Interaction of Differently Designed Immunoliposomes with Colon Cancer Cells and Kupffer Cells. An *in Vitro* Comparison

Gerben A. Koning[1, 5], Henriëtte W. M. Morselt[1], Arko Gorter[2], Theresa M. Allen[3], Samuel Zalipsky[4], Gerrit L. Scherphof[1] and Jan A. A. M. Kamps[1,6]

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Purpose. Evaluate the effectiveness of distal-end coupling of a tumor-specific antibody to liposomal polyethylene glycol (PEG) chains to improve target binding and reduce interference by macrophage uptake.

Methods. Monoclonal antibody CC52, specific for CC531 rat colon carcinoma, was coupled to the bilayer of PEG-liposomes (type I) or to the distal end of bilayer-anchored PEG-chains (type II). Uptake of both (radiolabeled)liposome types by CC531 cells and rat liver macrophages was determined.

Results. With increasing antibody density, both immunoliposome types showed increased binding to target cells, but type II liposomes displayed better target recognition than type I. Uptake by macrophages increased with antibody density for both liposome types. Lowest uptake by macrophages was found for type II liposomes at low antibody densities. Unexpectedly, not only for type I but also for type II liposomes, in which the antibody is coupled via its Fc moiety, uptake by macrophages was inhibited by aggregated IgG, indicating involvement of Fc receptors. Also polyinosinic acid, an inhibitor of scavenger receptors, reduced uptake of type II liposomes.

Conclusion. Although distal end coupling of antibodies to bilayer-anchored PEG chains in liposomes through the Fc moiety enhances target cell binding, it does not prevent the recognition by Fc receptors on macrophages.

KEY WORDS: Immunoliposomes; Poly(ethylene glycol); Colon cancer; Kupffer cells; Tumor specific antibody; Drug-targeting.

[1] Department of Cell Biology, Section Liposome Research, Groningen University Institute for Drug Exploration (GUIDE), Faculty of Medical Sciences, University of Groningen, A. Deusinglaan 1, 9713 AV, Groningen, The Netherlands.

[2] Department of Pathology, Leiden University Medical Center, P.O. Box 9600, 2300 RC Leiden, The Netherlands.

[3] Department of Pharmacology, University of Alberta, Edmonton, Canada T6G 2H7.

[4] ALZA Corporation, Palo Alto, California 94303-0802, USA.

[5] Present address: Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht University, P.O Box 80 082, 3508 TB Utrecht, The Netherlands.

[6] To whom correspondence should be addressed. J.A.A.M. Kamps, Department of Cell Biology, Faculty of Medical Sciences, University of Groningen, A. Deusinglaan 1, 9713 AV, Groningen, The Netherlands. Tel: 31 50 363 2655; Fax: 31 50 363 8171. (e-mail: j.a.a.m.kamps@med.rug.nl)

ABBREVIATIONS: CC52, Colon Carcinoma CC531 specific antibody; PC, Phosphatidylcholine; Chol, Cholesterol; PEG-DSPE, methoxypoly(ethylene glycol)$_{2000}$-distearoylphosphatidylethanolamine; MPB-PE, maleimido-4-(*p*-phenylbutyryl)phosphatidylethanolamine; Hz-PEG, hydrazide-PEG; BSA, bovine serum albumin; FCS, fetal calf serum; poly I, polyinosinic acid; TL, total lipid (phospholipids and cholesterol).

INTRODUCTION

Delivery of drugs to target cells by means of liposomes is hindered by the uptake of these particles by macrophages in liver and spleen. Attachment of antibodies to liposomes for targeting purposes may result in increased macrophage uptake and thus in an even lower availability of the drug for the target cells. Characterization of the effects of antibody coupling to liposomes on the target binding potential *vs.* uptake by macrophages is therefore a major issue.

PEG coupled to the liposomal surface increases the circulation half-life of liposomes (1). The method of coupling and the orientation of the antibody, combined with liposome size and the absence or presence of PEG, mainly determine the fate of systemically applied immunoliposomes. Efficient localization of immunoliposomes at sites not readily accessible, like tumor cells in solid tumors, is determined by blood circulation time and liposome size (2–5). Increased circulation time results in enhanced extravasation in the tumor, allowing increased anti-tumor activity.

Not only liver and spleen macrophages interfere with tumor-cell specific delivery. Following extravasation at the tumor site, tumor-associated macrophages may also compete with the target cells for uptake of the immunoliposomes (6).

Several reports describe decreased circulation half-lives following the coupling of antibodies to PEG-liposomes, especially when the antibody is attached to the distal end of the PEG-chains either via a hydrazone-bond (3,5,7), by means of PDP-PEG-DSPE (8) or by using a PEG-terminal carboxyl-group (9). Increased clearance rates paralleled enhanced uptake by (macrophages of) liver and spleen (7,8). Liposomes with antibodies attached to the distal end of the PEG-chains were cleared from circulation much faster than liposomes with the antibody coupled directly to the bilayer of PEG-DSPE containing liposomes (5,9). Circulation time of the latter was similar to that of control PEG-liposomes without antibody suggesting that the antibody was masked by the PEG-chains (5,9,10). Most antibody coupling methods result in a random orientation of the antibody on the liposomes. The resulting exposure of the Fc portion of the antibody facilitates recognition by Fc receptors on macrophages. Coupling of antibodies to the distal end of the PEG-chain by means of Hz-PEG-DSPE specifically involves the Fc portion of the antibody: in this way diminished Fc-exposition can be achieved (11).

