Prevention of Antibody-Mediated Elimination of Ligand-Targeted Liposomes by Using Poly(Ethylene glycol)-Modified Lipids

WAI MING LI, LAWRENCE D. MAYER, and MARCEL B. BALLY

Department of Pathology and Laboratory Medicine (W.M.L., M.B.B.) and Faculty of Pharmaceutical Sciences (L.D.M.), University of British Columbia, Vancouver, British Columbia, Canada; and Department of Advanced Therapeutics, British Columbia Cancer Agency, Vancouver, British Columbia, Canada (W.M.L., M.B.B., L.D.M.)

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ABSTRACT

One of the major obstacles in the development of ligand-targeted liposomes is poor lipid circulation longevity as a result of antibody-mediated elimination of these highly immunogenic carriers. Because studies from our laboratory suggest that it is not possible to reduce the immunogenicity of ligand-conjugated liposomes by using surface-grafted poly(ethylene glycol) (PEG), we investigated the usefulness of PEG in protecting hapten-conjugated liposomes from elimination by an existing immune response that was previously established against the hapten. Using biotin as a model hapten, a strong biotin-specific antibody response was generated in mice by using bovine serum albumin-biotin. When these animals were challenged with liposomes containing biotin-conjugated lipid (1 or 0.1%), these liposomes were rapidly eliminated. Incorporation of PEG-lipids into these liposomes substantially reduced biotin-specific antibody binding as measured using an in vitro antibody consumption assay. However, depending on the hapten concentration, significant reductions in antibody binding through the use of PEG-lipids may not be sufficient to protect these liposomes from rapid elimination in vivo. Complete protection of liposomes was only achieved when the biotin concentration on liposome surface was low (0.1%) and with 5 mol% of either 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-n-[methoxy(polyethylene glycol)-2000] or 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-n-methoxy(polyethylene glycol)-2000). The use of 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-n-[methoxy(polyethylene glycol)-2000] (up to 15 mol%) was not effective in protecting liposomes from rapid elimination in vivo, indicating the limited usefulness of this highly exchangeable PEG-lipid. In conclusion, our in vivo and in vitro data indicate that liposomes can be protected from antibody-mediated elimination by using the right type and concentration of PEG-lipids. This result has important implication in the development of ligand-targeted liposomes.

Active targeting can be achieved by conjugating macromolecules such as antibodies, peptides, and ligands of natural receptors onto liposome surfaces to improve the specificity of these drug carriers to disease sites (Vingerhoeds et al., 1994). Although active targeting of liposomes has met with some success both in vitro and in vivo (Park et al., 1995; Kirpotin et al., 1997; Gabizon et al., 1999), further development of ligand-targeted liposomes for in vivo use remains a challenge due to the immunogenicity of the drug carriers bearing surface ligands that function as antigenic haptons (Phillips and Emili, 1991; Phillips et al., 1994; Phillips and Dahman, 1995). Repeated administration of these liposomes becomes problematic because the pharmacokinetic and biodistribution behaviors of the carrier change after subsequent injections of the drug carrier (Shek and Heath, 1983; Phillips and Emili, 1991; Phillips et al., 1994; Harding et al., 1997; Tardi et al., 1997; Dams et al., 2000). Enhanced elimination of the liposomes is due to the generation of a humoral response and immunoglobulin binding to the liposomes in the plasma compartment. We and others have shown that surface-grafted poly(ethylene glycol) cannot reduce the immunogenicity of these liposomes but can enhance the immune response to targeting molecules bound to the surface of liposomes (Phillips and Dahman, 1995; Li et al., 2001a) or to the terminal moiety of the grafted PEG (Harding et al., 1997). However, it

ABBREVIATIONS: PEG, poly(ethylene glycol); BSA, bovine serum albumin; biotin-X-DSPE (Bx-DSPE), N-(((6-biotinoyl)amino)hexanoyl)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine; DSPC, 1,2-distearoyl-sn-glycero-3-phosphocholine; DSPE-PEG2000, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-n-[methoxy(polyethylene glycol)-2000]; DMPE-PEG2000, 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-n-[methoxy(polyethylene glycol)-2000]; DPE-PEG2000, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-n-methoxy(polyethylene glycol)-2000]; DSPE-PEG5000, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-n-[poly(ethylene glycol) 5000]; Chol, cholesterol; ELISA, enzyme-linked immunosorbent assay; ANOVA, analysis of variance; 1% Bx-lipo, DSPC/Chol liposomes containing 1% biotin.
has not been shown whether surface-grafted PEG can protect liposomes once an immune response has been generated. This investigation was aimed at determining the benefits of surface-grafted PEG in improving circulation longevity of ligand-targeted liposomes in mice with an existing immune response.

