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Immunogenicity and Rapid Blood Clearance of Liposomes Containing Polyethylene Glycol-Lipid Conjugates and Nucleic Acid

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ABSTRACT

Polyethylene glycol (PEG) is used widely in the pharmaceutical industry to improve the pharmacokinetics and reduce the immunogenicity of therapeutic and diagnostic agents. The incorporation of lipid-conjugated PEG into liposomal drug delivery systems greatly enhances the circulation times of liposomes by providing a protective, steric barrier against interactions with plasma proteins and cells. Here we report that liposome compositions containing PEG-lipid derivatives and encapsulated antisense oligodeoxynucleotide (ODN) or plasmid DNA elicit a strong immune response that results in the rapid blood clearance of subsequent doses in mice. The magnitude of this response is sufficient to induce significant morbidity and, in some instances, mortality. This effect has been observed in several strains of mice and was independent of sequence motifs, such as immunostimulatory CpG motifs. The ODN-to-lipid ratio and ODN dose was also determined to be important, with abrogation of the response occurring at a ratio between 0.04 and 0.08 (w/w). Rapid elimination of liposome-encapsulated ODN from blood depends on the presence of PEG-lipid in the membrane because the use of nonpegylated liposomes or liposomes containing rapidly exchangeable PEG-lipid also abrogated the response. These studies have important implications for the evaluation and therapeutic use of liposomal formulations of nucleic acid, as well as the potential development of liposomal vaccines.

DNA- and RNA-based therapeutics have tremendous potential for exquisite selectivity in the management of disease, yet these agents have several properties that restrict their application as therapeutic agents, including nuclease sensitivity, rapid plasma elimination, poor intracellular delivery, and hemodynamic toxicities (Levin, 1999; Wang et al., 2003). This has prompted significant research into the development of delivery technologies for this class of drugs. Long-circulating, sterically stabilized liposomes (SSL) containing amphipathic polyethylene glycol (PEG) have been used extensively over the past decade to improve the circulation lifetime of lipid vesicles and entrapped therapeutic agents, provide a steric barrier against interactions with plasma proteins and opsonins, improve disease-site delivery of therapeutic and diagnostic agents, and reduce liposome uptake by mononuclear cells of the reticuloendothelial system (Allen and Hansen, 1991; Senior et al., 1991; Boerman et al., 1997; Lasic et al., 1999). In this regard, SSL have been used in oncology (Goren and Gabizon, 1998; Lasic et al., 1999), antimicrobial (Bakker-Woudenberg and van Etten, 1998), and radiodiagnostic applications (Boerman et al., 1997; Goins et al., 1998). As such, this type of delivery system is a natural candidate to enhance the pharmacokinetic and pharmacodynamic properties of DNA- and RNA-based therapeutics (Lasic et al., 1999).

In the absence of encapsulated or surface-coupled proteins, SSL and other nonviral lipid delivery systems are generally considered to be nonimmunogenic (van Rooijen and van Nieuwmegen, 1980; Alving, 1992; Harding et al., 1997). The immunogenicity and rapid plasma elimination of protein-

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ABBREVIATIONS: SSL, sterically stabilized liposomes; PEG, polyethylene glycol; ODN, oligodeoxynucleotide(s); SCID, severe-combined immunodeficient; DSPC, distearoylphosphatidylcholine; DSPE, distearoylphosphatidylethanolamine; DODAP, 1,2-dioleoyl-3-*N*,*N*-dimethylammonium-propane; CH, cholesterol; biotin X-DSPE, *N*-[((6-biotinoyl)amino)hexanoyl]-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine; biotin-PEG_{2000}-DSPE, *N*-[ω-biotinoylamino (polyethylene glycol)$_{2000}$]-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine; PEG-$CerC_{14}$, 1-*O*-(2′-(ω-methoxypolyethyleneglycol)succinoyl)-2-*N*-myristoylsphingosine; PEG-$CerC_{20}$, 1-*O*-[2′-(ω-methoxypolyethyleneglycol)succinoyl]-2-*N*-arachidoylsphingosine; CHE, cholesteryl hexadecyl ether; ICAM, intercellular adhesion molecule; PO, phosphodiester; PS, phosphoro-

coupled liposomal systems after repeat injections have been well documented (Aragnol and Leserman, 1986; Phillips and Emili, 1991; Phillips and Dahman, 1995; Harding et al., 1997; Tardi et al., 1997) and typically result from antibody-mediated recognition of protein. T cell-independent antibody responses to liposomal glycolipid antigens such as ganglioside GM_1 have also been described (Freimer et al., 1993). These responses were characterized by elevations in antibody production primarily of the IgM class, with antibody recognition directed against the carbohydrate portion of the ganglioside.

