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Immunogenicity and pharmacokinetic attributes of poly(ethylene glycol)-grafted immunoliposomes

Jennifer A. Harding [a], Charles M. Engbers [a], Mary S. Newman [a], Neil I. Goldstein [b], Samuel Zalipsky [a,*]

[a] *SEQUUS Pharmaceuticals, Incorporated, 960 Hamilton Court, Menlo Park, CA 94025, USA*
[b] *ImClone Systems, Incorporated, 180 Varick Street, New York, NY 10014, USA*

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Abstract

Immunoliposomes composed of hydrogenated soy phosphatidylcholine, cholesterol, methoxypoly(ethylene glycol)-distearoyl phosphatidylethanolamine (mPEG-DSPE), and hydrazide-PEG-DSPE (mole ratio, 57:38:3.3:1.7) linked to periodate-oxidized chimerized mouse IgG (C225, anti-human epidermal growth factor receptor) were prepared by an optimized aggregation-free procedure. The antigen-binding activity of the immunoliposomes was well preserved. When injected intravenously into naive rats, the immunoliposomes (~ 18 IgG per 100 nm liposome) exhibited long circulation times (MRT = 8.5 h, Cl = 0.2 ml/h). Subsequent injections of the immunoliposomes into the same animals resulted in rapid clearance (MRT ≤ 0.7 h, Cl ≥ 7 ml/h), which was accompanied by a significant increase in anti-C225 specific titers. Upon repeated injection or coinjection with the parent liposomes free C225 consistently exhibited prolonged circulation without any increase in C225-specific antisera, but was cleared quickly when administered into animals that had been pretreated with the immunoliposomes. Screening of the immunoliposome induced antisera against human polyclonal IgG and C225-derived Fab′ fragment revealed that the immune response was specifically triggered by the constant human region of C225. These results demonstrate that the preparations of PEG-grafted immunoliposomes are more immunogenic than the free IgG component, which is of profound importance to the antibody-mediated liposomal drug delivery effort. © 1997 Elsevier Science B.V.

Keywords: Antibodies; Immunoliposomes; Polyethylene glycol (PEG); Immunogenicity; Pharmacokinetics

1. Introduction

Recent development of unilamellar lipid vesicles (liposomes) containing external surface-grafted poly(ethylene glycol) (PEG) chains holds a great promise for liposomal drug delivery [1]. PEG-grafted liposomes exhibit dose-independent long-circulating blood lifetimes, reduced uptake by organs of mononuclear phagocyte system (liver and spleen), and enhanced accumulation in tumors [2–5]. These

Abbreviations: PEG, poly(ethylene glycol); mPEG, methoxy-PEG; DSPE, distearoyl phosphatidylethanolamine; Hz, hydrazide; EGFR, epidermal growth factor receptor; HSPC, hydrogenated soy phosphatidyl choline; TMB, 3,3′,5,5′-tetramethylbenzidine; AUC, area under curve; MRT, mean residence time; NAM, *N*-acetylmethionine

* Corresponding author. Fax: 415-617-3080; E-mail: SamuelZ@SEQUUS.com

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properties are in striking contrast to those of classical (non PEG-containing) liposomal preparations, which are quickly removed from the circulation by the liver, limiting their use for systemic drug delivery. For similar reasons, antibody-mediated targeting of classical liposomes have been disappointing. In the last several years a number of advances have taken place in methodologies for preparation of PEG-grafted immunoliposomes (for review, see ref. [6]). It is believed that since PEG-liposomes persist in circulation for a long period of time there should be a better opportunity for the liposome-bound antibody to encounter its target and thus result in better accumulation at that site. Several approaches to the preparation of PEG-grafted immunoliposomes have been explored by various groups of investigators. We favor the methodology of linking antibodies to the distal ends of liposomal surface-grafted PEG chains for two reasons [7–10]. This should minimize interference of the polymer chains: (A) in antibody–antigen interaction, and also (B) in conjugation reactions, i.e. the coupling of antibodies to liposomes. Both phenomena were observed when antibodies were conjugated to polar head groups of phospholipid components of PEG-grafted liposomes [3,10,11]. To explore the distal end coupling, several end-group functionalized PEG–lipid conjugates were introduced recently [8–10,12–14]. These derivatives incorporated into liposomes allow for facile coupling of immunoglobulins, often with good preservation of antigen-binding activity. Thus the field of PEG-grafted immunoliposomes is now well positioned for accumulation of in vivo data which should provide a true measure of the value of this approach. A number of animal models have been already investigated with different formulations of PEG-immunoliposomes, examining such variables as particle size, coupling chemistry, antibody density, density of the grafted polymer, and the ability to deliver increased quantities of drug to the target sites [3,9–11,15–17]. Although immunogenicity of classical liposomes and immunoliposomes has been studied extensively [18–22] (for reviews, see refs. [23,24]), this important parameter as it pertains to the PEG-containing immunoliposomes has thus far received little attention [25]. [1]

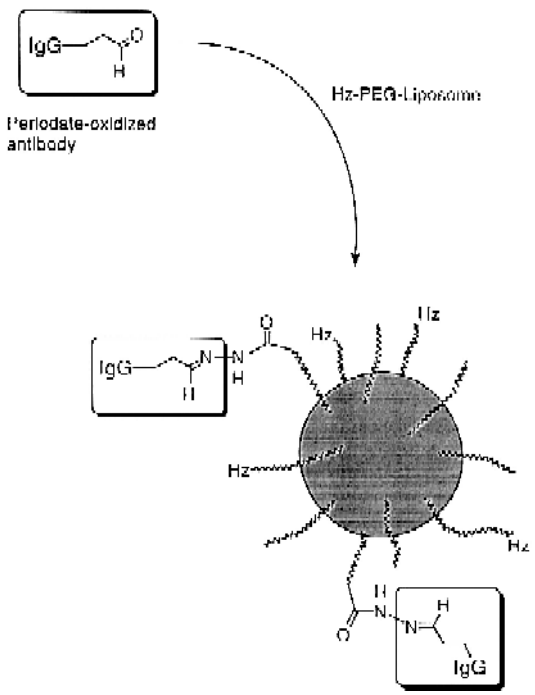

Fig. 1. Schematic depiction of the immunoliposome formation

It is relevant to note that the experience with PEG-proteins shows that the polymer conjugation often results in reduced immunogenicity [27,28] and in some cases even induces tolerance to the protein component of the conjugate [29].