Liposomes with surface-grafted PEG can be prepared by incorporating PEG-conjugated lipids into the liposome formulation. The use of PEG-lipids to prolong liposome circulation lifetime has been well documented (Allen et al., 1991; Woodle and Lasic, 1992; Woodle et al., 1994). The mechanism of improved liposome circulation longevity is proposed to be due to the steric effect of the grafted polymer that protects the liposome surface from the nonspecific adsorption of proteins and the associated elimination of the carrier by the mononuclear phagocytic system (Zeisig et al., 1996; Du et al., 1997; Miller et al., 1998). It has been previously shown that PEG-lipid can reduce the nonspecific binding of various proteins, including BSA, laminin, and fibronectin to glass surface (Du et al., 1997). Incorporation of PEG-lipids into liposomes can also prevent complement binding to liposome surface (Bradley et al., 1998). As well, endocytosis of liposomes in vitro by macrophages was shown to be reduced by incorporating PEG-lipids (Zeisig et al., 1996; Miller et al., 1998; Johnstone et al., 2001). In general, the protective effect of PEG-lipids depends on its polymer size as well as grafting density (Kenworthy et al., 1995; Zeisig et al., 1996; Du et al., 1997; Bradley et al., 1998; Miller et al., 1998; Needham et al., 1999). However, our laboratory has recently demonstrated that this protective effect could actually be due to selective binding of proteins that is mediated, in part, by the presence of surface-grafted PEG (Johnstone et al., 2001). This study and others clearly question the dogma that PEG incorporation provides effective protection against nonspecific serum protein binding. It is becoming apparent that PEG incorporation creates a selective barrier to protein binding and the amount and type of proteins bound to liposome with surface-grafted PEG is not well characterized. When considering the application of PEG-based surface protection for liposomal carriers designed to target defined in vivo targets, whether a cancer cell-specific surface marker or disease generated element that is normally not expressed in healthy tissue, a further dilemma arises. It is now well established that PEG incorporation can interfere with surface-surface interactions. This effect results in decreases in binding avidity between liposomes and their intended target.

Because specific antibody binding to liposomal ligands appears to be a critical step in antigen recognition and elimination by the immune system (Geiger et al., 1981; Hsu and Juliano, 1982), we propose that PEG-lipids can prevent opsonization by specific antibodies and thereby improve liposome circulation longevity. However, such protection must be achieved using materials and under conditions that will still support surface-surface interactions. In this study, the protective effect of PEG-lipid to inhibit specific antibody access to liposome surface ligand was investigated in vitro. This protective effect of PEG-lipid was correlated with the ability of PEG-lipid to prevent antibody-mediated clearance of liposomes in vivo.

Experimental Procedures

Materials. BSA-biotin, O-phenylethylene diamine, cholesterol, EDTA, and complete and incomplete Freund’s adjuvant were purchased from Sigma Chemical (St. Louis, MO). Monoclonal anti-biotin antibody was obtained from Biogenesis Ltd. (Sandown, NH). Peroxidase-linked anti-mouse Ig was purchased from Amersham Biosciences, Inc. (Piscataway, NJ). Maxisorp 96-well plates were obtained from Naige Nune International (Rochester, NY). [3H]-Cholesteryl hexadecyl ether was purchased from NEN Dupont Canada (Mississauga, ON, Canada). N-(6-Biotinylaminohexanoyl)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (Bx-DSPE) was obtained from Northern Lipids (Vancouver, BC, Canada). 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC); 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-n-[(poly(ethylene glycol) 2000) (DSPE-PEG2000); 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-n-[(poly(ethylene glycol) 2000) (DMPE-PEG2000); 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-n-[(poly(ethylene glycol) 2000) (DPPE-PEG2000); and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-n-[(poly(ethylene glycol) 5000) (DSPE-PEG5000) were purchased from Avanti Polar Lipids (Birmingham, AL).