In this paper we compare liposomes with varying amounts of the antibody CC52, specific to rat CC531 colon cancer cells with regard to their target-binding capacity and their uptake by Kupffer cells. The antibody was attached either directly to the bilayer of liposomes with or without mPEG-DSPE (type I) or to the distal end of bilayer-anchored PEG-chain (type II).

MATERIALS AND METHODS

Materials

N-succinimidyl-S-acetylthioacetate (SATA), sodium periodate, *N*-acetylmethionine, cholesterol (Chol), polyinosinic acid (poly I) and human IgG were obtained from Sigma (St. Louis, MO, USA). Egg yolk phosphatidylcholine (PC), ma-

leimido-4-(*p*-phenylbutyryl)phosphatidylethanolamine (MPB-PE) and poly(ethylene glycol)$_{2000}$-distearoylphosphatidylethanolamine (PEG_{2000}-DSPE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Hydrazide-PEG-DSPE (Hz-PEG-DSPE) was synthesized as described previously (12). [^{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.

Monoclonal Antibody

The monoclonal antibody CC52 (IgG_1), recognizing a surface antigen on CC531 colon adenocarcinoma cells was developed in the department of Pathology, Leiden University Medical Center, the Netherlands (13) and 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, Department of Cell Biology, Groningen, the Netherlands.

Liposomes

Liposomes were composed of PC/Chol (23:16 molar ratio). Liposomes to which CC52 antibodies were coupled contained 0.025 mol MPB-PE, to prepare type I immunoliposomes, or Hz-PEG-DSPE, to prepare type II immunoliposomes, per mol total lipid (TL) (cholesterol and phospholipid). Sterically stabilized liposomes were prepared by incorporating PEG_{2000}-DSPE in a molar ratio of 0.04 mol/mol TL; to Hz-PEG-DSPE containing liposomes (type II) an additional 0.015 mol PEG_{2000}-DSPE was added per mol of TL to obtain 0.04 mol PEG-DSPE per mol TL. When required, liposomes were labeled with trace amounts of [^{3}H]-cholesteryloleylether (^{3}H-COE) (1 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 liposomes containing MPB-PE (type I liposomes) or Hz-PEG-DSPE (type II liposomes), lipids were hydrated in pH 6.7 HN-buffer, or pH 5.5 NaAc-buffer (100 mM sodiumacetate, 70 mM NaCl), respectively. Liposomes were sized by repeated extrusion through polycarbonate filters with a pore size of 50 nm (Costar, Cambridge, MA, USA) using a high-pressure extruder (Lipex, Vancouver, Canada). Phospholipid phosphorus was determined by a phosphate assay after perchloric acid destruction (14). Total liposomal lipid 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, USA). The mean diameter of the liposomes was obtained from the volume distribution curves produced by the particle analyzer. Results of the liposome characterization are presented in Table I.

Coupling of Antibodies to Liposomes

The monoclonal antibody CC52 was coupled to MPB-PE-containing liposomes (type I liposomes) by a sulfhydryl-maleimide coupling method as described previously (15). CC52 antibody was coupled to liposomes containing Hz-PEG-DSPE (type II liposomes) via a hydrazone-linkage of the hydrazide moiety at the distal end of the PEG-chains and oxidized carbohydrates in the Fc-region of the antibody as described previously (5,7,16).

Table I. Characterization of Immunoliposomes

Liposome	Antibody	Oxidation conditions[a]		Coupling conditions[b]		Size before coupling (nm ± SD)	Size after coupling (nm ± SD)	Coupled antibody (μg/μmol TL)
		h	Temp	h	Temp			
MPB	CC52			4	RT	74 ± 28	167 ± 37	155
MPB-PEG	CC52			4	RT	70 ± 24	80 ± 32	28
	CC52			4	RT	72 ± 26	80 ± 35	33
	CC52			4	RT	82 ± 30	84 ± 37	38
	CC52			4	RT	72 ± 24	82 ± 34	46
	CC52			4	RT	71 ± 26	94 ± 38	58
	$mIgG_1$			4	RT	72 ± 24	81 ± 36	74
	$mIgG_1$			4	RT	82 ± 30	97 ± 36	92
Hz-PEG	CC52	1	RT	16	4°C	77 ± 30	84 ± 39	8
	CC52	1	RT	16	4°C	81 ± 28	92 ± 37	10
	CC52	1	RT	16	4°C	89 ± 32	93 ± 38	15
	CC52	1	28°C	16	28°C	74 ± 31	110 ± 39	52
	CC52	1	28°C	16	28°C	72 ± 32	106 ± 40	28
	$mIgG_1$	1	RT	16	4°C	89 ± 32	90 ± 38	5

Note: Antibody was coupled to MPB, MPB-PEG, or Hz-PEG liposomes as outlined in the Methods section, with antibody to lipid ratios of 2:1, 2:1, and 1:1 nmol antibody per μmol TL, respectively. In case of the Hz-PEG-liposomes, experimental conditions were varied as indicated in the table to obtain varying antibody densities. Liposome diameter before and after coupling was measured and the amount of antibody coupled was determined using a protein assay.

[a] For coupling to Hz-PEG-DSPE oxidation of antibodies was performed with 0.01 M $NaIO_4$ for the time and temperature indicated. RT = room temperature, 20°C.

[b] Coupling of antibodies to liposomes was performed at the temperature and for time indicated.

Immunoliposomes were characterized by determining protein content (17), phospholipid phosphorus (14) and particle size (Table I). Liposomes were stored at 4°C under nitrogen and used within two weeks after preparation.