In this manuscript we present the results of our experiments with preparations of liposomes bearing residues of chimeric monoclonal antibody to the EGFR (C225) covalently attached through their carbohydrate moieties to hydrazide end-groups of PEG chains grafted onto the liposomal surface (Fig. 1). In vivo experiments were designed to gain an insight into issue of immunogenicity of PEG-immunoliposomes. In particular, we studied immunoliposome formulations optimized to exhibit extended circulation lifetimes while retaining antigen binding ability. After repeated i.v. injections in rats these formulations consistently exhibited rapid clearance

[1] Another reference [26] in which α,ω-bis-stearoyl-PEG400 was used in immunoliposome formulations to enhance their adjuvanticity, was brought to our attention by one of the reviewers. The derivative used contained stearate ester positioned at both termini of short oligoethylene glycol fragment (average length 9 oxyethylene units). It is not capable of steric stabilization nor of conveyance of long-circulating properties to its liposomes. Thus the relevance of this PEG-derivative and its formulations to the current work is marginal.

from the bloodstream with concomitant increase in antisera titers specific to C225. To the best of our knowledge, this is the first study examining the immunogenicity of PEG-immunoliposomes constructed by a well-defined, aggregation-free chemistry. The results have important implications for antibody-mediated liposomal drug delivery.

2. Experimental procedures

2.1. Materials and methods

Hz-PEG-DSPE and mPEG-DSPE were synthesized from PEG and mPEG, both of molecular weight 2000 Da (Fluka) as described in detail elsewhere [12]. Sodium periodate, N^{α}-acetyl-methionine (NAM), cholesterol, desferoxamine mesylate, Human IgG, and BSA were purchased from Sigma (St. Louis, MO). Hydrogenated soy phosphatidylcholine (HSPC) was obtained from Lipoid (Ludwigshafen, Germany). The Sepharose CL-4B size exclusion gel was from Pharmacia. The radiochemicals $Na^{125}I$ and ^{67}Ga citrate were obtained from Amersham, Arlington, IL, and Syncor International Corp., Chatsworth, CA, respectively. The 96-well plates used for the ELISA assay were Immulon 1 from Dynatech Laboratories, Chantilly, VA. Human clinical grade C225 (monoclonal chimerized anti-EGFR) was produced by ImClone Systems, New York, NY. The secondary antibody, peroxidase-labelled goat anti-rat IgG, was purchased from Gibco BRL, Grand Island, NY. The turbo-TMB, Iodo-Gen tubes, ImmunoPure IgG1 Fab and $F(ab)_2$ preparation kit 44880 were obtained from Pierce Chemical Co., Rockford, IL. The 4–20% SDS-PAGE redi-gels and the Bradford protein assay reagent were from Bio-Rad (Hercules, CA). Amino acid analysis was performed on hydrolysed aliquots of C225-containing samples at the Protein Structure Laboratory of University of California, Davis. The particle size of liposomes and immunoliposomes was measured by dynamic light scattering (Coulter N4MD, Hialeah, FL). Phospholipid concentrations were measured by phosphorus determination [30].

2.2. Liposome preparation

Liposomes were formulated from HSPC, cholesterol (mole ratio, 57:38), and PEG–lipid conjugates (combined mPEG-DSPE and Hz-PEG-DSPE, 5 mole%). The molar ratio of the above components was maintained as 57:38:3.3:1.7 after the optimization of the conjugation protocol. The liposomes were prepared by solvent evaporation, hydration in 10 mM desferoxamine mesylate in isotonic saline (0.9% NaCl) solution, and extrusion through 0.2-, 0.1- and 0.05-μm membranes down to approximately 100 nm, according to the previously published protocols [31,32]. The external buffer was exchanged for 0.1 M acetate buffer pH 5.5 by dialysis.

Radiolabelling of liposomes as well as immunoliposomes with ^{67}Ga-oxine resulting in entrapment of the label by desferoxamine chelation in the liposomal aqueous compartment was achieved as previously described [33].

2.3. Iodination of antibody

Iodo-Gen tubes were prepared according to manufacturers instructions and stored at 4°C. The C225 antibody solution (200 μl, 1.6 mg/ml) in acetate buffer (0.1 M, pH 5.5) was added to a prerinsed Iodo-Gen, followed by $Na^{125}I$ (37 mBq). After incubation for 1 h the contents of the tube were transferred to a G25-80 spin column and spun for 2 min at 1500 rpm at room temperature to remove any unreacted ^{125}I.

2.4. Preparation of immunoliposomes

2.4.1. Small-scale optimization experiments (see Table 1)

A solution of C225 IgG (100 μl at 1.6 mg/ml) in acetate buffer (0.1 M, pH 5.5) was spiked with ^{125}I-labeled antibody (5 μl). It was then treated with an appropriate amount of sodium periodate stock solution (100 mM) and incubated under conditions indicated in Table 1. At the end of the oxidation period the excess periodate was quenched by the addition of NAM solution (10 μl at 500 mM). The oxidized antibody was mixed with hydrazide-containing liposomes (100 μl at 35 μmol phospholipid/ml) in the acetate buffer and the reaction mixture was incubated overnight at 6°C. The conjugate was separated from the free antibody on Sepharose CL-4B. Column fractions were counted on a gamma counter

Table 1
Optimization of conjugation conditions [a]

Hz content [b] (mole%)	Oxidation time (min)	Oxidation temperature (°C)	Periodate concentration (mM)	% of conjugated IgG [c]	% increase in size [d]
5	60	25	10	99	88
5	60	25	6	95	85
5	60	25	4	95	82
5	60	25	2	94	36
5	40	25	10	91	85
5	20	25	10	94	87
5	60	6	10	93	41
5	20	6	2	79	32
1.7	20	6	2	70	25
1.7	20	6	2	73	21