Preparation of Large Unilamellar Vesicles. Liposomes were prepared using the extrusion method of Mayer et al. (1986). Haptenized liposomes were prepared by incorporating Bx-DSPE (1 or 0.1%) into DSPC/Chol liposomes, with a molar ratio of DSPC/Chol/Bx-DSPE of 55:45:5 where X = 1 or 0.1. PEG-lipids were incorporated into DSPC/Chol liposomes containing 0.1% or 1% biotin (Bx-lipo) at a concentration of 2 to 10 mol% as specified in each experiment with liposomal cholesterol content remaining constant. To prepare liposomes, lipid mixtures (100 to 150 μmol of total lipid) were first dissolved in chloroform. [3H]-cholesteryl hexadecyl ether, used as a liposome label, was added to the lipid mixture to obtain a specific activity of 0.1 μCi/μmol. A lipid film was then formed from the lipid mixture by first drying under nitrogen gas and then under high vacuum for at least 3 h. Subsequently, the lipid film was hydrated in 1.5 ml of HEPES-buffered saline, pH 7.4, at 65°C. The resulting multimamellar vesicles were subjected to five freeze-thaw cycles and then extruded (10 times) through polycarbonate filters (Corning Separations, Acton, MA) and an extrusion device (LipiMembranes Inc., Vancouver, BC, Canada) at 65°C. Liposome size was determined by quasieisclastic light scattering by using a Nicomp 370 submicron particle sizer operating at a wavelength of 632.5 nm. The liposomes used in this study exhibited mean diameters of 100 to 120 nm, and a Gaussian distribution with a chi square value of less than 2.

Immunization. BALB/c mice 7 to 9 weeks of age were injected i.p with BSA-biotin (50 μg/mouse), mixed with incomplete Freund’s adjuvant, which was mixed with 10% complete Freund’s adjuvant, once per week for 3 weeks. One week after the last immunization, the mice were injected with various liposome formulations to monitor liposome elimination. At 1 and 2 h after liposome injection, 25 μl of blood was collected from the tail vein by nicking the tail and using a microcapillary tube presoaked with a 200 mM EDTA solution. The blood collected was added to 200 μl of 200 mM EDTA solution and then centrifuged to separate the cellular components from plasma. The [3H]radioactivity from liposomes in the supernatant containing plasma was then determined with 5 ml of Pico-Fluor 40 scintillation cocktail and using a Beckman LS 3801 scintillation counter. At 4 h, mice were terminated by CO2 asphyxiation and whole blood was collected by cardiac puncture. Because there is no difference in the method of blood collection for the determination of blood liposome levels, 25 μl of blood collected from cardiac puncture was used to determine plasma liposome levels at the 4-h time point. The remaining blood was used for the determination of antibody levels by using enzyme-linked immunosorbent assay (ELISA). Importantly, all animal studies were conducted according to the protocols approved by the University of British Columbia’s Animal Care Committee, which follows the current guidelines established by the Canadian Council on Animal Care.
Anti-Biotin Antibody Quantification by Using ELISA. Plates (96-well) were coated with BSA-biotin (0.02 μg/ml) at 4°C overnight and then blocked with 10% calf serum for 1 h at 37°C. Plasma samples diluted in phosphate-buffered saline containing 0.05% Tween 20 and 1% BSA (1:50–1:500 dilution; 50 μl) were added to wells in the ELISA plate and incubated for 1 h at 37°C. Subsequently, secondary antibody (horseradish peroxidase-conjugated anti-mouse Ig; 1:1000, 100 μl) was added and incubated for an additional 1 h. After each incubation step, the plate was washed three times with wash buffer (phosphate-buffered saline with 0.05% Tween 20). For color development, o-phenylenediamine was used as substrate. o-Phenylenediamine diluted in phosphate-carbonate buffer (pH 5.0; 0.4 mg/ml; 150 μl) was added to each well and incubated for 20 min at room temperature. To stop the enzyme reaction, 40 μl of 3 M H₂SO₄ was added to plate and incubated for another 10 min after which absorbance at 490 nm was read using a Dynex microplate reader.