Nonmethylated bacterial DNA and synthetic oligodeoxynucleotides (ODN) can stimulate mononuclear cells and lymphocytes in vitro and in vivo, resulting in the secretion of interleukin-6, interleukin-12, interferon-γ, and IgM (Krieg, 2002). This effect has been primarily attributed to CpG and palindromic sequence motifs, but phosphorothioate ODN all exhibit some degree of immune stimulation in vivo (Monteith et al., 1997). Given the relative inefficiency of nucleic acid-based therapeutics and the likely requirement for frequent administration, we evaluated the potential immunogenicity and circulation properties of SSL containing synthetic ODN, plasmid DNA, or ribozyme on repeated injections. Liposome elimination from the circulation was used as a convenient indicator of immunogenicity in vivo. The results presented in this study indicate that the presence of encapsulated nucleic acid in lipid vesicles containing surface-associated PEG stimulates an immune response against the carrier, irrespective of sequence motifs or DNA chemistry, and induces morbidity and rapid plasma elimination of subsequent administrations. The data further demonstrate that this response is directed specifically against the PEG-lipid.

Materials and Methods

Mice. Female 7- to 8-week-old ICR, C57BL/6, and BALB/c mice were obtained from Harlan (Indianapolis, IN). BALB/c *nu/nu* and BALB/c SCID-Rag2 mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained under pathogen-free conditions. All animals were quarantined for at least 1 week prior to use. All procedures involving animals were performed in accordance with the guidelines established by the Canadian Council on Animal Care.

Chemicals and Lipids. Distearoylphosphatidylcholine (DSPC), polyethylene glycol-conjugated distearoylphosphatidylethanolamine (PEG_{2000}-DSPE) and 1,2-dioleoyl-3-*N*,*N*-dimethylammoniumpropane (DODAP) were purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol (CH) was purchased from Sigma-Aldrich (St. Louis, MO). Biotin-X-DSPE and biotin-PEG_{2000}-DSPE were purchased from Northern Lipids (Vancouver, BC, Canada). PEG-$CerC_{14}$ and PEG-$CerC_{20}$ were synthesized and purified as described previously (Wheeler et al., 1999). [^{3}H]Cholesteryl hexadecyl ether (CHE) was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA).

ODN and Plasmid DNA. The designations and 5′ to 3′ sequences of the ODN used in these studies were as follows: hICAM, GCCCAAGCTGGCATCCGTCA (3′-untranslated region of human ICAM-1 mRNA); mICAM, TGCATCCCCCAGGCCACCAT (3′-untranslated region of murine ICAM-1 mRNA); EGFR, CCGTGGTCATGCTCC (human epidermal growth factor mRNA, receptor-translation termination codon region); c-myc, TAACGTTGAGGGGCAT (initiation codon region of human/mouse c-myc proto-oncogene mRNA; and c-mycC, TAAGCATACGGGGTGT (c-myc scrambled control). Phosphodiester (PO) and phosphorothioate (PS) ODN were purchased from Hybridon Specialty Products (Milford, MA). The backbone composition was confirmed by ^{31}P NMR. All ODN were analyzed for endotoxin by the manufacturer VLuc) was produced in *Escherichia coli*, isolated, and purified as described previously (Wheeler et al., 1999).