Cell Culture

CC531 colon adenocarcinoma is a 1,2-dimethylhydrazine-induced carcinoma of the colon of WAG/Rij-rats (18). Cells were maintained in 75-cm^2 culture flasks (Costar, Cambridge, MA, USA) in RPMI 1640 medium with 25 mM Hepes (Gibco BRL, Breda, The Netherlands) supplemented with 10% heat-inactivated fetal calf serum (Gibco), fresh L-glutamine (2 mM) and penicillin/streptomycin (100 U/ml and 100 μg/ml, respectively) (Gibco) at 37°C in a humidified atmosphere containing 5% CO_2 in air. Cells were subcultured at 80% confluency. In experiments 1×10^5 cells were plated in 24-wells plates (Costar) and left to adhere and grow for two days. One hour before the start of the experiment FCS-containing medium was replaced by fresh medium without FCS.

Animals

Specific pathogen-free (SPF) male WAG/Rij rats weighing 200-220 g were purchased from Harlan CPB (Zeist, The Netherlands), kept under clean conventional conditions and had access to food and water *ad libitum*.

Kupffer Cell Isolation and Culture

Kupffer cells were isolated after pronase digestion of the liver as described before (19) and purified by centrifugal elutriation. 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) and 5×10^5 cells/well were plated in 24-wells plates and maintained at 37°C in a humidified atmosphere containing 5% CO_2 in air. 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.

Association of Liposomes with CC531 or Kupffer Cells

To determine the association of liposomes either with CC531 or Kupffer cells, cells were incubated with ^{3}H-COE-labeled CC52-liposomes for 3 h in the absence of FCS. Unless stated otherwise all incubations were performed at 37°C. Incubations were terminated by removing the incubation medium followed by thorough washing with ice-cold PBS. Cells were lysed with 0.4 M NaOH. Cell-associated radioactivity was measured by liquid scintillation counting and was normalized for the amount of cellular protein (20) using BSA as a standard. Association of liposomes is expressed as nmol TL per mg cell protein.

The involvement of the Fc receptor on Kupffer cells in the uptake of immunoliposomes was studied by determining the effect of heat-aggregated human IgG (1 mg/ml), or bovine serum albumin (BSA) (1 mg/ml) as a control, on cell uptake (19). Heat-aggregated IgG, a multivalent Fc-exposing complex is a known inhibitor of Fc receptor mediated interactions, which is much more effective than monovalent free IgG molecules(19). Heat-aggregated IgG was obtained by incubating human IgG for 30 min at 63°C. Large aggregates were removed by centrifugation for 15 min. at 10000 × g. Involvement of the scavenger receptor system on Kupffer cell uptake of immunoliposomes was studied by determining the effect of polyinosinic acid (poly I) on cell uptake at a concentration of 20 μg/ml (21).

Statistics

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

RESULTS

Immunoliposome Characterization

Table I compiles preparation conditions, compositions, size and antibody densities of the type I and type II and control liposomes we used. In addition to the CC531-specific CC52 antibody, irrelevant murine $IgG_1$was used. With CC52 the highest antibody density attained on type I liposomes without PEG was 155 μg/μmol lipid. On type I PEG-liposomes, CC52-density was 3- to 5-fold lower under the same reaction conditions, probably as a result of steric hindrance by the PEG-chains. The density of CC52 on type I PEG-liposomes varied from 28 to 46 μg/μmol; of the irrelevant IgG up to 92 μg/μmol could be coupled to the liposomes. The standard protocol temperature of 4°C allowed only low amounts of antibody to be coupled to type II liposomes. By increasing the temperature during antibody oxidation and the coupling reaction, antibody levels were achieved close to those obtained with type I PEG-liposomes (28 to 52 μg/μmol). High antibody densities on type I liposomes without PEG resulted in a size increase from 80 up to 166 nm, which may be a result of aggregation and/or cross-linking of some of the immunoglobulins on the liposome surface. Antibody coupling to type I PEG-liposomes produced only a slight size increase, indicating that the antibody, which is coupled to the bilayer in between the PEG-molecules, only slightly protrudes from the PEG-layer. By contrast, type II liposomes increased in size from 80 nm to 110 nm when relatively large amounts of CC52 were coupled. In view of the location of the antibody, i.e., at the distal end of the PEG-chains, this is what could be expected.

Association of Immunoliposomes with CC531 Colon Cancer Cells

Figure 1 presents the association of the two immunoliposome types with CC531 tumor cells as a function of liposome concentration. The presence of the antibody (CC52) resulted in a marked increase in the association with CC531 cells for both liposome types: depending on antibody density, the association of CC52-immunoliposomes with the target cells increased 3- to 18-fold as compared to control liposomes without antibody or liposomes with an irrelevant $mIgG_1$ attached. Binding at 4°C varied from 35–75% of binding at 37°C (not shown).

