PEG (41 μg/μmol) (filled bars), IgG_1-MPB-PEG (69 μg/μmol) (dotted bars), PEG (open bars), CC52-Hz-PEG (14 μg/μmol) (horizontally hatched bars), CC52-Hz-PEG (44 μg/μmol) (hatched bars), or IgG_1-Hz-PEG (5 μg/μmol) (cross-hatched bars). Values are presented as a percentage of injected dose (means ± SEM, $n = 3$ to 6, as indicated in Table II).

Table II. Pharmacokinetics of PEG-Immunoliposomes in Tumor-Bearing Rats

Liposome type	Antibody density (μg/μmol TL)	AUC 0–24 h (% I.D. × h/ml)	$t_{1/2}$ a (h)	$t_{1/2}$ b (h)	*n*	*p*-value[a]
CC52-MPB-PEG	41 ± 7	2055 (±275)	0.42 (±0.18)	19.2 (±2.3)	6	0.74
IgG_1-MPB-PEG	69 ± 15	1234 (±282)	0.29 (±0.06)	13.0 (±2.0)	4	0.04
CC52-Hz-PEG	14 ± 1	1169 (±146)	0.20 (±0.05)	14.2 (±1.0)	6	0.005
CC52-Hz-PEG	44	56 (±4)	0.17 (±0.05)	29.5 (±3.9)	3	0.0001
IgG_1-Hz-PEG	5 ± 0	1824 (±465)	0.55 (±0.16)	14.8 (±2.3)	4	0.777
PEG	—	1946 (±156)	0.54 (±0.19)	16.6 (±1.7)	6	—

Twenty-one days after inoculation of CC531 colon cancer cells into the portal vein rats were injected i.v. with PEG-(immuno)liposomes labeled with ^{3}H-COE (10 μmol TL/kg body weight). Serum disappearance was determined during 24 h (Fig. 2a). From these values the area under the % of injected dose vs. time curve and the circulation half-life were calculated as mentioned in the methods section. Values are means ± SEM of three to six experiments as indicated.

[a] Statistical significance of difference of AUC compared to AUC of PEG-liposomes. P-values < 0.05 are considered significant.

Ab/μmol TL) accumulated to a significantly higher extent in the tumor nodules relative to PEG-liposomes ($p = 0.007$). However, the liver uptake of these liposomes was also high, resulting, in fact, in the lowest tumor:liver ratio of all liposome formulations tested, 0.38.

With the exception of the Hz-PEG-liposomes with high antibody density, the uptake in liver tissue of the immunoliposomes and of the PEG-liposomes was only slightly higher than the tumor uptake, which is a remarkable finding in view of the large differences in morphology of both tissues.

Our observations indicate that, firstly, there is no antibody-specific accumulation of tumor-targeted immunoliposomes in the tumor tissue. Secondly, attachment of either tumor-specific or nonspecific antibodies to MPB-PEG-liposomes increases uptake in both liver and tumor tissue, with a modest prevalence in the latter. This is in contrast to CC52-Hz-PEG-liposomes, which showed tumor:liver ratios comparable to control PEG-liposomes.

Obviously, the increased immunoliposome accumulation in the tumor is caused by a mechanism other than specific antigen recognition. One such mechanism may be recognition of the Fc portion of the antibody molecules by Fc receptors on tumor-associated macrophages or monocytes, expressing high-affinity Fc receptors (24,25). To test this, we incubated CC52-MPB-PEG-immunoliposomes with trypsin-treated Kupffer cells because this treatment is known to expose high-affinity Fc-receptors (24,25). Figure 4 shows that a 30-min trypsin treatment does enhance uptake of CC52-MPB-PEG immunoliposomes by Kupffer cells by 30%. This is consistent with an involvement of the high-affinity Fc-receptors in the uptake of these liposomes by macrophages.

