

Hypersensitivity and Loss of Disease Site Targeting Caused by Antibody Responses to PEGylated Liposomes

Adam Judge, Kevin McClintock, Janet R. Phelps, and Ian MacLachlan*

Protiva Biotherapeutics, 100-3480 Gilmore Way, Burnaby, BC, Canada V5G 4Y1

*To whom correspondence and reprint requests should be addressed. Fax: +1 604 630 5103. E-mail: ian@protivabio.com.

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The systemic application of nucleic acid drugs requires delivery systems that overcome the poor pharmacokinetics, limited biodistribution, and inefficient uptake of nucleic acids. PEGylated liposomes show considerable promise because of their intrinsic ability to accumulate at disease sites and facilitate transfection of target cells. Unlike many viral vectors, PEGylated liposomes are generally considered to be nonimmunogenic. We have developed a PEGylated liposome for the systemic administration of plasmid DNA that achieves high levels of selective gene expression at distal tumor sites. Here we report that the *in vivo* efficacy and safety of these systems can be severely compromised following repeat administration. This phenomenon is characterized by a loss of disease site targeting, accelerated clearance from the blood, and acute hypersensitivity. These effects are fully attributable to a surprisingly robust, long-lived antibody response generated against polyethylene glycol (PEG) that results from the strong adjuvant effect of the plasmid payload. Importantly, immunogenicity may be substantially reduced by modifying the alkyl chain of the PEG–lipid conjugate, thereby allowing successful repeat dosing of the modified plasmid formulations without adverse side effects. Immunogenicity is a relevant concern for a number of nonviral delivery systems given the potent immunostimulatory properties of many nucleic acid drugs.

Key Words: non-viral vectors, immunogenicity, nucleic acid-based drugs, liposomes, antibody responses, polyethylene glycol, hypersensitive reactions

INTRODUCTION

Liposomes are an attractive drug delivery system for a diverse array of therapeutic agents due to their relatively high stability in the blood and intrinsic ability to extravasate into tissues with increased vascular permeability, such as solid tumors and sites of inflammation [1,2]. This so-called “passive disease site targeting” can be facilitated by the incorporation of polyethylene glycol (PEG) into liposomes to provide a steric barrier against opsonization and clearance by the reticuloendothelial system (RES) [3,4]. These attributes have been exploited in the field of oncology by a number of liposomal chemotherapeutic [2,5] and scintigraphic agents [1]. PEGylated liposomes also show significant potential for developing nucleic acids as therapeutic agents, particularly in applications requiring systemic administration. Lipid encapsulation of RNA or DNA provides protection from intravascular nuclease degradation, passive targeting to disease sites and can enhance the intracellular delivery of nucleic acids that are otherwise poorly taken up by cells [6].

Administration of many nucleic acids can cause activation of the mammalian immune system, leading to the release of interferons and proinflammatory cytokines. In the case of DNA, immune stimulation is triggered primarily by the recognition of unmethylated CpG sequence motifs by Toll-like receptor-9 (TLR9) [7] located within the endosomal compartment of antigen-presenting cells (APC), including B cells [8,9]. Similar immune recognition pathways are also activated by exogenous single [10,11] and double-stranded RNA [12] through TLR7/8 and TLR3, respectively. In this context, we [13] and others [14,15] have recently reported that synthetic siRNA, under development as a therapeutic mediator of RNA interference, can also induce potent immune stimulation. These immune responses elicited by nucleic acids can be greatly potentiated by the use of delivery vehicles that facilitate cellular uptake [13,16]. Although the immunomodulatory effects of CpG DNA are now being harnessed therapeutically in oncology and allergy applications [17], in many cases immune activation represents an additional hurdle to drug development

due to the significant toxicities associated with excessive cytokine release and the potential for the drug carrier to be rendered immunogenic.

It has long been recognized that liposomes can act as immunological adjuvants as a result of their particulate nature, efficient uptake by APC, and ability to crosslink surface receptors [18], and this property is enhanced when immunostimulatory agents such as CpG DNA are incorporated into the liposomes [19,20]. This has been exploited in the design of liposomal vaccines that generate strong antibody (Ab) responses against weakly immunogenic antigens grafted onto the liposome surface. It is therefore unsurprising that immunogenicity has proven to be a major obstacle in developing receptor-targeted liposomes that incorporate antibodies, peptides, or receptor ligands on their surface to enhance target cell uptake [21–23]. The addition of a PEG coating to these liposomes typically has a minor effect on reducing their immunogenicity [19,21,23].