[a] Detailed procedure is given in Section 2.
[b] Liposomes composed of HSPC, cholesterol, and Hz-PEG-DSPE in molar ratio of 57:38:5, except for the last two entrees, where liposomes contained both mPEG-DSPE and Hz-PEG-DSPE (3.3 and 1.7 mole%, respectively) without alterations in the other components.
[c] Determined from the integration of chromatographic trace (see Fig. 2).
[d] Increase in particle size of the parent liposomes (~ 100-nm diameter) resulting from the conjugation process.

and the percent of conjugated antibody was calculated by dividing the counts in the liposomal peak by the total counts loaded on the column (Fig. 2). Particle size was determined by dynamic light scattering.

2.4.2. Preparation of immunoliposomes (preparative procedure)

A solution of C225 antibody (5.5 ml, at 3 mg/ml) was incubated with sodium periodate (2 mM, final concentration) in acetate buffer (0.1 M, pH 5.5) at 6°C, for 20 min. NAM (0.5 ml of 500 mM) was added to quench the excess periodate [32]. Hydrazide-liposomes (5.6 ml at 47 μmol phopholipid/ml) in the same buffer were added to the solution, and the mixture was incubated overnight at 6°C. Separation of the immunoliposomes from the free antibody was performed on a 2.5 × 80 cm Sepharose CL-4B size exclusion column (Pharmacia). The immunoliposome fractions were pooled and the average number of antibody molecules per liposome was calculated from the results of amino acid analysis and phosphate analysis as summarized in Table 2.

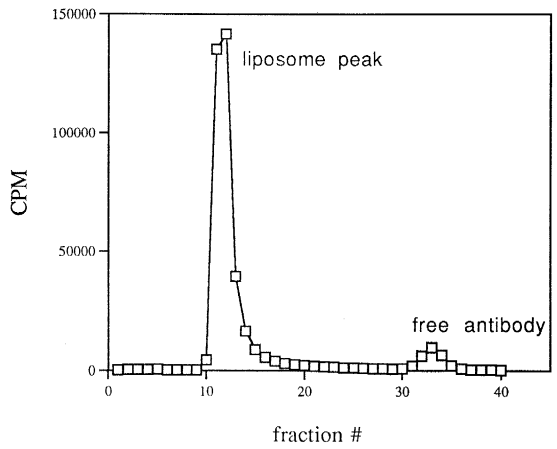

Fig. 2. Size-exclusion chromatography separation on Sepharose CL-4B column of the ^{125}I-labeled C225 antibody eluting in liposomal peak from the free unconjugated protein.

2.5. Determination of antigen-binding activity

DU145 (androgen-independent prostate carcinoma; EGFR-positive) and A431 (epidermoid carcinoma; EGFR-positive) were obtained from the ATCC (Rockville, MD) and routinely grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (Intergen, Purchase, NY), 2 mM L-glutamine and antibiotics.

FACS analysis: DUI45 cells (human prostate carcinoma, androgen-independent EGFR expressing) were removed from T-flasks with 2 mM EDTA, washed with serum-free Dulbecco's modified Eagle's medium containing 1% BSA (D-BSA) and added to glass tubes at a concentration of $(0.5–1.0) \times 10^6$

Table 2
Determination of immunoliposome composition by Amino Acid Analysis (AAA)

Amino acid	Residues/C225	nmol/AAA injection	nmol IgG/AAA injection
Asp	118	7.041	0.0597
Thr	104	6.508	0.0626
Ser	161	9.759	0.0606
Pro	96	5.56	0.0579
Ala	67	3.656	0.0546
Val	119	7.003	0.0588
Ile	38	2.253	0.0593
Leu	109	6.49	0.0595
Tyr	58	3.405	0.0587
Phe	48	2.888	0.0602
Lys	82	5.387	0.0657
Arg	38	2.204	0.0580

An aliquot of immunoliposome solution was hydrolysed in 6 N HCl, and then subjected to a standard amino acid analysis protocol. The following amino acids were excluded from the calculation: Glu, Gly, Met, His. The amino acid composition of C225 is listed in the second column. The values in the third column obtained directly from the AAA integration, were divided by the corresponding values in the second column. This provided the amount of each amino acid in nmol IgG units (fourth column). Averaging of the numbers in the right column provided the amount of 0.06 nmol IgG per AAA injection. This value adjusted for dilutions leads to the concentration of the immunoliposome solution aliquots of which were submitted for both phosphate and amino acid analysis (2.4 nmol IgG/ml = 10 μmol phospholipid/ml). Thus the mole ratio of IgG/phospholipid was $2.4 \cdot 10^{-4}$ (38.4 μg protein/μmol phospholipid). Assuming 75,000 phospholipid molecules per vesicle of 100 nm, this ratio corresponds to 18 IgG residues/liposome.

cells/tube. For direct binding studies, C225, immunoliposomes, parent liposomes, or irrelevant human IgG (Sigma) were added at a concentration of 20 μg protein/tube for 60 min on ice. After washing, goat anti-human IgG conjugated to FITC (Tago, Burlingame, CA) was added for an additional 30 min on ice. For competitive inhibition experiments, cells were first incubated with immunoliposomes, parent liposomes, human IgG, or C225 at a concentration of 20 μg protein/tube for 30 min on ice followed by FITC-labelled C225 (5 μg/tube; a gift of Dr. N. Giorgio, ImClone Systems) for an additional 60 min. Both sets of samples were analyzed for cell surface fluorescence using a Coulter Epics ELITE sorter. Results are presented as the Mean Fluorescence Intensity (MFI) which is an indirect measure of antigen expression and relative affinity [34,35]. MFI is determined by dividing the percent positive cells by the mean channel of fluorescence.