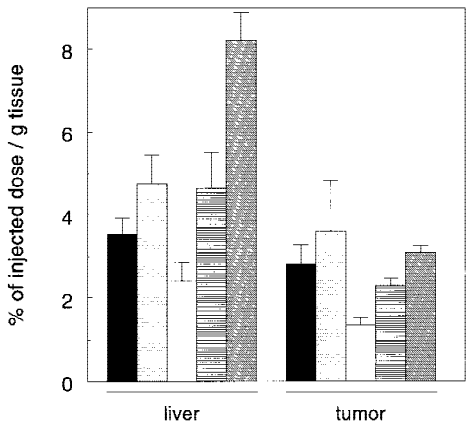

Fig. 3. Specific uptake of immunoliposomes in liver and tumor tissue. Tumor accumulation of the liposomes from Fig. 2 was corrected for the tumor weight and depicted as a percentage of injected dose per gram of tissue and compared with liver uptake. The following liposome types were studied (antibody density in μg antibody/μmol of total lipid in parentheses): CC52-MPB-PEG (31–50 μg/μmol) (filled bars), IgG_1-MPB-PEG (48–92 μg/μmol) (dotted bars), PEG (open bars), CC52-Hz-PEG (14 μg/μmol) (horizontally hatched bars), or CC52-Hz-PEG (44 μg/μmol) (hatched bars).

DISCUSSION

Pharmacokinetic parameters of differently designed immunoliposomes were studied in normal rats and rats with hepatic metastases of colon cancer. We observed a number of striking differences between normal and tumor rats as well as between the various PEG-containing immunoliposome formulations. In healthy animals blood elimination rates of PEG-immunoliposomes were almost log-linear and only slightly affected by attachment of a monoclonal antibody, but

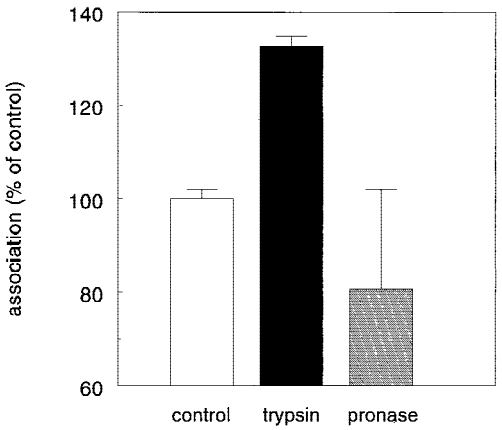

Fig. 4. Effect of treatment with proteolytic enzymes on the association of CC52-MPB-PEG-liposomes with isolated Kupffer cells *in vitro*. Kupffer cells were treated for 30 min with trypsin or pronase and subsequently incubated for 3 h with 200 nmol CC52-MPB-PEG-liposomes. Results are presented as a percentage of the association (nmol lipid/mg cell protein) of the control incubations (without proteolytic enzymes).

in tumor-bearing animals all liposome formulations displayed biphasic elimination characteristics with a rapid initial clearance phase, followed by a much slower phase. Increased antibody densities on the immunoliposomes led to increased clearance in both normal and tumor-bearing animals, but this was more pronounced for the liposomes in which the antibody was coupled to the distal end of the PEG chains via a hydrazone linkage than for the liposomes with antibody conjugated directly to the bilayer by means of the MPB-PE linker. Also, the effect of antibody density on Hz-PEG-immunoliposome was more pronounced in the tumor-bearing than in the normal animals. The increased elimination rate of all immunoliposome formulations in tumor-bearing animals could only partly be accounted for by uptake into the liver tumor nodules, where uptake varied between 2 and 11% of injected dose. The rapid (initial) elimination of immunoliposomes in the tumor animals was also clearly reflected in enhanced uptake by normal liver tissue. This may be explained by the increased number and activity of macrophages that has been shown to accompany tumor growth in this organ (15,28).

We had expected the Hz-PEG-immunoliposomes to display a lower affinity toward macrophages than the MPB-PEG-immunoliposomes, because the former are coupled by means of the Fc portion of the antibody whereas the latter are coupled to the liposomal surface in a random manner allowing better exposure of the Fc moiety and thus interaction with Fc receptors on macrophages. In fact, we have previously shown that of the two types of immunoliposomes the hydrazide-coupled formulation is considerably less sensitive to Fc-receptor-mediated uptake by isolated Kupffer cells than the MPB-PE coupled formulation (8). Because, in particular at the higher antibody densities, the hydrazide-coupled liposomes show rapid elimination, we propose that the more extensive peroxidation of the antibody, required to achieve high antibody density, confers increased affinity toward, for example, scavenger receptors on the macrophages (G.A. Koning et al, unpublished observations) and thus enhanced uptake by these cells. Future studies should be aimed at more effectively preventing exposure of the Fc-portion of the antibody at the liposomal surface. This may be accomplished by using the so-called multilayer liposome (4) with the antibodies present at the terminal ends of PEG-chains and shielded by the presence of even longer PEG-chains. The long PEG-chains need to be shed from the liposomes on arrival at the target site, allowing the antibody to interact with the target cells. A different way to prevent Fc-mediated uptake is by coupling antibody fragments lacking the Fc-portion to the liposomes (1,11). A final possibility lies in the use of intact monoclonal antibodies with diminished affinity for the Fc-receptor as has been recently demonstrated for IgG4 (29).