We have developed stable plasmid lipid particles (SPLP) as a nonviral systemic vector for the expression of therapeutic pDNA at disease sites such as tumors and sites of inflammation [24,25]. SPLP consist of a PEGylated liposome that fully encapsulates a single copy of plasmid DNA, thereby conferring protection from nuclease degradation and extended blood circulation times following systemic administration [24,25]. Here we report that the *in vivo* efficacy and safety of these systems can be severely compromised following repeat administration due to a surprisingly robust Ab response against PEG that arises from the primary administration. Importantly, the immunogenicity of the PEGylated liposomes can be significantly reduced by modification of the PEG–lipid component, allowing for the safe and effective re-administration of the formulated pDNA. Our findings raise important concerns regarding the potential immunogenicity of delivery vehicles currently under consideration for use with immunostimulatory nucleic acid-based drugs, including pDNA, siRNA, and antisense oligodeoxynucleotides.

RESULTS AND DISCUSSION

Disease Site Targeting and Blood Clearance of PEGylated Liposomes

To demonstrate the utility of these systems for the delivery of nucleic acids to distal tumor sites, we encapsulated a CMV–luciferase reporter plasmid into SPLP containing 10 mol% PEG-S-DSG (Luc-SPLP). A single intravenous (iv) administration of Luc-SPLP (5 mg/kg pDNA) into A/J mice bearing subcutaneous Neuro2a tumors on the hind flank resulted in significant reporter gene expression at the distal tumor site 48 h after administration (Fig. 1A). Transgene expression within other, nontarget organs including the liver, lungs, spleen, kidney, and heart was low (Fig. 1A). We obtained similar results in a CT26 liver metastases model in Balb/C mice (not shown).

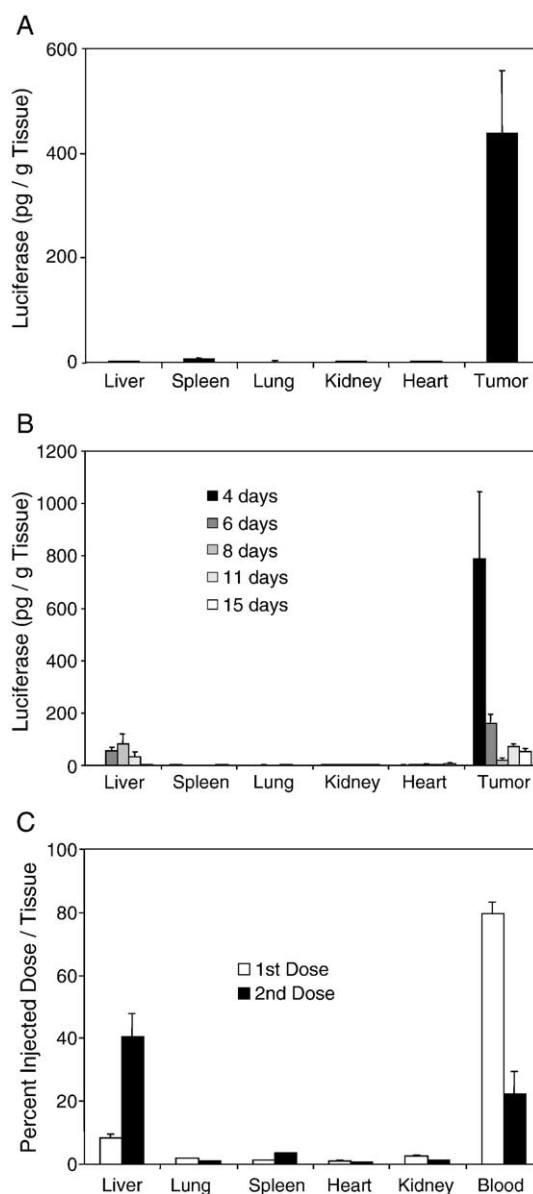


FIG. 1. Repeat administration of PEGylated liposomes is associated with loss of tumor-targeted transgene expression and accelerated blood clearance. (A) Luciferase expression in the tumor and nontarget tissues 48 h after a single iv administration of Luc-SPLP (100 μ g pDNA) containing PEG-S-DSG in Neuro2a tumor-bearing A/J mice. Transgene expression is expressed as pg Luciferase/g tissue. Data represent means \pm SD; $n = 5$ mice. (B) Luciferase expression following repeat administration of Luc-SPLP. Mice were treated with Luc-SPLP 4 to 15 days prior to a second treatment with Luc-SPLP (see key for treatment intervals). Luc expression was determined 48 h after second treatment. Data are presented as in (A). (C) Biodistribution of SPLP 1 h after first or second iv administration. ICR mice were treated with unlabeled SPLP containing 100 μ g pDNA. 7 days later, naive (first dose) or pretreated animals (second dose) received ^3H -labeled SPLP (100 μ g pDNA). Blood and major tissues were collected 1 h after radiolabeled SPLP administration and specific activity was determined. Values are expressed as percentage of injected dose/tissue (means \pm SD; $n = 4$ mice).