Encapsulation of ODN. Stabilized antisense-lipid particles (SALP) composed of DSPC/CH/DODAP/PEG-$CerC_{14}$ or PEG-$CerC_{20}$ (molar ratio, 25:45:20:10) and encapsulated PS ODN were prepared as described previously (Semple et al., 2000). For PS ODN, 300 mM citrate buffer was used to dissolve the antisense, whereas 20 mM citrate, pH 4.0 was used for PO ODN and plasmid formulations. The lower citrate concentration was required to facilitate efficient charge interactions between DODAP and the PO ODN or plasmid, resulting in optimal encapsulation efficiencies (Stuart et al., 2004). At higher citrate concentrations, these charge interactions are presumably shielded, and significant reductions in encapsulation efficiencies are observed. Phosphorothioate-modified ODN bind strongly to cationic molecules, and encapsulation efficiencies are much less sensitive to differences in buffer and salt concentrations (Stuart et al., 2004). Plasmid formulations were not extruded, resulting in ~200-nm particles. DSPC/CH (molar ratio, 55:45) and DSPC/CH/PEG_{2000}-DSPE (molar ratio, 50:45:5) vesicles were prepared from dry lipid films by aqueous hydration in HBS (20 mM Hepes and 145 mM NaCl, pH 7.4). Similarly, ODN encapsulation was achieved by hydration of 100 mg of lipid with 100 mg of ODN in 1.0 ml HBS, followed by five cycles of freeze-thawing and extrusion through two stacked 100-nm filters (Semple et al., 2000). The resulting particles were approximately 110 to 140 nm in diameter, as judged by quasi-elastic light scattering using a model 370 NICOMP submicron particle sizer (Particle Sizing Systems, Santa Barbara, CA). [^{3}H]CHE, a nonexchangeable, nonmetabolizable lipid marker, was incorporated into all vesicle compositions to monitor lipid levels in the blood (Stein et al., 1980).

Liposome Elimination from the Circulation. Mice received a single intravenous (lateral tail vein) dose of empty liposomes (50 mg/kg lipid) or liposome-encapsulated ODN (50 mg/kg lipid and 10 mg/kg ODN, unless otherwise specified) containing [^{3}H]CHE (~1 μCi/mouse). Dosing occurred weekly unless otherwise noted. Blood (25 μl) was collected 1 h postinjection by tail nicking unanesthetized mice, using a sterile scalpel (tails were wiped with 70% ethanol prior to nicking), and placed in 200 μl of 5% EDTA. The blood was then digested (SOLVABLE, PerkinElmer Life and Analytical Sciences), decolorized and analyzed for radioactivity according to the manufacturer's instructions. The tail nicking procedure allowed all repeat-injection data to be collected from the same group of animals. A comparison of this procedure with blood (~0.5 ml) collected weekly by terminal cardiac puncture on anesthetized (ketamine/xylazine) mice produced equivalent results.

ELISA. Groups of mice (n = 20 initially) were injected weekly (i.v.) with SALP (PEG-$CerC_{20}$) containing c-myc ODN or empty liposomes of the same lipid composition. One week after each injection, a subgroup of animals (n = 5) was anesthetized (ketamine/xylazine), blood was collected from each animal by cardiac puncture (~0.5 ml), and the animals were subsequently euthanized. Plasma was collected for each animal after centrifuging the blood for 15 min at 1200g in a refrigerated centrifuge (4°C). Individual plasma samples were pooled (n = 5), and serial dilutions were analyzed by ELISA as described below. The results are expressed for the 1:800 plasma dilution, which was the lowest dilution that exhibited minimal nonspecific binding to control wells.

The presence of liposome-reactive antibody was evaluated by ELISA using biotinylated liposomes bound to streptavidin-coated microplates. Liposome formulations bound to microtiter plates included: DSPC/CH/DODAP/PEG-$CerC_{20}$/biotin-PEG_{2000}-DSPE (molar ratio, 24.5:45:25:5:0.5), DSPC/CH/biotin-X-DSPE (molar ratio, 54.5:45:0.5), and DSPC/CH/PEG_{2000}-DSPE/biotin-PEG_{2000}-DSPE (molar ratio, 49.5:45:5:0.5). Biotinylated liposomes (10 nmol of total lipid/well) were incubated overnight at 4°C in clear SILENUS streptavidin-coated microwell plates (Chemicon International, Temecula, CA). Plates were washed, blocked with phosphate-buffered saline containing 3% bovine serum albumin and 0.1% Tween 20, and

liposome (containing entrapped ODN)-treated animals for 1 h at room temperature. Following multiple washes with phosphate-buffered saline/0.1% Tween 20, IgM binding was evaluated by incubation with horseradish peroxidase-conjugated rat anti-mouse IgM monoclonal antibody (1:1000 dilution; BD Biosciences PharMingen, San Diego, CA). The plates were washed and subsequently incubated with 3,3′,5,5′-tetramethylbenzidine substrate (Sigma-Aldrich) and read at 450 nm.