2.6. Fab′ fragment preparation

The C225-derived Fab′ fragment was obtained using the ImmunoPure IgG1 Fab and $F(ab')_2$ preparation kit (Pierce, Rockford, IL) following the instructions provided with the kit. Briefly, a solution of C225 antibody (1 mg in 0.5 ml digestion buffer containing 10 mM cysteine) was loaded onto a Ficin immobilized column and incubated for 5 h at 37°C. The Fc portion and undigested antibody were removed by protein A column chromatography. The purity of the Fab fragment was checked by SDS-PAGE using 4–20% polyacrylamide gels in electrophoresis buffer (25 mM Tris, 250 mM glycine, 0.1% SDS, pH 8.3). Samples were electrophoresed under non-denaturing conditions at 100 V for 2 h and visualized by Coomasie blue staining. This revealed a single band of 50–55 kDa corresponding to the Fab′ product. Under reducing conditions SDS-PAGE produced single-band of approximately 25 kDa. The concentration of the Fab product was determined by Bradford protein assay, using BSA as a standard. The yield of the process was 0.28 mg (42%).

2.7. Determination of anti-C225 antibody titers and cross-reactivity of antisera

The antibody titers in rat serum samples were analyzed by sandwich ELISA at room temperature using Immulon 1 microtiter plates. First the plates were incubated with a tested protein (C225 antibody, C225-derived Fab′, human IgG, or irrelevant protein, BSA) solution (10 μg/ml, 100 μl per well) for 2 h. The plates were washed twice with PBS, treated with blocking buffer (1% normal rat sera in PBS) for 2 h, and then washed again twice with PBS. Diluted sera (1:100) from immunized animals was added (100 μl per well). The plates were incubated for 1 h, and then washed with PBS/0.5% Tween-20 to remove the unbound protein. Then a peroxidase-labelled goat anti-rat IgG solution (100 μl, diluted 1:20,000 in the blocking buffer) was added to each well. The plates were incubated again for 1 h. The unbound antibody

Table 3
Tissue distribution at 24-h post-dose of C225, parent liposomes and conjugates thereof in percent of injected dose per tissue [a]

Sample	Liver	Spleen	Blood
Parent liposomes [b]	11.0 ± 1.5	8.4 ± 1.1	45.1 ± 3.6
Free C225 [c]	3.0 ± 0.2	0.2 ± 0.0	39.1 ± 4.7
Immunoliposomes:			
10 IgG/vesicle	32.5 ± 4.0	11.9 ± 1.6	16.3 ± 1.7
18 IgG/vesicle	21.8 ± 2.2	24.3 ± 1.7	3.7 ± 0.6
24 IgG/vesicle	30.1 ± 2.6	18.0 ± 4.9	3.1 ± 0.9
40 IgG/vesicle	34.5 ± 3.9	6.1 ± 0.9	0.6 ± 0.1

[a] Other tissues sampled (heart, kidneys, lungs, skin, bone and muscle) routinely contained less than 1.5% of the injected dose.
[b] Repeated injections or coinjections with C225 antibodies yielded the same tissue distribution.
[c] Repeated injections or coinjections with parent liposomes yielded the same tissue distribution.

was removed by washing with the PBS/Tween-20 solution. The signal was measured by addition of substrate, Turbo-TMB solution (100 μl) to each well and incubating for 10 min. The reaction was terminated by addition of sulfuric acid (2 M, 50 μl per well). The plates were read at 450 nm on a plate reader.

2.8. Animal experiments

In vivo studies were conducted using adult male Sprague Dawley rats (250–400 g) that were dosed by bolus tail vein injection. Single-dose pharmacokinetic and biodistribution studies utilized 4 animals per experiment. Subsequent repeated-dose immunogenicity studies utilized 16–20 animals per experiment to enable interim sacrifices (4–6 rats per sacrifice) after each dose for tissue distribution evaluations. The appropriately radiolabelled samples (C225, parent liposome, mixture thereof, or immunoliposome) prepared in isotonic saline were administered intravenously at a dose of approximately 10–20 μmol phospholipid/kg body weight. Blood levels at various times were determined by retro-orbital bleeding while tissues were obtained surgically 24-h post-dose. Tissue and blood levels of ^{67}Ga and/or ^{125}I radioactivity were determined using a Beckman 5500 gamma counter (Table 3). Radioactivity in blood and tissue samples were converted into percent of injected dose units. For immunogenicity experiments the animals were subjected to three repeated i.v. injections on days 0, 14, and 28. Serum samples were obtained at selected time points (see Fig. 7) by collecting whole blood on ice, and removing the blood cells by cen-

Table 4
Pharmacokinetic parameters

Sample	$MRT_{0\rightarrow}$ (h)	$AUC_{0\rightarrow}$ (% dose h/ml)	$T_{1/2}\,\beta$ (h)	Cl (ml/h)
Parent liposomes [a]	37.6 ± 1.0	2686.0 ± 165.5	19.1 ± 0.6	0.037 ± 0.002
Free C225 [b]	39.5 ± 9.4	2942.0 ± 605.2	27.7 ± 6.5	0.035 ± 0.007
Immunoliposomes:				
10 IgG/vesicle	20.3 ± 2.1	1104.9 ± 106.4	14.3 ± 1.6	0.091 ± 0.009
18 IgG/vesicle [c]				
Dose 1	8.6 ± 0.5	495.4 ± 51.0	6.4 ± 0.4	0.20 ± 0.02
Dose 2	0.7 ± 0.2	13.9 ± 1.1	0.5 ± 0.1 [d]	7.23 ± 0.54
Dose 3	0.4 ± 0.1	12.8 ± 1.6	0.3 ± 0.1 [d]	7.92 ± 0.88
24 IgG/vesicle	9.3 ± 0.6	362.0 ± 83.6	7.0 ± 0.5	0.29 ± 0.07
40 IgG/vesicle	7.6 ± 0.6	98.1 ± 14.9	6.5 ± 0.8	1.04 ± 0.16