Antibody Consumption Assay. Antibody binding to liposomes was measured indirectly in an antibody consumption assay based on a competitive ELISA method. Biotinylated liposomes added to the ELISA assay described above would compete for the monoclonal anti-biotin antibody and therefore inhibit the antibody binding to immobilized antigen. The inhibition is then a measure of antibody consumption by the liposomes added to the assay. In the consumption assay, various formulations of biotinylated liposomes were incubated in the ELISA plate with the monoclonal antibody for 1 h at 37°C. The concentration of the monoclonal anti-biotin antibody was kept low (1:8000) to ensure its concentration was not sufficient to saturate specific binding to immobilized antigen. Antibody consumption is determined by comparing the absorbance of sample wells with the maximum absorbance obtained without liposome and is calculated as follows: % antibody consumption = \[\left(1 - (A - A_p)/(A_o - A_p)\right) \times 100\%\], where A is absorbance of sample well, A₀ is background absorbance (without antibody), and Aₚ is maximum absorbance.

Statistical Analysis. Two-way ANOVA was performed to detect differences among treatment groups in liposome elimination experiments. Newman-Keuls tests were preformed as post hoc analysis for two-way ANOVA. A p value of < 0.05 was considered significant.

Results

Antibody-Mediated Clearance of Biotinylated Liposomes. Mice were immunized using biotin-BSA conjugate as described under Experimental Procedures to induce an immune response against biotin. One week after the last immunization, biotin-specific antibody levels were measured in plasma. The results, summarized in Fig. 1, indicate that there were significant levels of circulating anti-biotin IgG with mean values estimated to be in excess of 2400 ng/50 μl. Injection of the carrier protein (BSA) 1 week before the first immunization substantially reduced the biotin-specific antibody response to values less than 800 ng/50 μl. This was expected due to specific epitopic suppression by the protein conjugate (Schutze et al., 1989), confirming the immune response was specific for the hapten, not the protein carrier.

The circulation longevity of our model targeted liposomes (biotinylated liposomes) was measured after i.v. injection into mice 1 week after the last immunization with BSA-biotin, a time point where the animals maintained significant anti-biotin IgG levels (Fig. 1). As shown in Fig. 2, when 1% Bx-lipo were injected i.v. into control mice previously given saline, 3.62 ± 0.38, 3.18 ± 0.17, and 2.54 ± 0.26 μmol/ml plasma remained at 1, 2, and 4 h, respectively. The level at 1 h can be used to estimate that more than 80% of the injected dose remained in the circulation at this time point.

When the same liposome formulation was injected into mice immunized with BSA-biotin, less than 0.5% of the injected dose remained in the plasma at the same 1-h time point,
indicating that these liposomes were rapidly eliminated from the circulation due to the existing immune response against biotin (Fig. 2). To test the protective effect of PEG-lipids on these liposomes, 5 mol% of DSPE-PEG2000 was incorporated into the formulation and these were injected i.v. into animals with preexisting circulating anti-biotin IgG. The inclusion of PEG-lipid significantly increased liposome levels remaining at 1 and 2 h (Fig. 2). However, these liposome levels (less than 20% of the injected dose) were significantly lower compared with the levels in saline-treated mice, indicating minimal protection of the haptenized liposomes from immune recognition and elimination. When the PEGylated Bx-liposomes were injected into saline control mice, the elimination profile obtained was comparable to that of the non-PEGylated formulation, indicating that inclusion of 5 mol% DSPE-PEG2000 did not influence the rate of nonspecific elimination of liposomes in the time frame measured. It should be noted that at the lipid dose used (3.3 μmol/mouse), the effect of PEG on circulation lifetime is not observed at time periods less than 4 to 6 h after i.v. injection.

**Protection of Liposomes with Low Hapten Density.** The minimal liposome protection by 5% PEG-lipid was probably a result of insufficient hapten shielding due to either too much hapten or insufficient PEG grafting density. To investigate the protective effect of PEG-lipids on liposomes bearing reduced levels of surface hapten, the experiment with Bx-liposomes was repeated with a formulation prepared with 10 times less biotin (0.1 mol% biotin-DSPE). As shown in Fig. 3, Bx-liposomes with 0.1 mol% biotin exhibited rapid elimination from the circulation, similar to liposomes with high hapten density (1 mol%). There was less than 0.5% of the injected 0.1% Bx-liposomes remaining in the plasma 1 h after i.v. injection. Inclusion of 5 mol% of DSPE-PEG2000 in 0.1% Bx-liposomes was, in contrast to the 1% Bx-liposome, able to completely protect these liposomes from rapid elimination (Fig. 3), where the circulation lifetime of these liposomes was not significantly different from DSPC/Chol liposomes. The protective effects of DSPE-PEG2000 were comparable to that obtained with DPPE-PEG2000. This is consistent with data indicating that DSPE-PEG2000 and DPPE-PEG2000 are retained well in the membrane after i.v. administration (Li et al., 2001b). When the concentration of PEG-lipid (DPPE-PEG2000) was lowered to 2%, the protective effect of this lipid was no longer evident. The circulating levels for these liposomes were significantly higher than the non-PEGylated formulation (0.48 ± 0.22 versus 0.014 ± 0.004 μmol/ml at 1 h after injection) but were significantly lower than those measured for nonhaptenated DSPC/Chol liposomes (2.6 ± 0.21 μmol/ml). Collectively, these data indicate that complete protection of liposomes from rapid elimination can be achieved using sufficient amount of PEG-lipid; however, hapten density is an important factor in determining the effectiveness of PEG-lipids.