Results

Blood Levels of PEG Liposomes Containing Encapsulated ODN. We have recently developed and characterized a novel lipid formulation that can encapsulate antisense ODN in the aqueous space of lipid vesicles with high encapsulation efficiency and ODN-to-lipid ratios (Semple et al., 2000). This formulation contains a PEG-ceramide lipid coating that is required for the formulation step, and we have therefore called these vesicles SALP. Previous studies have shown that liposomes containing PEG covalently coupled to ceramide derivatives with 20 carbon acyl chains, a relatively nonexchangeable lipid anchor, have circulation profiles indistinguishable from the same liposome compositions containing PEG_{2000}-DSPE, the most commonly used PEG-lipid anchor (Harding et al., 1997; Woodle, 1998; Semple et al., 2000). Typically, these formulations are long-circulating, with 75 to 95% and 30 to 40% of the administered dose expected to remain in the circulation at 1 and 24 h, respectively. Thus, we initially compared the repeat dosing pharmacokinetics (PK) of SALP to various lipid formulations of antisense ODN, including traditional long-circulating, SSL, and DSPC/CH liposomes.

For liposomes and lipid-based delivery systems, monitoring the circulation properties of repeated injections is a convenient surrogate measure of carrier immunogenicity since this parameter is invariably altered by an immune response (Aragnol and Leserman, 1986; Phillips and Emili, 1991; Harding et al., 1997; Tardi et al., 1997; Goins et al., 1998). To evaluate the impact of repeated administrations on the circulation times of PEG liposomes containing hICAM ODN, the blood levels of DSPC/CH/PEG_{2000}-DSPE liposomes or SALP (PEG-$CerC_{20}$) were evaluated 1 h postinjection after weekly intravenous administrations. As expected, no differences in elimination were observed for empty vesicles over several administrations (Fig. 1a). Surprisingly, rapid elimination (<20% of the injected dose remained in the blood at 1 h) of ODN-containing vesicles was observed following the second and subsequent injections. This effect was accompanied by pronounced morbidity and, in some instances, resulted in death of the animal within 30 min postinjection. This rapid elimination phenomenon with PEG liposomes and ODN was observed in several strains of mice, including outbred ICR mice and inbred BALB/c and C57BL/6 strains (results not shown). To determine whether an immune component was involved in this response, these same studies were performed in T cell-deficient BALB/c nude mice and B and T cell-deficient BALB/c SCID-Rag2 mice. A rapid elimination response was observed in BALB/c nude mice (Fig. 1b) but not in BALB/c SCID-Rag2 mice (Fig. 1c), suggesting that the response depends on the presence of B cells and immunoglobulin.

Influence of Nucleic Acid Composition on Clearance of PEG Liposomes. Since it has been shown that PS ODN