[a] Repeated injections or coinjections with C225 antibodies yielded the same clearance curves and pharmacokinetic parameters.
[b] Repeated injections of ^{125}I-labeled C225 antibodies or coinjections with parent liposomes yielded the same clearance curves and pharmacokinetic parameters.
[c] Repeated injections of immunoliposomes yielded significantly different circulation times after the second and third administration.
[d] The clearance rate of the immunoliposomes after the second and third injections was mono-expontial.

trifugation after allowing 20 min for clotting. Pharmacokinetic analysis of concentration vs. time data was performed using noncompartmental methods with the RSTRIP program (Micromath, Salt Lake City, UT). The software provides values for areas under the zero (AUC) and first moment (AUMC) concentration curves from which the derived parameters (Table 4) were calculated. Mean residence time (MRT), the time it takes to eliminate 63.2% of the administered dose, was derived by the formula: MRT = AUMC/AUC, while total clearance (Cl) was determined by dividing dose by AUC.

3. Results and discussion

3.1. Preparation of immunoliposomes

Immunoliposomes were prepared by coupling periodate-oxidized C225, IgG to hydrazide-PEG-DSPE containing liposomes. As schematically depicted in Fig. 1, hydrazone attachments are formed between aldehyde-bearing oxidized IgG and hydrazide end groups on the periphery of the liposome. Immunoglobulin conjugation utilizing the specific reactivity of their oxidized carbohydrate residues has been known to produce active adducts [36–38]. This is explained by the fact that the conjugation occurs site-specifically via the oligosaccharide moieties positioned on the Fc portion of the glycoprotein molecule, far away from the antigen binding sites [38,39]. Our initial experiments showed that the coupling conditions used in some of our previous studies with other immunoglobulins [7,11,15,40,41] produced severe aggregation of the immunoliposomes. This was manifested by considerable increase in the particle size of the product. By gradually moderating the conditions of the conjugation reaction, as summarized in Table 1, we arrived at a final procedure which was used for the remainder of this study (last two entries in Table 1). These optimization experiments were monitored by particle size measurements and size-exclusion chromatography, which allowed us to determine how much of the total ^{125}I-IgG was eluted in the liposomal peak (Fig. 2). Since the starting liposomes were on average 100 nm in diameter, and IgG molecule can be viewed roughly as a 100-Å sphere [42], an approximately 20% increase in size as a result of conjugation was judged as reasonable. The final optimized conditions included oxidation of the antibody at 6°C, with 2 mM periodate for 20 min and then conjugation with liposomes containing only 1.7 mole% of Hz-PEG-DSPE (the rest of the components, HSPC, cholesterol, mPEG-DSPE, in molar ratio 58:38:3.3). These oxidation conditions closely approximate those reported to specifically modify sialic acid residues, and they are unusually mild for conjugation of immunoglobulins [39].

3.2. Single-dose pharmacokinetics of immunoliposomes

Varying the amount of oxidised C225 and its ratio to hydrazide-bearing liposomes we prepared samples containing 10, 24, and as many as 40 IgG residues per vesicle. Pharmacokinetic characteristics of these preparations and the parent liposomes were evaluated in rats after radiolabelling the liposomal aqueous compartment with ^{67}Ga-desferal [33]. As can be seen from Fig. 3A increasing the amount of bound antibody per vesicle facilitated faster clearance from blood circulation. Labelling the IgG with ^{125}I-label in addition to the ^{67}Ga in the aqueous compartment, and following the fate of both markers in blood showed identical pharmacokinetic profiles (Fig. 3B). This result suggests that the immunoliposomes remained intact for the duration of the experiment. Tissue distribution at 24 h post injection from these experiments is summarized in Table 3, while the pharmacokinetic parameters are summarized in Table 4. These data show that the hepatosplenic uptake was markedly increased for faster-clearing immunoliposome formulations. Moreover, these results are in agreement with the previous observations that attachment of increasing amounts of immunoglobulin [9,10,41], or even low-molecular-weight peptide [32], to the periphery of PEG-grafted liposomes results in faster clearance from systemic circulation. In order to balance prolonged circulation lifetime with a reasonably high antibody density, based on the pharmacokinetic curves depicted in Fig. 3A, we decided to use 10–24 IgG per vesicle in the subsequent studies.

3.3. Antigen binding activity

Preservation of antigen-binding activity of the immunoliposomes was corroborated by both direct and

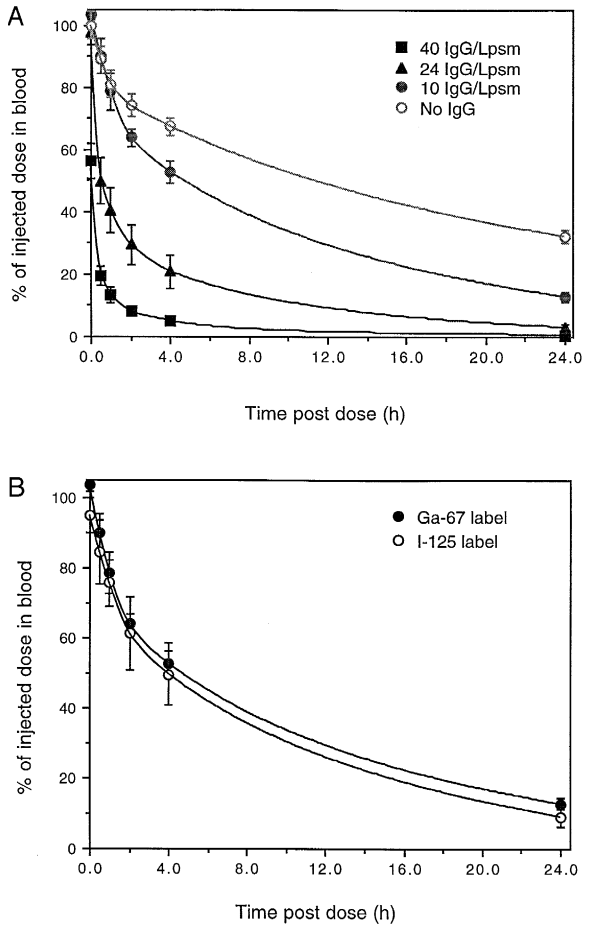

Fig. 3. Pharmacokinetic profiles of parent and C225-bearing liposomes (mean ± SD). Four rats per experiment. (A) Blood clearance rates of ^{67}Ga-labeled liposomes carrying various amounts of C225 antibody. (B) Comparison of plasma clearance curves obtained by following ^{67}Ga-aqueous compartment label, and ^{125}I-conjugated C225 label of immunoliposomes containing ≈ 10 IgG residues per liposome.