Previous studies have shown that DMPE-PEG2000 is a highly exchangeable PEG-lipid that is not retained well in the liposomal bilayer. Exchange rates in the order of minutes have been measured both in vitro and in vivo when this lipid is incorporated in neutral liposomes (Holland et al., 1996; Li et al., 2001b). The protective effect of this PEG-lipid on Bx-liposomes was also investigated in this study. The 0.1% Bx-liposomes containing either 5, 10, or 15 mol% DMPE-PEG2000 were prepared and injected into mice previously immunized with BSA-biotin as mentioned above. As shown in Fig. 4, DMPE-PEG2000 had little protective effect on these liposomes. Inclusion of 15 mol% DMPE-PEG caused a significant increase in liposome levels at 1 h after i.v. injection compared with the non-PEGylated formulation (0.57 ± 0.31 versus 0.020 ± 0.006 μmol/ml plasma). However, this level of liposomal lipid represents only 12% of the injected dose and is significantly less than that observed after injection of the nonhaptenized formulation (DSPC/Chol). The limited effectiveness of DMPE-PEG2000 in protecting Bx-liposomes from rapid elimination in vivo is probably due to the rapid loss of this PEG-lipid out of the liposomal bilayer (Holland et al., 1996; Li et al., 2001b).

**Assessment of Antibody Binding to Bx-Liposomes.** The protective effect of surface-grafted PEGs on biotin-labeled liposomes injected into mice with an established immune response is probably due to the prevention of antibody binding to biotin on the liposome surface. To determine the ability of PEG-lipids to prevent antibody binding to Bx-liposomes, an in vitro assay was used to measure biotin-specific antibody consumption by liposomes. In the assay, the binding of a monoclonal anti-biotin antibody to immobilized biotin (on ELISA plate) was measured as a function of liposome concentration in the assay. Bx-liposomes binding to the anti-biotin antibody was represented as a decrease in antibody consumption by liposomes. Inclusion of 5% of the hapten recognition and elimination. When the PEGylated Bx-liposomes were injected into saline control mice, the elimination profile obtained was comparable to that of the non-PEGylated formulation, indicating that inclusion of 5 mol% DSPE-PEG2000 did not influence the rate of nonspecific elimination of liposomes in the time frame measured. It should be noted that at the lipid dose used (3.3 μmol/mouse), the effect of PEG on circulation lifetime is not observed at time periods less than 4 to 6 h after i.v. injection.
the ELISA assay. As shown in Fig. 5A, the addition of non-haptenized DSPC/Chol liposomes to the anti-biotin antibody assay did not result in any significant loss of antibody binding as measured using the ELISA assay, even when present at concentrations as high as 25 mM. In contrast, 50% of the anti-biotin antibody was “consumed” by the addition of 10 μM 1% Bx-liposomes (Fig. 5A). Inclusion of 5 mol% DSPE-PEG2000 in 1% Bx-liposomes did effect a significant change in antibody binding and reduced antibody consumption by more than 1000-fold compared with the non-PEGylated formulation (i.e., 1000-fold increase in liposome concentration was needed to achieve the same level of antibody consumption). This is an interesting result when considering that the presence of 5 mol% DSPE-PEG2000 had little protective impact on the circulation lifetime of 1 mol% biotinylated liposomes injected into mice with an established anti-biotin response (Fig. 2). Incorporation of 10% DSPE-PEG2000 did not have a substantial effect in further reducing antibody consumption as shown by the antibody consumption curve which is similar to that of the formulation containing 5 mol% PEG-lipid. The effectiveness of PEG-lipids having a longer PEG polymer (mol. wt. 5000) was also investigated using this assay. The results suggest that DSPE-PEG5000 was not as effective as DSPE-PEG2000 in reducing antibody consumption.