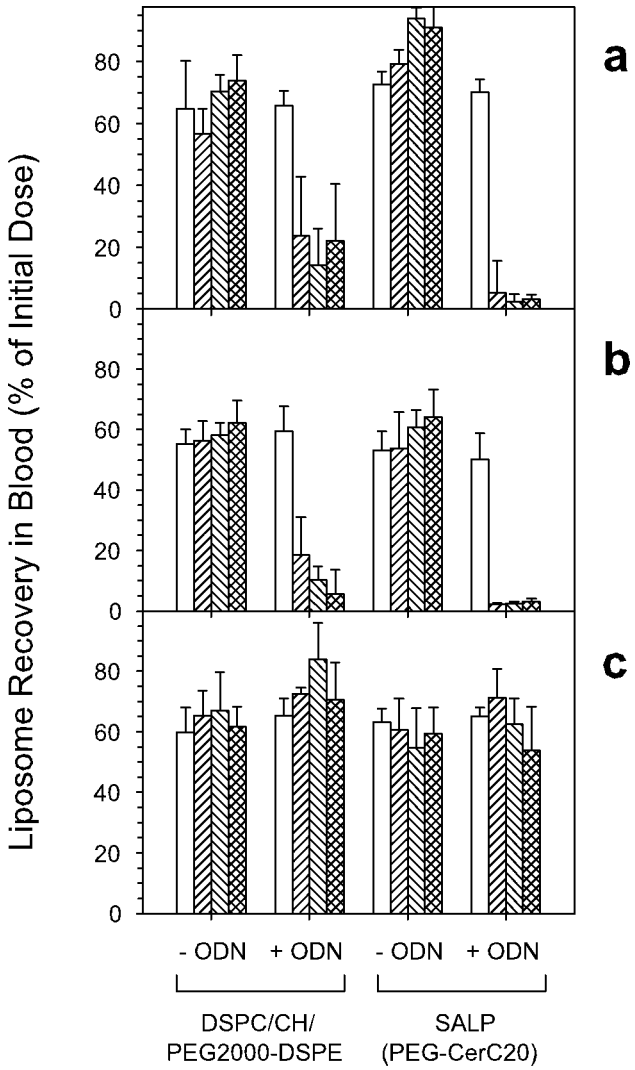

Fig. 1. Circulation levels of PEG liposomes on repeat administrations in immune-competent BALB/c mice (a), and immune-compromised BALB/c nude (b) and BALB/c SCID-Rag2 mice (c). Mice were injected i.v. with empty DSPC/CH/PEG_{2000}-DSPE liposomes, DSPC/CH/PEG_{2000}-DSPE liposomes containing hICAM PS ODN, empty SALP (PEG-$CerC_{20}$), or SALP (PEG-$CerC_{20}$) containing hICAM ODN. Lipid doses were 50 mg/kg. The ODN/lipid ratios for the DSPC/CH/PEG_{2000}-DSPE and SALP (PEG-$CerC_{20}$) were 0.058 and 0.20, respectively. Injections were administered weekly, and the circulation levels at 1 h postinjection were monitored by the lipid label [^{3}H]CHE. The bars represent the first (open bars), second (back slash), third (forward slash), and fourth (cross-hatched) injection. All bars represent the mean and standard deviation of eight mice.

immunostimulatory and/or nonantisense effects (Stein and Krieg, 1997), the influence of oligonucleotide sequence was evaluated by encapsulating a variety of PS ODN in SALP (PEG-$CerC_{20}$). Interestingly, all PS ODN encapsulated in SALP (PEG-$CerC_{20}$) induced morbidity in mice and were rapidly removed from the circulation on repeat administrations (Fig. 2a). This was also observed for ODN that did not contain CpG or G-quartet motifs (mICAM) or contained CpG only (hICAM and EGFR), CpG and G-quartet (c-myc), or G-quartet only (c-mycC).

Having demonstrated sequence independence of the rapid elimination phenomenon, the dependence on nucleic acid chemistry and structure was evaluated. PS or PO ODN, ribozyme, or plasmid DNA was encapsulated in the SALP