competitive binding assays using DU145 cells (EGFR-positive, androgen-independent prostate carcinoma). Both methods produced results consistent with each other (Fig. 4). Similar antigen-binding activities were observed with A431 cells (EGFR-positive, epidermoid carcinoma, data not shown). The direct assay results suggest somewhat lower activity of the immunoliposomes in comparison to the free C225. Although the possibility that the antigen-binding affinity was diminished by the conjugation cannot be excluded, in light of the mildness of our conjugation conditions it seems more likely that the binding of the liposomal conjugate to the cells was affected by the size of the conjugate or accessibility of its Fc residues. The latter factor is important in quantitation of the EGFR-positive cell-bound immunoconjugate using goat anti-human FITC-linked IgG. In fact detection of antigen binding using this method might be in contrast to what could have been expected, because it suggests that despite our use of Fc-directed site-specific conjugation method, the Fc residues of the resulting immunoliposomes were still exposed to a measurable extent.

3.4. Immunogenicity studies

All the above pharmacokinetics experiments (Fig. 3) involved only a single i.v. injection per formula-

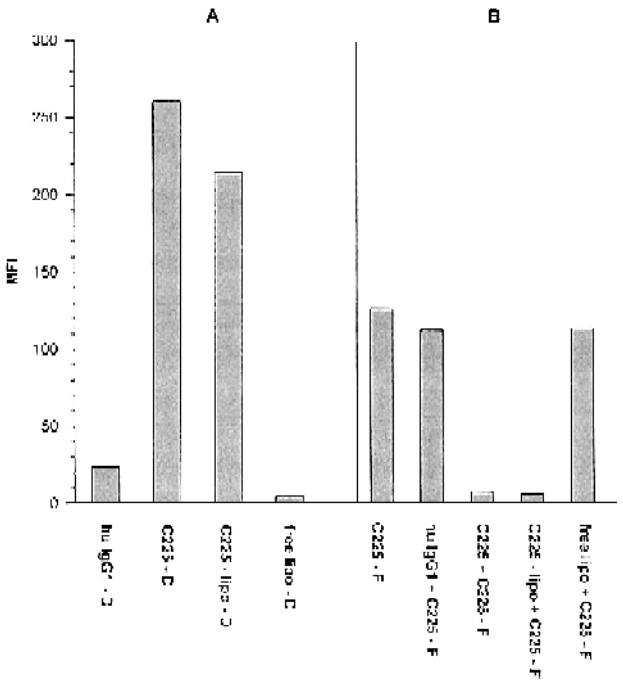

Fig. 4. Antigen-binding activity of immunoliposomes (≈ 13 IgG residues per vesicle of 100 nm) to EGFR-positive DU145 cells. The cells were treated, as detailed in experimental section, with: (A) for direct binding assay: human IgG1 (hu IgGl-D), C225 (C225-D), immunoliposomes (C225-lipo-D), or parent liposomes (free lipo-D) and probed with goat ant-human IgG conjugated to FITC; and (B) for competitive assay: human IgGl (hu IgGl + C225-F), C225 (C225 + C225-F), immunoliposomes (C225-lipo + C225-F), or parent liposomes (free lipo + C225-F) and probed with FITC conjugated C225 (C225-F). The C225-F-labeled bar represents positive control. Samples were analyzed by flow cytometry and the results are expressed as the Mean Fluorescence Intensity (MFI).

tion per animal. Under these conditions the immune response, even if induced, would not have an opportunity to influence the pharmacokinetics of the immunoliposomes since measurements were only taken over a 24-h period. Although single injections of immunoliposomes might be adequate for diagnostic purposes, to be useful for targeted drug delivery repeated administrations in many instances would be advantageous. In light of these considerations and to address the issue of immunoliposomes' immunogenicity and its relationship to their pharmacokinetic behavior, the following experiments were designed. Animals were given three injections of either the immunoliposomes, or free C225, or a mixture of parent liposomes and C225, each formulation given on days 0, 14, and 28. A separate group of four rats was treated with immunoliposomes, on day 0, and then injected with free C225 on days 14 and 28. Pharmacokinetic curves generated from these experiments are shown in Figs. 5 and 6. Blood samples taken from the tested animals at various time points were used for determination of anti-C225 titers. These results are summarized in Fig. 7. These experiments demonstrate that the subsequently injected immunoliposomes were cleared much faster from the bloodstream than the ones administered initially (see the clearance rate constants and other pharmacokinetic parameters in Table 4). This accelerated clearance correlated with significant increase in C225-specific antisera in the animal plasma. In contrast, repeated injections of the free immunoglobulin or a mixture of C225 with the parent liposomes did not trigger any detectable response as demonstrated by the overlapping clearance curves (Fig. 5B and 6) and negligible anti-C225 titers generated (Fig. 7A). Further evidence indicating that the injection of immunoliposomes was responsible for the anti-C225 specific response came from the experiments subjecting immunoliposome-pretreated animals to injections with free C225. As can be seen from Fig. 5B, administration of the free immunoglobulin showed the same pharmacokinetic behavior after each injection, except when injected into immunoliposome-pretreated animals. The latter group of rats had a similar anti-C225 serum reactivity to animals treated with immunoliposomes only (Fig. 7B). These results would be expected from C225 preimmunized animals. Note that all the animals subjected to the three repeated injection regiments,