An increase in the antibody density on type I PEG-liposomes from around 30 μg/μmol to 46 μg/μmol resulted in

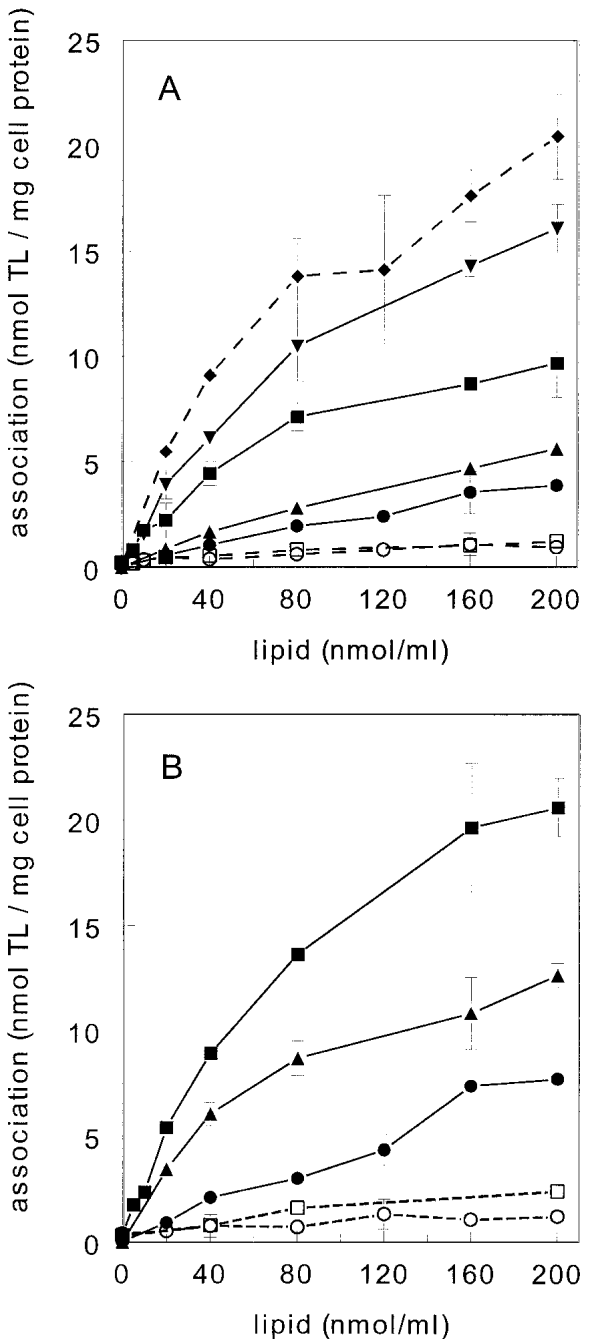

Fig. 1. Association of immunoliposomes with CC531 colon carcinoma cells as a function of liposomal lipid concentration. Varying amounts of the CC531-specific monoclonal antibody CC52 were attached either to MPB-PE-containing (type I) or Hz-PEG-DSPE-containing (type II) ^{3}H-COE-labeled liposomes with or without PEG. (A) CC52-MPB-PEG-liposomes (type I-PEG) with 28 (▲), 33(●), 38(■) or 46 (▼) μg CC52 / μmol TL and CC52-MPB-liposomes (type I) with 155 μg/μmol (♦); control type I liposomes without CC52 (○) and liposomes with an irrelevant IgG_1 (□). (B) CC52-Hz-PEG-immunoliposomes (type II) with, 8 (●), 15 (■) or 28 (▲) μg CC52 / μmol TL; control Hz-PEG-liposomes (type II) without CC52 (○) and liposomes with an irrelevant IgG_1 (□).

an approximately 4-fold increase in the association with CC531 cells. The high antibody density of 155 μg/μmol, which could only be obtained on the surface of type I liposomes without PEG, did not result in a notable increase in the association with the tumor cells compared to type I PEG-liposomes with 46 μg/μmol. This suggests that maximal binding to CC531 cells is achieved at antibody densities of approximately 50 μg/μmol (Fig 1A).

In all experiments the association of the type II liposomes with the CC531 cells reached the same level as that of type I liposomes at much lower antibody densities (Fig 1B). This demonstrates the superior antigen recognition by antibodies attached at the PEG-terminus. The association of type II liposomes did not show the proportionality with antibody density which we saw for the type I liposomes: at a density of 28 μg/μmol the association of the liposomes with the CC531 cells was lower than at 15 μg/μmol. We ascribe this to partial damage of the antibody due to the increased severity of the oxidative conditions required to obtain the higher coupling ratio.

Association of Immunoliposomes with Kupffer Cells

Figure 2 represents the uptake of different liposome formulations by liver macrophages *in vitro*. Uptake of type I liposomes without PEG was 35-fold higher than that of control liposomes without antibody. Incorporation of PEG-DSPE in the liposomal bilayer reduced uptake of type I immunoliposomes 17-fold. Uptake of type I control liposomes without antibody was inhibited by more than 50% if PEG-DSPE was incorporated in the bilayer. Clearly, the presence of the PEG chains strongly contributes to the diminished interaction of the immunoliposomes with the Kupffer cells.

Although uptake by Kupffer cells of both liposome types was clearly enhanced by the presence of antibodies and