To examine whether tumor-selective transgene expression was maintained following multiple treatments, we subjected mice to a subsequent administration of Luc-SPLP at increasing time intervals after the initial treatment. A second administration of Luc-SPLP given 4 days after the first resulted in Luc expression within the tumor that was comparable to that observed after a single treatment (Fig. 1B). However, when the interval between injections was extended to 6 days or greater, tumor transfection resulting from the second SPLP treatment was significantly reduced. Loss of transgene expression in the tumor after multidosing was accompanied by increased expression in the liver, suggesting that the pharmacokinetic or biodistribution profile of the second SPLP dose was adversely affected (Fig. 1B). Changes in tumor growth rate or other tumor-specific changes induced by the first SPLP treatment were unlikely to be the cause of reduced transgene expression since expression was also attenuated following injection intervals of 11 days or greater, which required initial SPLP administration to occur prior to tumor seeding. SPLP treatment had no effect on tumor growth rates (data not shown).

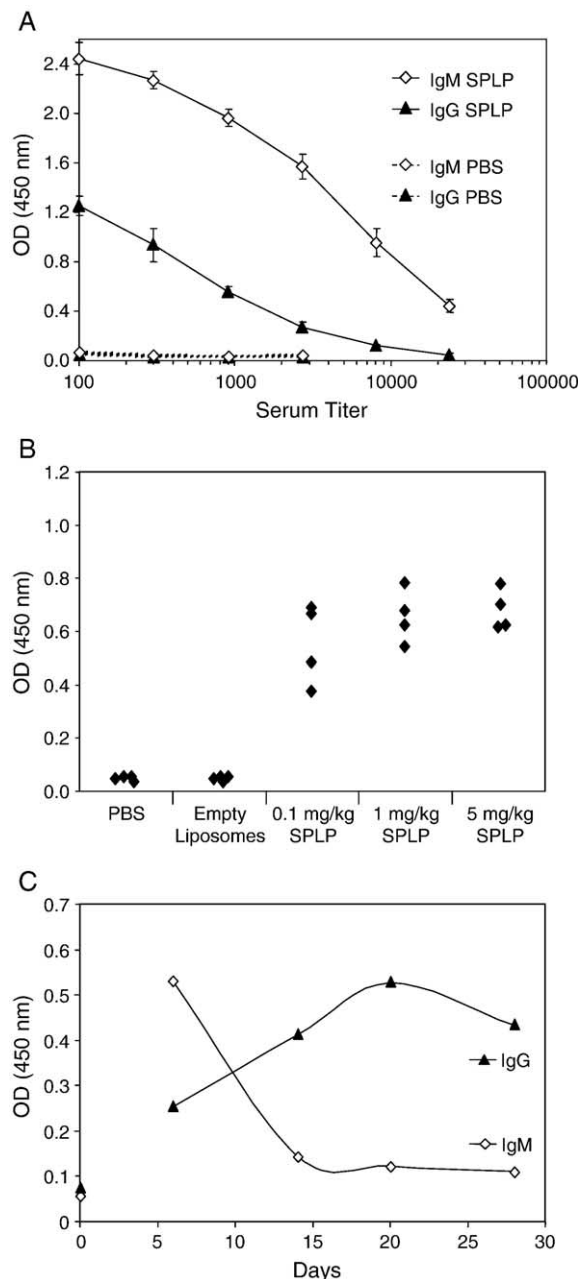
To determine if the dramatic change in gene expression profiles was caused by altered blood clearance or biodistribution of the second SPLP treatment, we incorporated a nonexchangeable radiolabeled lipid marker [26] into SPLP. One hour after a single iv injection of radiolabeled SPLP 80% of the injected dose remained within the blood of naive mice (Fig. 1C), consistent with an expected blood circulation half-life of 12–14 h for liposomes containing PEG-C18 lipids such as PEG-S-DSG [24,25]. Less than 10% of the labeled liposomes had accumulated in the livers of these animals over the first hour after administration (Fig. 1C). By contrast, in mice treated with SPLP 7 days earlier, only 25% of the second SPLP dose remained in the blood 1 h after administration. This was accompanied by significant accumulation of SPLP in the liver and, to a lesser extent, the spleen, implying involvement of the RES in the accelerated blood clearance (Fig. 1C). These results indicate

that changes in gene expression pattern after multiple SPLP administrations are likely attributable to rapid elimination of the PEGylated liposomes from the blood and their redistribution to the liver.