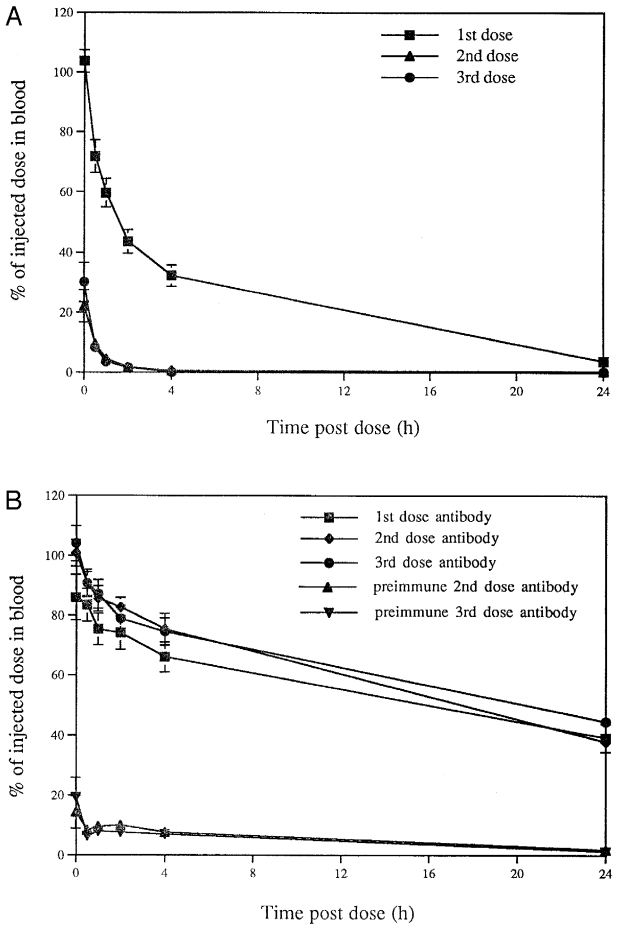

Fig. 5. Blood clearance curves obtained following each dose of (A) three repeated injections (on days 0, 14 and 28) of ^{67}Ga-labeled immunoliposomes ($\approx$18 C225 residues per vesicle of 100 nm); and (B) three repeated injections (days 0, 14 and 28) of ^{125}I-labeled free C225-antibody (3 upper curves), and two injections (days 14 and 28) of free ^{125}I-labeled C225-antibody into animals, which received one injection (day 0) of the immunoliposomes (lower 2 curves). Four rats per experiment (mean $\pm$ SD).

appeared to be healthy, gained weight and exhibited no abnormal behavior.

These results are in accord with the observations previously made by Phillips et al. [25] who prepared avidin–biotin conjugated liposomes bearing GK1.5 rat IgG, and reported significant levels of anti-GK1.5 neutralizing antibodies forming after i.v. administration of these immunoliposomes in mice, regardless of whether mPEG–lipid components were present in the formulations. In contrast, we used site-specific conjugation approach yielding homogeneous immunoliposomes of a well-defined composition. Thus despite

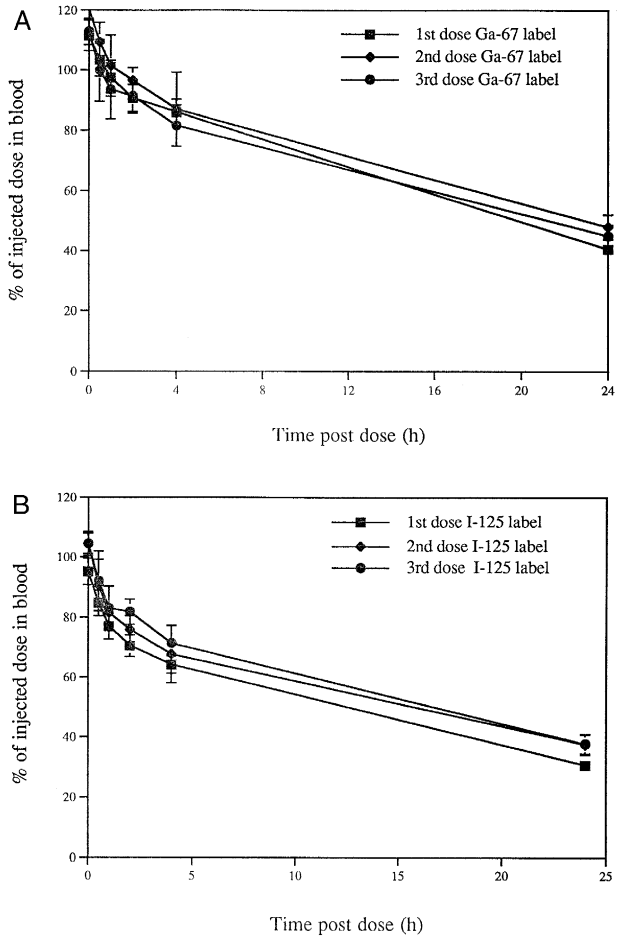

Fig. 6. Blood clearance curves obtained following three coinjections of ^{125}I-labeled free C225-antibody and ^{67}Ga-labeled parent liposomes. Four rats per experiment (mean ± SD). (A) Pharmacokinetic profiles of each injection following ^{67}Ga-label of liposomes. (B) Pharmacokinetic profiles of each injection following ^{125}I-label of C225.

the significant differences in our experiments in methodology, composition, and homogeneity of the immunoliposome preparations, both studies confirm the immunogenic properties of PEG-containing immunoliposomes.