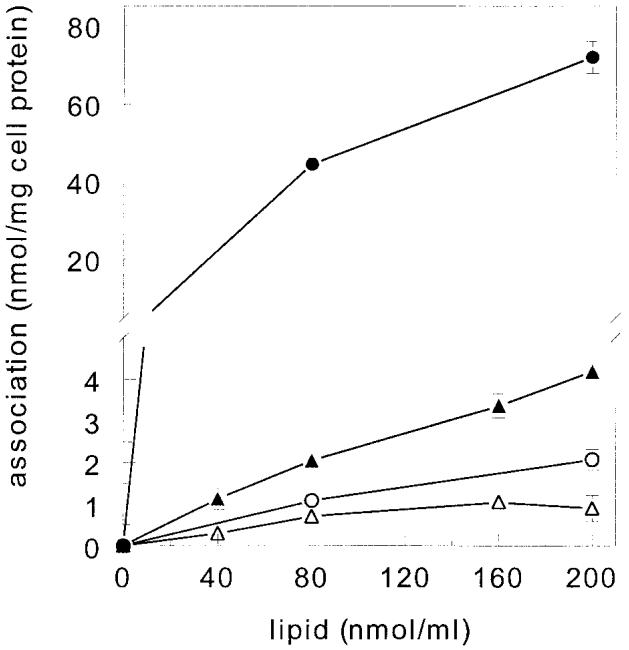

Fig. 2. Effect of the attachment of antibodies and PEG to liposomes on the association with Kupffer cells *in vitro*. In vitro cultured isolated Kupffer cells were incubated for 3h with varying concentrations of ^{3}H-COE-labeled type I liposomes (155 μg/μmol) (●), control type I (without antibody) liposomes (○), CC52-MPB-PEG-liposomes (type I) (33 μg/μmol) (▲) or MPB-PEG-control liposomes (Δ).

showed saturation kinetics, there was no direct proportionality between antibody density and uptake for type I liposomes. For example, an increase in antibody density from 33 to 58 μg/μmol (Fig. 3A) led to a more than 5-fold increase in uptake for the type I liposomes. Unexpectedly, the levels of association of type I liposomes with 28 μg/μmol were found in between those of 33 and 58 μg/μmol, an observation we ascribe to the different Kupffer cell batches used in these experiments. In Table II we therefore compared the levels of association of type I and II liposomes when using the same batch of Kupffer cells. For type II liposomes (Fig. 3B) a more clear relation was observed between the antibody density and Kupffer cell uptake; an increase in density from 8 to 28 μg/μmol even caused a more than 10-fold increase in uptake by the cells. These observations altogether emphasize the critical importance of the presence of antibodies and especially for type II immunoliposomes the antibody density in determining the "affinity" of the immunoliposomes for the macrophages. The steep increase in cellular uptake with antibody density on the type II liposomes may be related to protein modification caused by the rigorous oxidative reaction conditions required for high antibody densities on these liposomes.

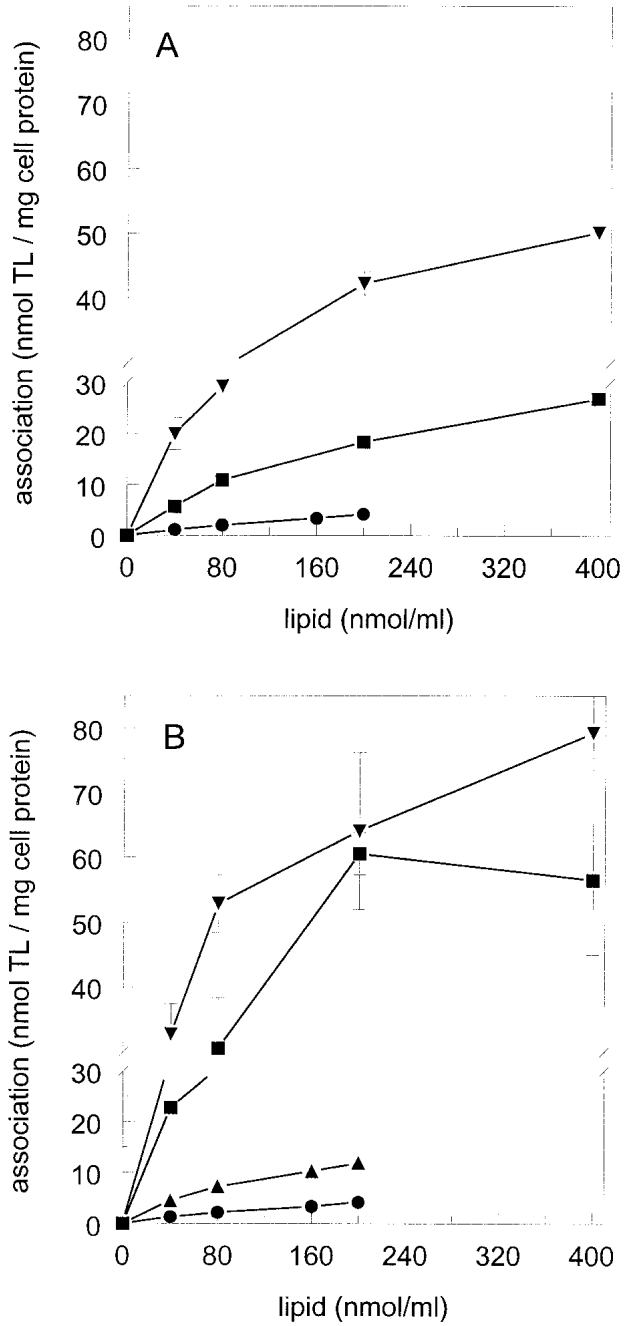

Fig. 3. (A) Effect of antibody density on the association of type I immunoliposomes with Kupffer cells. Kupffer cells were incubated for 3 h with varying amounts of ^{3}H-COE-labeled liposomes containing 28 (■), 33 (●) or 58 (▼) μg CC52 / μmol TL (B) Effect of different antibody densities on type II immunoliposomes on the association with Kupffer cells. Kupffer cells were incubated for 3 h with varying amounts of ^{3}H-COE-labeled immunoliposomes containing 8 (●), 10 (▲), 28 (■) or 52 (▼) μgCC52 / μmol TL.