Although tumor localization of the liposomes used in this study remains limited at maximally 11% of injected dose for the rapidly clearing Hz-liposomes with high antibody density, specific uptake per gram tissue shows values of 2–4% of injected dose per gram of tumor tissue for all the immunoliposomes tested, *i.e.,* of the same order of magnitude as liver uptake. This is notable in view of the apparently poor vascularization of the tumor nodules as compared to the surrounding liver tissue. Tumor uptake seems to be enhanced in a nonspecific manner by the presence of antibodies on the liposome surface. The lack of specificity of liposome uptake by the tumor tissue strongly suggests that the antibody-induced enhancement of liposome uptake is not due to specific recognition of the tumor cells, but rather to interaction with tumor-associated macrophages. From a recent study in which we assessed the localization of tumor cell-directed MPB-immunoliposomes within the tumor we concluded that the immunoliposomes were mostly not associated with tumor cells but rather localized in tumor associated cells, probably macrophages (30). The interaction with the macrophages may be mediated by Fc-receptors and scavenger receptors (8,9). Especially in tumor areas, expression of high-affinity Fc-receptors on activated macrophages and monocytes present in such areas (15), may be involved. The presence of proteolytic enzymes may enhance such expression (24,25). In our experiments, the involvement of high-affinity Fc receptors in the uptake of immunoliposomes is indicated by the experiment with trypsin-treated Kupffer cells, which showed that the trypsinized cells take up larger quantities of immunoliposomes than nontreated cells.

Increased immunoliposome clearance in tumor-bearing animals compared to healthy ones was demonstrated before by Lopes de Menezes et al. (2) for i.p. injected B-cell lymphoma in mice. The contribution of either liver uptake or specific tumor cell targeting to the increased immunoliposome clearance was not explicitly determined, but the observation that increased clearance was only observed for tumor cell-specific immunoliposomes and not for irrelevant control pointed toward strong involvement of tumor cell binding (2).

In the present study we used two radiolabels: the nonexchangeable, nonmetabolizable ^{3}H-cholesteryl-oleylether and the dipalmitoyl derivative of ^{3}H-labeled FUdR. The latter was used in view of our intention to deliver this prodrug to the CC531 tumor cells. We previously demonstrated that immunoliposomes carrying a specific antibody against these tumor cells are able to selectively deliver the prodrug to the tumor cells without internalization of the liposome as such (3,17). The plasma elimination experiments demonstrate that the prodrug remains tightly associated with the liposomes while in circulation, considering the similarity in results with the two labels. When internalized by cells (mostly macrophages) in liver and spleen, however, much less of the FUdR label was recovered than of the cholesteryloleylether label in these two organs, due to the efficient hydrolytic removal of the palmitoyl residues and the subsequent release of most of the resulting product, free FUdR, from the cells. FUdR is rapidly eliminated from the blood (19,27), so this release of label does not contribute significantly to plasma radioactivity levels.

Taken together, our results show that the presence of tumor growth in the body substantially influences pharmacokinetics of PEG (immuno)liposomes, and that high antibody densities on the liposomal surface cause a substantial increase in elimination rate from the circulation and a concomitantly increased uptake by, predominantly, the liver. By choosing appropriate immunoliposome characteristics, regarding the density and spatial orientation of the antibody, PEG-immunoliposomes were developed with a long-circulating capacity. Moreover, they were shown to retain the anticancer prodrug FUdR-dP during circulation. Liposome uptake by metastatic colon tumor nodules in the liver was enhanced by attachment of antibodies to the liposomal surface to levels comparable to uptake in liver tissue, a rather remarkable finding in view of the differences in morphology of both tissues. These results show the potency of long-circulating immunoliposomes to lo-

calize to considerable extents in liver metastatic nodules. However, the enhanced uptake of immunoliposomes in the tumor can be achieved with tumor-specific and tumor-irrelevant antibodies alike. We take this to indicate that uptake of immunoliposomes by the metastatic tumor nodules is more likely to be achieved by tumor-associated macrophages than by the tumor cells *per se*. This view is corroborated by our earlier observation that isolated Kupffer cells readily take up the immunoliposome formulations of the current study by means of both Fc and scavenger receptor-mediated processes (8,9).

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