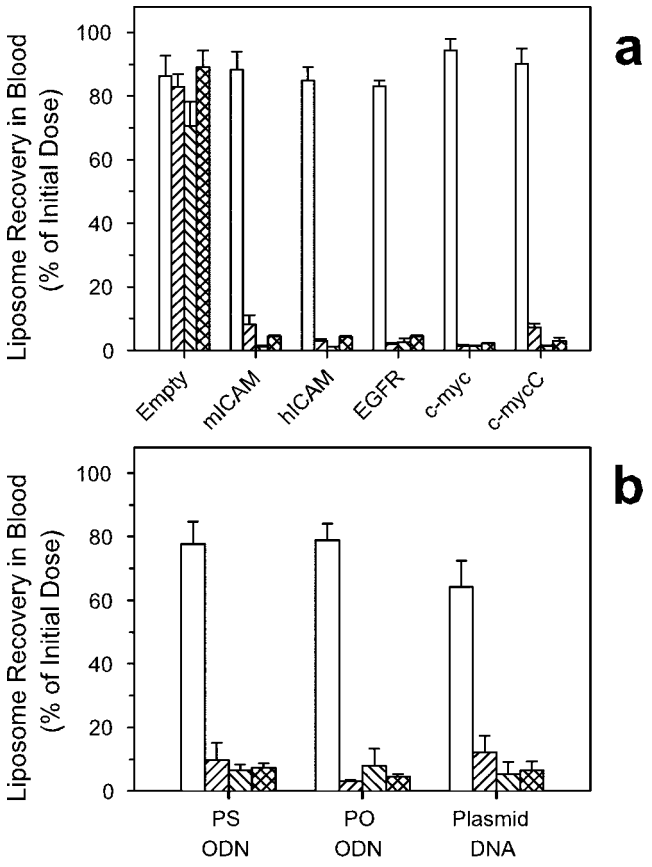

Fig. 2. Influence of nucleic acid sequence (a) and structure (b) on blood clearance of SALP (PEG-CerC_{20}). ICR mice were injected i.v. with SALP (PEG-CerC_{20}) containing PS ODN of various nucleotide sequences (a). PO hICAM ODN and bacterial plasmid DNA were also evaluated (b). The lipid dose was adjusted to 50 mg/kg, and the ODN/lipid ratio for each formulation was ~0.20. Injections were administered weekly, and the circulation levels at 1 h postinjection were monitored by the lipid label [^{3}H]CHE. The bars and numbers of animals are indicated in the legend to Fig. 1.

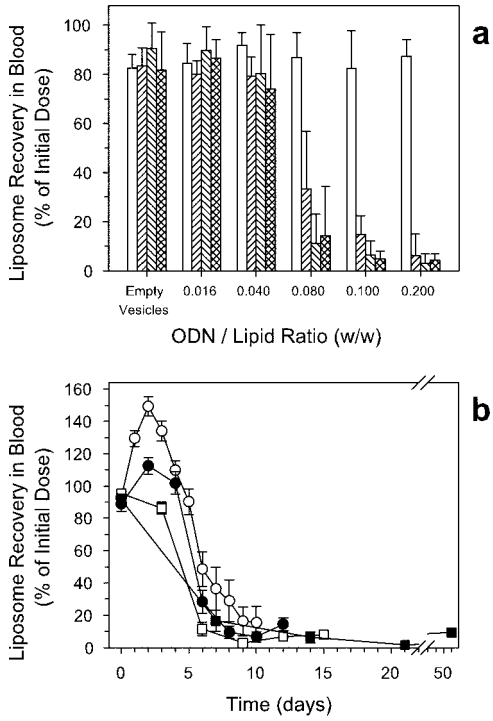

Fig. 3. Factors influencing the onset of rapid clearance of PEG liposomes containing nucleic acid. The influence of the DNA-to-lipid ratio (a) and dosing schedule (b) were evaluated in ICR mice. For the DNA-to-lipid ratio study, mice were injected weekly (i.v.) with SALP (PEG-CerC_{20}) containing hICAM PS ODN at various ODN/lipid ratios. The bars and numbers of animals are indicated in the legend to Fig. 1. In the dose schedule study, mice were injected i.v. with SALP (PEG-CerC_{20}) containing hICAM PS ODN at various dosing schedules: daily (○), every 2 days (●), every 3 days (□), and weekly (■). In both studies, the lipid dose was adjusted to 50 mg/kg/dose, and circulation levels at 1 h postinjection were monitored by the lipid label [^{3}H]CHE.

1 h postinjection were monitored after weekly injections (Fig. 2b). Rapid elimination of the carrier was observed on the second and subsequent injections, indicating that closed circular, double-stranded DNA was as effective at inducing the rapid elimination as ODN with free 5′ and 3′ ends. Similarly, the more nuclease-sensitive PO ODN also induced an immunogenic response, as did ribozyme (results not shown).

Impact of Antisense/Lipid Ratio and Dose Schedules. In Fig. 1, the magnitude of blood clearance for DSPC/CH/PEG_{2000}-DSPE vesicles containing encapsulated ODN was less pronounced than for SALP (PEG-CerC_{20}). The major difference between these two formulations was the encapsulation procedure and the resulting ODN-to-lipid ratio. To test whether the amount of encapsulated ODN influences the generation of the immune response, ODN was encapsulated in SALP (PEG-CerC_{20}) at different ODN/lipid ratios, and the circulating level of vesicles was monitored after weekly injections of 50 mg/kg lipid. Interestingly, at ODN/lipid ratios less than 0.04 (w/w), no apparent changes in elimination were observed, whereas marked decreases in circulation levels were observed at ratios greater than 0.08 (w/w) (Fig. 3a). Since the lipid dose was constant in these studies, the ODN-to-lipid ratio and/or the total ODN dose administered signif-