Involvement Scavenger- and Fc-Receptors in Immunoliposome -Kupffer Cell Interaction

We determined the effect of aggregated human IgG, as a competitor for the Fc-receptor, on the binding and uptake of immunoliposomes by macrophages (Fig. 4). Figure 4A shows the results with MPB-coupled PEG-immunoliposomes. Aggregated IgG inhibited the association of type I liposomes by more than 50% as compared to incubations without inhibitor ($p = 0.006$), while similar amounts of bovine serum albumin (BSA) had no significant effect. This indicates a significant role for Fc-mediated recognition of this type of liposome. The effect of aggregated IgG on uptake of type II liposomes (Fig. 4B) was less pronounced than for the type I but still substantial (more than 30% for the low density and almost 20% for the high density). Also here, equivalent amounts of BSA had no significant effect on the uptake of type II immunoliposomes.

Association of both type I and type II liposomes with Kupffer cells at 4°C was only 15 to 25% of that at 37°C (not shown). Also at 4°C aggregated IgG inhibited the association of either type of immunoliposome.

The apparently less important role of Fc-mediated uptake by Kupffer cells for type II immunoliposomes than for type I immunoliposomes probably relates to the site-specific linkage of the antibody molecule via its Fc-portion to the terminal end of the PEG-chain, rendering this part of the IgG molecule less accessible. Nonetheless, considerable uptake by Kupffer cells was still observed for type II liposomes, especially at relatively high antibody densities, suggesting the involvement of at least one additional uptake mechanism.

Since the Fc-mediated coupling of the antibody to the PEG-chains requires its oxidation with periodate, we speculate that, in analogy to oxidized LDL (22) the antibody may interact with scavenger receptors. In the experiment presented in Fig. 5 we tested this by blocking these receptors with polyinosinic acid (poly I). Figure 5 A and B demonstrate that poly I inhibits the uptake of type II immunoliposomes. The inhibitory activity of poly I was stronger at high (Fig. 5B) than at low (Fig. 5A) antibody density. Even in the presence of aggregated IgG, poly I further reduced the uptake. These results suggest the involvement of the scavenger receptor system in the uptake of type II liposomes.

In Table II a comparison is made between the uptake of the two main immunoliposome types used in this study by CC531 tumor cells and liver macrophages, respectively, for

Table II. Relative Uptake of Immunoliposomes with Varying Antibody Density by CC531 and Kupffer Cells

Liposome	Ab-density (μg/μmol)	Liposome concentration (nmol/mL)	CC531 Association (nmol/mg)	KC Association (nmol/mg)	Association ratio CC531/KC
CC52-MPB[a]	155	80	13.8 (±1.8)	44.4 (±2.3)	0.31 (±0.06)
		200	20.4 (±2.0)	72.9 (±5.9)	0.28 (±0.05)
CC52-MPB-PEG[a]	33	80	2.0 (±0.3)	2.1 (±0.2)	0.95 (±0.26)
		200	3.9 (±0.4)	4.2 (±0.1)	0.93 (±0.13)
CC52-Hz-PEG[a]	8	80	3.0 (±0.1)	2.2 (±0.1)	1.37 (±0.13)
		200	7.7 (±0.5)	4.2 (±0.6)	1.84 (±0.37)
CC52-MPB-PEG[b]	28	80	2.8 (±0.3)	10.9 (±1.5)	0.26 (±0.06)
		200	5.6 (±0.2)	18.4 (±1.6)	0.31 (±0.04)
CC52-Hz-PEG[b]	28	80	8.7 (±0.8)	30.5 (±7.8)	0.28 (±0.11)
		200	12.6 (±0.6)	60.5 (±3.3)	0.21 (±0.02)

Note: The targeting capacity of the immunoliposomes to CC531 is given by the association ratio in the last column, representing the amount of immunoliposomes associating with CC531 cells vs. Kupffer cells. Values higher than 1 indicate higher levels of interaction with target cells than with Kupffer cells.

[a,b] Experiments with the same letter were performed with one batch of freshly isolated rat Kupffer cells allowing intraexperimental comparison between different liposome types.

two liposome concentrations. The ratio of tumor cell over Kupffer cell uptake was calculated. Clearly, only the type II immunoliposomes with low antibody density (8 μg/μmol) produced a ratio higher than 1, i.e., an association in favor of the tumor cells. For the type I liposomes, with an antibody density of 33 μg/μmol, a ratio close to 1 was observed. With higher antibody densities both liposome types lose their preferential association with CC531 cells.

DISCUSSION

Kupffer cells together with the splenic macrophages are largely responsible for the competitive removal from the blood circulation of liposomes designed for (tumor) cell specific targeting. Therefore, we chose this cell type for an *in vitro* study on the balance between uptake by (tumor) target cells and uptake by macrophages of liposomes containing covalently coupled target-cell-specific antibodies.

Of the two antibody coupling methods we used, one resulted in a randomly oriented attachment of the antibody directly to the bilayer and the other in a non-random, Fab'-exposing orientation of the antibody, linked via its Fc-portion, at the distal end of bilayer-anchored PEG.

Especially at low antibody densities, coupling of the antibody to the distal end of the PEG-chains resulted in superior target cell interaction when compared with coupling directly to the bilayer. This can be explained either by a larger fraction of antibody displaying the proper orientation for interaction with the cell-surface antigen or by reduced steric interference of the PEG-chains with the antibody-antigen interaction (9,11,23).

Increasing the antibody density on type II immunoliposomes to more than 30 μg/μmol lipid led to a reduction in target cell interaction. We attribute this to the experimental conditions required to attain such high antibody densities. Elevated temperature and prolonged incubation times are likely to result in progressive oxidative damage of the antibody molecules, as has been described before (16) and this may jeopardize antigen recognition.