To determine the effectiveness of PEG-lipids to prevent antibody binding to liposomes bearing a low hapten density (0.1 mol% biotin), antibody consumption by these formulations was measured (Fig. 5B). It should be noted that antibody consumption by 0.1% Bx-liposomes was less efficient compared with the formulation containing 1% biotinylated lipid. The 10-fold reduction in biotinylated lipid concentration resulted in a greater than 2-log shift in the antibody consumption curve. When 5 mol% of either DSPE-PEG2000 or DPPE-PEG2000 was incorporated into 0.1% Bx-liposomes, antibody consumption was reduced to the same level as that of nonhaptenized DSPC/Chol liposomes, suggesting complete inhibition of antibody binding to these liposomes. When 2 mol% DPPE-PEG2000 was used, there was a shift in the antibody consumption curve, but an antibody consumption level of 25% was still observed at a liposome concentration of 50 mM (Fig. 5B), suggesting insufficient liposome protection. As shown in Fig. 3, these liposomes were rapidly removed from circulation after i.v. administration into mice with an established antibody response. Thus, it can be concluded that

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**Fig. 4.** Elimination profiles of Bx-liposomes containing DMPE-PEG lipids. The protective effect of DMPE-PEG lipids was investigated by monitoring the elimination of Bx-liposomes containing 0% (●), 5% (▲), 10% (▼), or 15% (●) DMPE-PEG2000 in mice previously immunized with BSA-biotin. Liposome levels in the blood were measured at 1, 2, and 4 h postliposome injection as described under Experimental Procedures. Values represent mean ± S.D. of sample size n = 4. The data was analyzed using two-way ANOVA and a p value of <0.05 was considered significant. *, significantly higher than all other groups.

**Fig. 5.** Antibody consumption by liposomes. Biotin-specific antibody binding to Bx-liposomes was measured indirectly in a competitive ELISA assay as described under Experimental Procedures. A, antibody consumption by liposomes containing 1% biotin as a function of liposome concentration. The effect of PEG-lipids on shielding the liposomal antigen was investigated using 10% DSPE-PEG2000 (●), as well as 5% (▲) and 10% (▼) DSPE-PEG2000. B, antibody consumption by liposomes containing 0.1% biotin as a function of liposome concentration. The effect of incorporating 5% DSPE-PEG2000 (▲), 5% DPPE-PEG2000 (●), and 2% DPPE-PEG2000 (▼) into 0.1% Bx-liposomes on antibody consumption was investigated.
even low levels of antibody consumption provides a good predictor of increased liposome elimination in mice that have an existing humoral immune response generated against the liposomal hapten.

Discussion

The objective of this study was to investigate whether surface-grafted PEG polymers can protect hapten-bearing liposomes from elimination after i.v. administration in mice with an established immune response against the hapten. To our surprise, PEG-lipid incorporation at levels considered to provide optimal steric stabilization properties (5 mol% PEG<sub>2000</sub>) was only effective when used in liposomes bearing a low concentration of the associated hapten. An antibody consumption assay was used to determine that incorporation of PEG-modified lipids engendered a significant decrease in antibody binding to haptenized liposomes. However, only under conditions where PEG completely prevented antibody binding to liposomes, there was effective protection of the liposomes in vivo. There are two important conclusions that we have reached on the basis of these results. First, at a fixed PEG-grafting density, a density that is sufficient to achieve steric stabilization, the polymer’s ability to inhibit antibody binding to haptenized liposomes is dependent on hapten density. Second, when injecting liposomes with a surface-associated antigen, the PEG-grafting density sufficient to cause a two-log reduction in antibody binding is not sufficient to ensure that the liposomes are not eliminated rapidly from the plasma compartment. These two conclusions will be discussed in turn.