Standard dosing schedules for antisense ODN therapies typically involve repeated injections or infusions. The relevance of the dosing schedule on circulating levels of SALP (PEG-CerC_{20}) was examined by injecting mice daily or every 2, 3, or 7 days. Liposome levels in the blood were evaluated 1 h after each injection (Fig. 3b). For daily injections, the plasma levels of circulating carrier increased over the first three injections, which was not surprising given that 30 to 40% of a given dose of PEG-coated liposomes remains in the circulation 24 h postinjection (Allen and Hansen, 1991; Harding et al., 1997; Woodle, 1998; Semple et al., 2000); however, this increase was followed by a dramatic decline in the circulation levels of subsequent doses. In all dosing schedules, rapid elimination of subsequent doses was observed beginning 4 to 6 days after the initial dose and was maintained for at least 50 days.

PEG-Lipid Involvement in the Response. To evaluate the importance of PEG-lipid on the generation of the immune response observed in Fig. 1, PS ODN was encapsulated in 100-nm DSPC/CH vesicles or in SALP containing PEG-CerC_{14}. The CerC_{14} lipid anchor has shorter acyl chains than PEG-CerC_{20} and exchanges more rapidly out of the lipid bilayer ($t_{1/2}$, ~1.1 h in vitro) (Wheeler et al., 1999). No differences in the circulation levels of these vesicles, whether empty or containing encapsulated ODN, were observed on repeated administrations (Fig. 4a). This was in striking con-

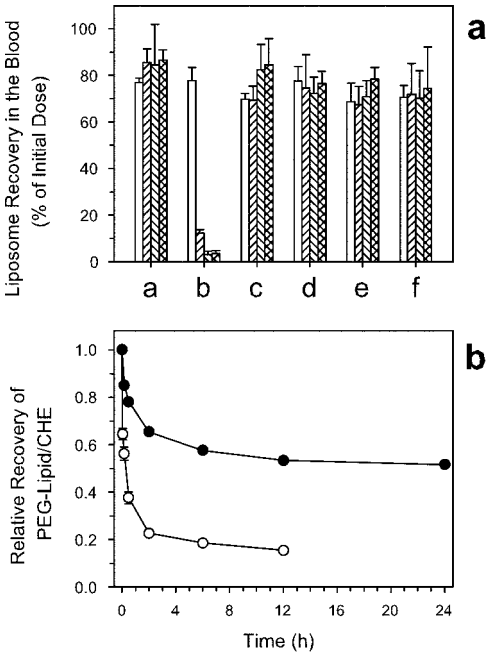

Fig. 4. Role of PEG-lipid in the rapid elimination of liposomes containing ODN. Panel a, mice injected i.v. with empty SALP (PEG-CerC_{20}) (a), SALP (PEG-CerC_{20}) (b), empty SALP (PEG-CerC_{14}) (c), SALP (PEG-CerC_{14}) (d), empty DSPC/CH liposomes (e), or DSPC/CH containing hI-CAM PS ODN [ODN-to-lipid ratio, 0.081, (w/w)] (f). The lipid dose was adjusted to 50 mg/kg/dose, and the circulation levels at 1 h postinjection were monitored by the lipid label [^{3}H]CHE. The bars and numbers of animals are indicated in the legend to Fig. 1. Panel b, time course for exchange of PEG-CerC_{20} (●) and PEG-CerC_{14} (○) evaluated by monitoring the ratio of [^{3}H]PEG-ceramide to [^{14}C]CHE in the plasma of mice over 24 h. The symbols represent the mean and standard deviation of six mice.