Here we observed that both types of PEG-immunoliposomes associate with the tumor cells to much higher extents at 37 °C than at 4 °C. This difference cannot be attributed to a distinction between adsorption (4°C) and internalization (37°C) as in earlier work we observed that CC52-immunoliposomes are not internalized by the CC531 tumor cells (6,24). Since we are not aware of any evidence indicating that antibody-antigen interaction is strongly temperature-dependent, the observed temperature effect may be related to an effect on the mobility of either the antigen in the cell membrane or of the antibody in the liposomal bilayer. The antibody coupled to the distal end of the PEG chains is likely to be in a more flexible position than that coupled directly to the bilayer. Nonetheless, the temperature dependence was the same for both immunoliposome types. Therefore, we consider it more likely that the temperature effect involves antigen accessibility and is at the level of the cell membrane rather than at the level of the liposomal bilayer.

Irrespective of the coupling method, antibody coupling also enhanced the association of liposomes with Kupffer cells. This can be attributed in part to interaction with Fc receptors on the macrophages, as described for large unilamellar vesicles by Derksen *et al.* (19). PEG can only partially prevent this effect.

The incorporation of PEG-DSPE in the control liposomes without antibody caused a 2-fold decrease in the uptake by the Kupffer cells. It is noteworthy that this PEG effect was obtained in the absence of serum. This indicates that prolonged circulation time caused by PEG cannot be explained fully on account of diminished adsorption of opsonic factors (25,26). Direct interference with liposome-cell interaction may play an additional role, compatible with a mechanism we proposed earlier for the interaction of negatively charged liposomes with proteins on the cell surface of Kupffer cells (27).

Unexpectedly, the uptake of the immunoliposomes with correctly oriented antibody was higher than that of liposomes with the randomly coupled antibody, particularly at high antibody densities. Only at a low antibody density Kupffer cell uptake was moderate. The substantial inhibition of uptake by aggregated IgG indicates that even in the case of immunoli-

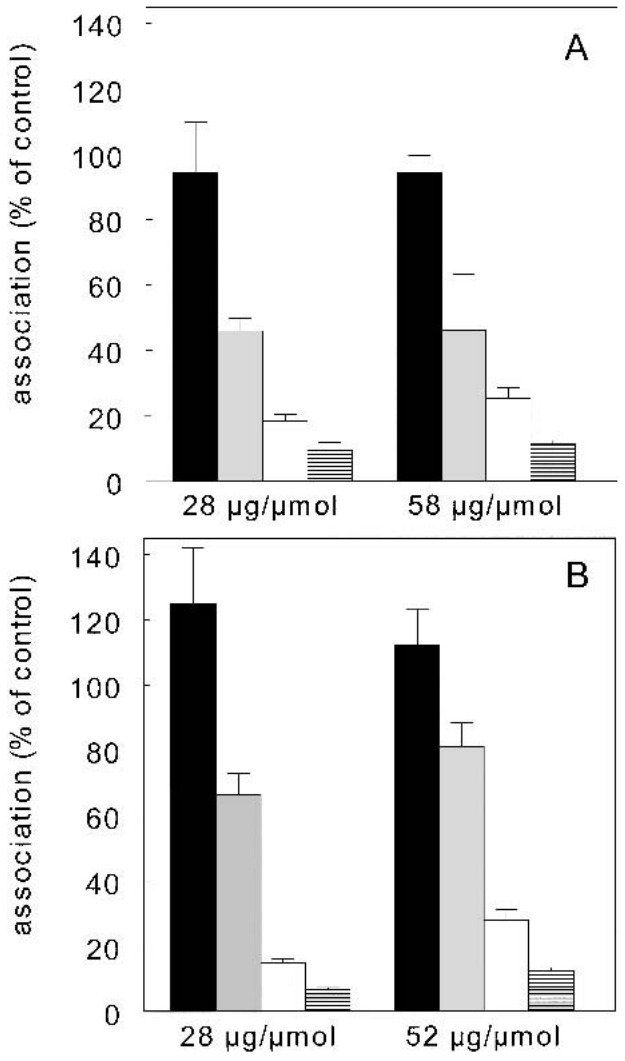

Fig. 4. Effect of aggregated IgG on Kupffer cell uptake of immunoliposomes. Kupffer cells were incubated with radiolabeled PEG-immuno-liposomes in presence of aggregated IgG (1 mg/ml) and amount of lipsome uptake was determined (hatched bars). BSA (1mg/ml) was used as a control (black bars). Also the association at 4°C (open bars) and the effect of aggregated IgG at 4°C (horizontally-hatched bars) were determined. Results are expressed as a percentage of control incubations without inhibitors at 37°C. (A) Kupffer cells incubated with 80 nmol type I immunoliposomes at an antibody density of 28 or 58 µg/µmol. (B) Kupffer cells incubated with 80 nmol type II immunoliposomes at an antibody density of 28 or 52 µg/µmol.