Steric stabilization of neutral liposomes by using PEG-lipids has been extensively studied in the past (Allen et al., 1991; Woodle and Lasic, 1992; Woodle et al., 1994). These earlier studies indicate that PEG-lipid concentration as low as 5 mol% was enough to dramatically improve circulation longevity of neutral liposomes. The stealth properties of these liposomes are thought to be attributed to reduced mononuclear phagocytic system uptake. This study and another recent study (Chiu et al., 2001) using liposomes with a reactive moiety suggest that the surface-shielding properties of PEG depend on the binding avidity of the penetrating molecule for the reactive surface. This, in turn, can be determined by 1) the type of interaction, 2) the valency of the interaction, and 3) the accessibility of the reactive moiety. For liposomes containing a reactive moiety, such as an antigen or charged lipid, there is additional energy to provide for the penetrating molecules to move through the polymer barrier due to the high binding affinity. In contrast to the non_specific binding of proteins to neutral liposome surface, these specific interactions are mediated by the sum of many non_covalent forces, including van der Waals force, hydrophobic force, hydrogen bonds, and electrostatic force, which make the binding affinity much higher. For liposomes containing phosphatidylserine, it was shown that a PEG-grafting density of 15 mol% is required to protect these liposomes from rapid elimination in plasma as well as from prothrombin binding to liposome surface in vitro (Chiu et al., 2001).

In the current study, our results indicate that as low as 5 mol% PEG<sub>2000</sub> was sufficient to prevent antibody-mediated elimination of biotinylated liposomes when biotin concentration was low (0.1%). However, our data also suggest that complete antigen shielding by PEG-lipids may not be achievable with a high ligand concentration. This may be attributed to the much higher binding avidity when the ligand concentration was high. The antigens on liposomes having 1% biotin would certainly be close enough to each other for bivalent binding of the antibody. This may be sufficient to ensure that the antibody is avidly bound to the liposome surface. For liposomes with 10 times less antigen (0.1% biotin), the estimated distance between antigens is 24 nm (assuming 68,000 lipid molecules in the outer leaflet of a 100-nm liposome; Hutchinson et al., 1989), a distance still possible for bivalent binding assuming the hinge region of the antibody is flexible (Amzel and Poljak, 1979; Alzari et al., 1988). However, considering antigen lateral mobility in the bilayer, and that not all liposomal haptens are in a conformation available for binding (Petrossian and Owicki, 1984), bivalent binding would be less for liposomes with a much lower antigen density. This may be the reason for the large difference in antibody consumption between liposomes with 1% biotin and 0.1% biotin observed in the current study (Fig. 5). It should also be noted that the inability of higher mol% PEG-lipids to completely shield liposomes containing 1% biotin may be due to the geometric arrangement of the reactive moiety. The inclusion of a six-carbon linker in the biotinylated lipid, which extends the biotin molecules away from the lipid surface, can make them more accessible for antibodies to bind bivalently.

Our results also indicate that the use of a longer PEG polymer (mol. wt. 5000) cannot improve the shielding from antibody when the ligand concentration is high. The lack of a better protection by using a longer polymer, compared with the same mol% of PEG<sub>2000</sub>, may be explained by the high flexibility of the polymer, allowing antibodies to be “trapped” in the densely packed layer of PEG in the brush conformation, thus affecting the off-rate of the antibody. In addition, the actual amount of PEG-lipids incorporated may not be as high as 10 mol% because the maximum allowable PEG-lipid contents in liposome tend to decrease with increasing polymer size (Beugin et al., 1998). Another confounding factor not considered in these studies is the influence of enhanced non_specific protein binding to PEGylated liposomes (Johnstone et al., 2001), an effect that will be particularly relevant for in vivo studies. The additional shielding by these nonspecific proteins may be enough to protect liposomes from immune recognition when biotin concentration was low, but not when biotin concentration was high.

The fact that ligand concentration is an important parameter in determining the effectiveness of PEG in shielding liposome surface has special implications in the design of liposomes for targeting. Although many studies suggest the benefit of having more ligand molecules for targeting, our results suggest that ligand concentration should be balanced between the benefit of specific targeting and rapid elimination caused by immune recognition. Previous studies with antibodies have indicated that 40 targeting molecules per liposome would be optimal for targeting (MARUyama et al., 1995; KIRPOTIN et al., 1997). For our biotinylated liposomes containing 0.1% biotin, it is estimated that 68 biotin molecules are present on each liposome, assuming 68,000 lipid molecules are in the outer lipid monolayer (Hutchinson et al., 1989). This concentration of biotinylated lipid has been previously shown to be equally effective in targeting streptavi-
in vitro compared with the formulation containing 1% biotin. Moreover, by using the biotin-streptavidin targeting approach, it has been shown that specific targeting in vivo can be achieved using liposomes with approximately 50 copies of streptavidin as targeting ligand (Longman et al., 1995).