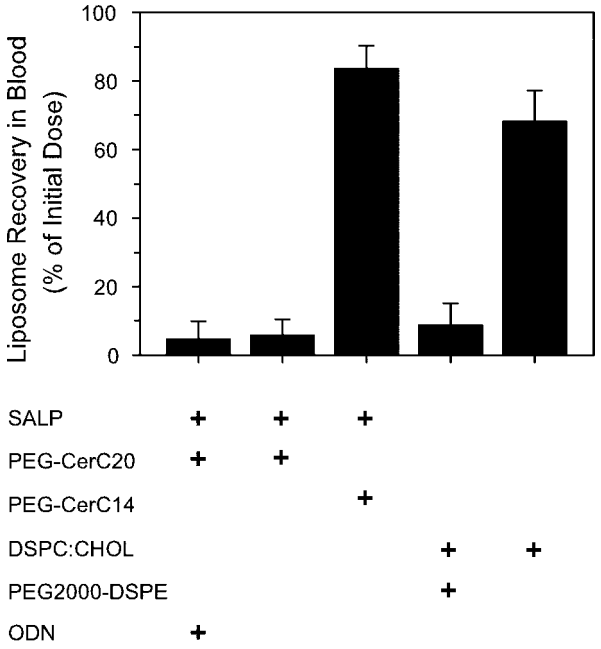

Fig. 5. Crossover studies in presensitized mice. Mice were injected i.v. with SALP (PEG-CerC_{20}) for a total of four weekly injections. In a subsequent, final injection, mice received either SALP (PEG-CerC_{20}), empty SALP (PEG-CerC_{20}), empty SALP (PEG-CerC_{14}), empty DSPC/CH/PEG_{2000}-DSPE vesicles, or empty DSPC/CH liposomes. In each instance, the lipid dose was 50 mg/kg/dose, and the circulation levels at 1 h postinjection were monitored by the lipid label [^{3}H]CHE. Each bar represents the mean and standard deviation of eight mice.

after a second injection. PEG-CerC_{14} exchanged out of the carrier immediately after injection, with greater than 50% loss of PEG-lipid in approximately 3 min (Fig. 4b). This same level was not achieved for PEG-CerC_{20} until 24 h. Since neither empty DSPC/CH vesicles nor SALP containing rapidly exchangeable PEG-lipid exhibited any differences in circulation levels on repeat administrations, we conclude that the presence of PEG-lipid in the external monolayer of the vesicles was critical for the rapid elimination of the carrier from the circulation.

The role of PEG-lipid was further examined in crossover studies in which mice were sensitized with weekly injections (total of four) of SALP containing PEG-CerC_{20} and encapsulated ODN. This was followed with a fifth injection of empty vesicles of varying compositions. Crossover injections of empty DSPC/CH/PEG-CerC_{20} or DSPC/CH/PEG_{2000}-DSPE vesicles were rapidly eliminated from the circulation, whereas crossover injections of DSPC/CH vesicles or SALP containing PEG-CerC_{14} exhibited prolonged circulation times (Fig. 5). The rapid clearance of DSPC/CH/PEG_{2000}-DSPE vesicles from the circulation indicates that the PEG moiety, and not the lipid anchor, was the critical component recognized in this response. Similarly, the use of empty vesicles that had never been exposed to ODN indicates that potential residual surface-associated ODN was not responsible for mediating elimination. Similar results were observed in crossover studies in which, instead of SALP (PEG-CerC_{20}), mice were pretreated with DSPC/CH/PEG_{2000}-DSPE liposomes containing encapsulated ODN (results not shown).

liposome-reactive antibody in the blood, mice were treated four times (weekly injections) with SALP (PEG-CerC_{20}) or empty DSPC/CH/DODAP/PEG-CerC_{20} liposomes, and the pooled plasma of these animals was examined by ELISA using various biotinylated liposomes bound to streptavidin-coated microplates. When the pooled plasma from each group of mice was incubated with plates containing DSPC/CH/DODAP/PEG-CerC_{20} or DSPC/CH/PEG_{2000}-DSPE, increases in liposome-reactive IgM were observed 1 week following the first injection and generally increased over the four injections (Fig. 6). However, when the same plasma was incubated in plates containing DSPC/CH (i.e., no PEG-lipid), very minimal IgM binding was observed, indicating the involvement of antibody in the clearance of PEG-liposomes containing ODN, as well as providing additional confirmation that the response is directed against PEG and not the other lipids comprising the formulation. The plasma from mice treated with empty PEG liposomes showed negligible reactivity in any of the plates.

Discussion

The majority of efficacy studies that use antisense oligonucleotides have required repeated dosing to observe biological activity in animal models. This is principally necessitated by the rapid plasma elimination of polyanionic DNA and minimal antisense ODN delivery to the disease site. To increase the disease site delivery of antisense ODN, we chose to evaluate pegylated lipid-based formulations of antisense ODN. Interestingly, we found that repeated injections of PEG liposomes containing ODN, plasmid DNA, or ribozyme

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