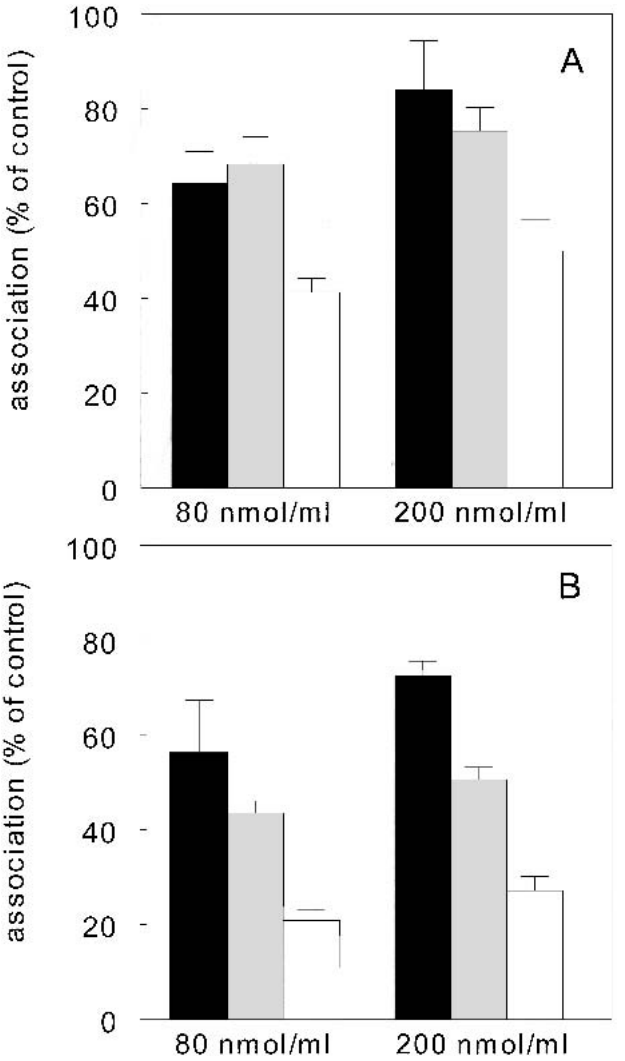

Fig. 5. Effect of poly-inosinic acid on Kupffer cell uptake of immunoliposomes. The involvement of scavenger receptors on Kupffer cells in the uptake of type II immunoliposomes was studied by inhibiting these receptors using polyinosinic acid (poly I). Results are expressed as percentage of control values obtained without inhibitors. Kupffer cells were incubated for 3 h at 37°C with 80 or 200 nmol type II immunoliposomes in the presence of: Poly I (20 µg/ml) (black bars), aggregated IgG (1 mg/ml) (hatched bars) or a combination of Poly I and aggregated IgG (open bars). (A) Effects at a low antibody density (10 µg/µmol). (B) Effects at a high antibody density (28 µg/µmol).

posomes with the antibody in the correct orientation, Fc receptors still recognize the Fc portion of the antibody, although to a lower extent than for the immunoliposomes with the randomly coupled antibody.

It is likely that the reaction conditions required to obtain higher antibody densities by this coupling method caused oxidative damage to the CC52 antibody. Indeed, experiments using polyinosinic acid as an inhibitor of scavenger receptor activity (21) confirmed the involvement of these receptors in the uptake of type II immunoliposomes, in which the oxidized antibody may serve as a ligand for the scavenger receptors on Kupffer cells, as has been shown for instance for oxidized LDL (22).

The high uptake by Kupffer cells of the type II immunoliposomes at high antibody density is compatible with *in vivo* studies reporting substantially decreased circulation times for such liposomes at increasing antibody density (5,7). Harding *et al.* also reported the development in rats of an immune response against the constant domain of the hydrazide-coupled human/mouse chimerized antibodies (7). This is consistent with the notion that this part of the molecule remains accessible following coupling to the PEG chains, both to receptors on the Kupffer cells and to the immune system. Lopes de Menezes *et al.*, on the other hand, did not observe changes in pharmacokinetics in mice of Hz-PEG-immunoliposomes with a murine antibody specific for B-cell lymphoma attached at antibody densities up to 40 µg/µmol

TL (28). This may be a reflection of the beneficial effects on pharmacokinetics of the use of auto-antibodies. Also differences in sensitivity of different antibody species to the oxidative conditions involved in the coupling procedure may have an effect, especially with respect to inducing scavenger receptor affinity. Maruyama *et al.* also observed decreased circulation time and enhanced liver uptake of PEG-immunoliposomes with the antibody coupled to the PEG chains distally (9).

Prevention or reduction of Fc-moiety exposure might be achieved by coupling the antibodies to shorter PEG-chains in liposomes containing PEG-DSPE with a regular chain length, i.e., with a molecular weight of 2000. Obviously, complete removal of the Fc region and coupling of the Fab' or $F(ab')_2$ fragments would provide an even more rigorous solution of Fc-receptor recognition (2,29). The preparation of antibody fragments, however, requires additional steps in the coupling procedure resulting in loss of antibody and in loss of affinity (29). Few studies using this approach have been carried out thus far. No differences in pharmacokinetic behavior between PEG-liposomes with or without distal end coupled Fab'-fragments were reported (2,30), but considerable amounts of liposomes (2) or of liposome-encapsulated doxorubicin were still retrieved in the liver (29).

In conclusion, in this paper we demonstrate that the presence of antibodies, coupled in different ways to liposomes, not only enhances the interaction with antibody-specific target cells, but also results in a substantial increase in the affinity of the liposomes for macrophages such as Kupffer cells. The delicate balance between association with target cells and association with Kupffer cells tends to shift toward the latter when antibody density is increased. Only the type II immunoliposomes with a relatively low amount of antibody show a significantly higher ratio of uptake by the target cells relative to Kupffer cells and in this sense display better targeting capacity than type I immunoliposomes. The results presented emphasize the significance of the amount of coupled antibodies, and the position and orientation of the antibody molecules at the liposomal surface on the balance between *in vivo* target-specific delivery and competitive uptake by macrophages.

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