Another important conclusion drawn from our study is that complete protection of the liposomal ligand is critical for ensuring long circulation lifetime of the carrier. Our results show that PEG-lipid can substantially decrease antibody binding. However, depending on the ligand concentration, a 600-fold decrease in antibody consumption may not be enough to sufficiently protect liposomes from rapid elimination in vivo (Table 1). Even at a low level of antibody binding (20% consumption), achieved with 2 mol% PEG2000 in liposomes containing 0.1 mol% biotin, there was minimal liposome protection in plasma (Fig. 5). This finding indicates that opsonization of liposome by circulating antibodies is a critical step in antibody-mediated clearance of liposomes with targeting ligands or hapten and even low levels of antibody binding to a liposome-bound epitope will be sufficient to accelerate liposome elimination.

One of the key factors determining the success of active targeting is liposome access to target site. For tissue-localized solid tumors in extravascular sites, liposome circulation lifetime becomes the most critical factor to consider because liposomes, which are rapidly eliminated from the blood compartment, have a very low probability to extravasate and reach the target site. Thus, we believe that circulation longevity is one of the most important criteria for targeted delivery systems. Although it is important to evade the immune system when liposomes are in the circulation, it would be necessary to reexpose liposome-associated targeting ligands to achieve targeting after localizing in the region where the target cells are. Studies conducted in our laboratories also indicated that PEGylated liposomes accumulate in solid tumor tissue at a reduced efficiency compared with conventional liposomes (Parr et al., 1997). The use of exchangeable PEG-lipids may help to address both of these issues. We have tested three PEG-lipids of varying acyl chain lengths and found DMPE-PEG, having the shortest acyl chain (C14:0), provided the least liposome protection. Less than 13% of the liposomes remained in the circulation at 1 h after liposome injection, a result that was similar to haptenated liposomes containing no PEG-lipids. With such rapid elimination, there would be very little liposome accumulation in the target tissue, considering liposome extravasation is the rate-limiting step. DSPE-PEG is known for its retention in the liposomal bilayer, and is thus probably not an ideal PEG-lipid for re-exposing liposomal ligands. It remains to be tested whether DPPE-PEG, with an intermediate exchange rate (Li et al., 2001b), can be released from liposomes in the time frame that is suitable for maximal target tissue localization as well as optimal dissociation to reveal the surface-associated targeting ligand.

In conclusion, PEG-lipids play a significant role in improving the circulation longevity of ligand-targeted liposomes. To design liposomes for active targeting, one should pay attention to the type and the concentration of PEG-lipids. As well, the concentration of the targeting ligand should be considered because it plays a role in determining the effectiveness of PEG-lipids in protecting liposomes from immune recognition.

Acknowledgments
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References

### TABLE 1

<table>
<thead>
<tr>
<th>[Biotin]a</th>
<th>[PEG-lipid]b</th>
<th>Lipid Remaining at 1 h after Injectionc</th>
<th>C0 d</th>
<th>[μmol/mL plasma]</th>
<th>[mM]</th>
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<tbody>
<tr>
<td>0 0.1 0.1</td>
<td>0.1 0.1 0.1</td>
<td>2.6 ± 0.21 0.0148 ± 0.0045 0.48 ± 0.23 0.23 ± 0.3</td>
<td>&gt;25</td>
<td>0.131</td>
<td>20.3</td>
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<td>1 1 1</td>
<td>0 0.1 0</td>
<td>0.019 ± 0.004 0.909 ± 0.52</td>
<td>&lt;0.0025</td>
<td>1.5</td>
<td></td>
</tr>
</tbody>
</table>

a Biotinylated lipid concentration in liposomes measured in mol%.
b PEG-lipid concentration in liposomes measured in mol%.
c Plasma lipid concentration measured in mice with an established anti-biotin antibody response obtained from Figs. 2 and 3.
d Lipid concentration required to mediate 20% consumption obtained from Fig. 5.
Targetability of novel immunoliposomes modified with amphipathic poly(ethylene glycol)s conjugated at their distal terminals to monoclonal antibodies. Biochim Biophys Acta 1234:74–80.


Address correspondence to: Dr. Wai Ming Li, Department of Advanced Therapeutics, Vancouver Cancer Research Center, British Columbia Cancer Agency, 601 W. 10th Ave., Vancouver, BC V5Z 1L3, Canada. E-mail: mli@bccancer.bc.ca