The C225 antibody is a chimerized mouse IgG and thus composed of a constant human portion (Fc) and variable mouse portions (Fab). It was important to find out to what extent the observed immune response was elicited by each of these regions of the conjugated C225. For this purpose selected serum samples from immunoliposome-treated animals were tested for binding to polyclonal human IgG and the C225-derived Fab fragment (Fig. 8). The signals obtained from human IgG-coated ELISA plate wells were almost the same as from intact C225-coated wells. In contrast, little antiserum binding was observed to the Fab portion of the antibody. These results demonstrate that the immune response to C225-liposomes was triggered primarily by the constant human region of the covalently attached antibody. This indicates that the observed potentiation of C225 immunogenicity as a result of its fixation to

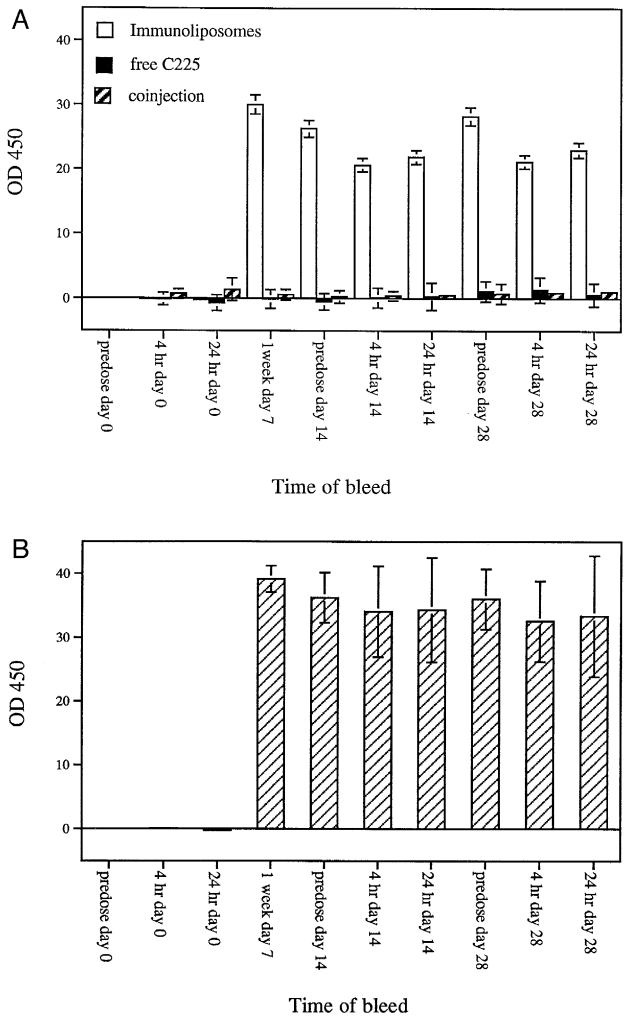

Fig. 7. Anti-C225 specific titers obtained by ELISA. (A) Analysis of plasma samples from three groups of rats treated with either immunoliposomes, or free C225, or with coinjection of C225 with the parent liposomes. (B) C225 specific antisera detected in plasma of animals treated first with immunoliposomes (day 0) and then twice (days 14 and 28) with free C225.

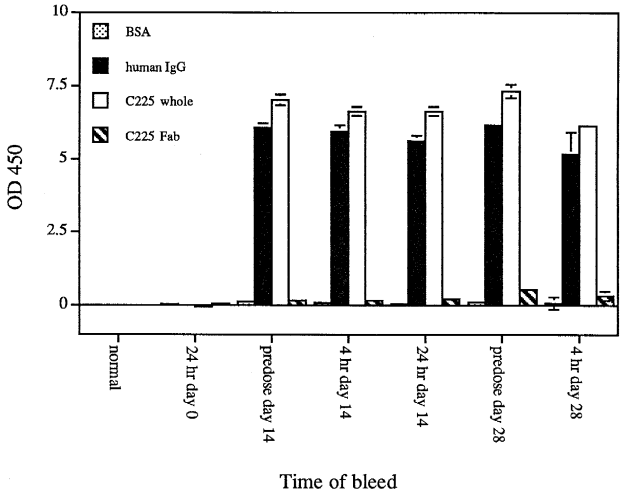

Fig. 8. ELISA analysis of antiserum binding to human polyclonal IgG, C225-derived Fab'. For comparison binding to intact/whole C225, and an irrelevant protein, BSA are shown. Plasma samples obtained from C225-liposome treated rats at indicated times.

PEG-liposomes was almost exclusively due to the Fc portion of the C225 molecule and little if any was due to the Fab parts.

3.5. Summary and conclusions

Our results demonstrate a straightforward methodology for preparation of PEG-grafted liposomes bearing C225 IgG residues attached to the termini of the external surface-grafted polymer chains. The methodology described has several promising attributes: (1) efficient conjugation procedure under very mild conditions; (2) composition of the immunoconjugates can be readily controlled; and (3) antigen-binding activity of the conjugates is well preserved. Although repeated i.v. administration of free chimerized mouse IgG, C225, into rats did not trigger an immune response, a single injection of C225-containing immunoliposomes did. This suggests that a potentiation of immunogenic properties occurred as a result of the covalent fixation of the IgG to the polymer-grafted liposomes. This conclusion was further corroborated by C225-specific IgG titers. The antiserum recognized readily human polyclonal IgG, yet it hardly recognized the Fab fragment of C225, indicating that the immunogenicity potentiation was specific to the constant human, Fc region of C225. The appearance of C225-specific antibodies in the immunoliposome-treated rats was manifested by a dramatically accelerated clearance from the bloodstream of the subsequently injected immunoliposomes or free C225. Somewhat surprisingly, in our experiments the free C225 IgG behaved as an antigen, but was not immunogenic. We would like to emphasize that at this point it is not clear how general the phenomenon of enhanced immunogenicity of PEG-liposome linked antibody is. Further experimentation should shed light on this question. Moreover, since we detected only a slight immune response to the Fab portion of conjugated C225, there might be further merit in evaluating Fab-fragments as targeting moieties for PEG-liposomes [43]. Our observations indicate that the potential for induction of immune response should be seriously considered in the development of long-circulating formulations of immunoliposomes as drug delivery vehicles, since at least the whole antibody conjugates of PEG-liposomes are probably immunogenic. The immunogenicity or lack thereof is bound to be crucially important in influencing the longevity in blood circulation of such constructs and their ability to reach their intended target sites.

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