Anti-PEG Antibodies Can Be Generated in Response to PEGylated Liposomes

The loss of tumor targeting and altered biodistribution in multidose studies with SPLP suggested an immune-mediated clearance mechanism. To examine this possibility, we developed a modified ELISA [27] to detect antibodies

FIG. 2. PEGylated liposomes can induce long-lived antibody responses against PEG. (A) DSG-SPLP induces IgM and IgG antibodies reactive against PEG-S-DSG. ICR mice were treated with either PBS (vehicle control; dashed lines) or DSG-SPLP containing 100 μ g pDNA (solid lines). Serum IgM and IgG antibodies reactive against PEG-S-DSG (anti-PEG Ab) were measured 7 days later by a modified ELISA. Pooled serum ($n = 4$ mice) was assayed in duplicate by serial dilution. Values represent mean ODs of three individual assays \pm SD. Similar results were obtained in C57BL/6j and C3H/HeN mice. (B) Anti-PEG Ab responses are induced by low doses of SPLP. Mice were treated with SPLP at 100, 20, or 2 μ g pDNA (1500–30 μ g total lipid, 600–12 μ g PEG-S-DSG) or empty liposomes of identical composition (equivalent to SPLP dose of 100 μ g pDNA). Total anti-PEG IgG (H+L) levels were assessed in serum 7 days later. Data are OD values from serum diluted 1:100 from individual animals in each group. (C) Duration of the anti-PEG IgM and IgG response. ICR mice were treated with SPLP (100 μ g) on day 0. Mice were test bled at the indicated times after treatment up to day 28. Anti-PEG IgM and IgG levels are expressed as mean OD values from pooled serum samples ($n = 4$) diluted 1:100 at each time point.



against the lipid components of PEGylated liposomes. Seven days after a single iv injection of SPLP we detected significant levels of IgM and IgG reactive against the PEG-S-DSG component of SPLP (anti-PEG Ab) in the serum of treated mice (Fig. 2A). These Ab were reactive against other PEG-conjugated lipids and nonreactive against the native, unconjugated lipid, suggesting that the antigenic epitope was the PEG moiety itself rather than the lipid anchor. We did not detect increased Ab reactivities against the other three lipid components of SPLP (not shown). Anti-PEG Ab were generated by relatively low doses of liposomal pDNA. Mice treated with 0.1 mg/kg SPLP (approximately 2 μ g pDNA, 30 μ g total lipid, 12 μ g PEG-S-DSG) developed significant levels of anti-PEG Ab 7 days after administration (Fig. 2B). However, treatment with empty liposomes demonstrated that the generation of anti-PEG Ab to SPLP was entirely dependent on the encapsulated pDNA within the liposome (Fig. 2B). We determined serum levels of anti-PEG IgM and IgG over 4 weeks after a single SPLP treatment. Anti-PEG IgM levels peaked 7 days after SPLP administration and then declined rapidly to reach near pretreatment levels by day 14 (Fig. 2C). In contrast, anti-PEG IgG increased for 20 days after treatment and remained elevated through day 28.

These data demonstrate that the encapsulation of immunostimulatory pDNA within SPLP is sufficient to render the PEGylated delivery vehicle immunogenic. This manifests as a surprisingly robust, long-lived humoral immune response to PEG that is sufficient to cause accelerated blood clearance and loss of disease site targeting upon subsequent re-administration. Liposomes incor-

porating immunostimulatory molecules are known to act as potent adjuvants that can promote Ab responses against weakly immunogenic antigens [19,20], including lipids [27,28], displayed on the outer surface of the liposome. Reports have also shown that Ab responses against PEG can be raised when PEGylated proteins are used in conjunction with aggressive immunization regimens [29,30]. Therefore, despite PEG being typically regarded as nonimmunogenic, it is clear from the current study, as implied in the recent report by Semple *et al.* [31], that PEG itself can act as an antigenic epitope in a drug formulation when presented in the context of a strong adjuvant such as a liposome containing an immunostimulatory payload.

Since pDNA can act as a polyclonal B cell activator [7–9], we examined the B cell proliferative response to SPLP *in vivo* to determine if the production of anti-PEG Ab is part of a generalized polyclonal Ab response. When we assessed proliferation by flow-cytometric analysis of bromodeoxyuridine (BrdU) incorporation [32], we observed only a small increase in the proportion of IgM⁺ B cells proliferating in the spleen following SPLP administration (Fig. 3A) compared to cells recovered from PBS (Fig. 3B) or empty liposome-treated control mice (Fig. 3C). Within the IgM⁺ population, however, a distinct subset of plasmablasts was expanded specifically in SPLP-treated mice. These cells represented up to 3% of the total IgM⁺ population and were defined by their expression of the plasma cell marker syndecan-1 [33] and incorporation of BrdU over the 3 days following SPLP treatment (Fig. 3A). The majority of plasmablasts exhibited reduced expression of the B cell marker B220 (not shown).

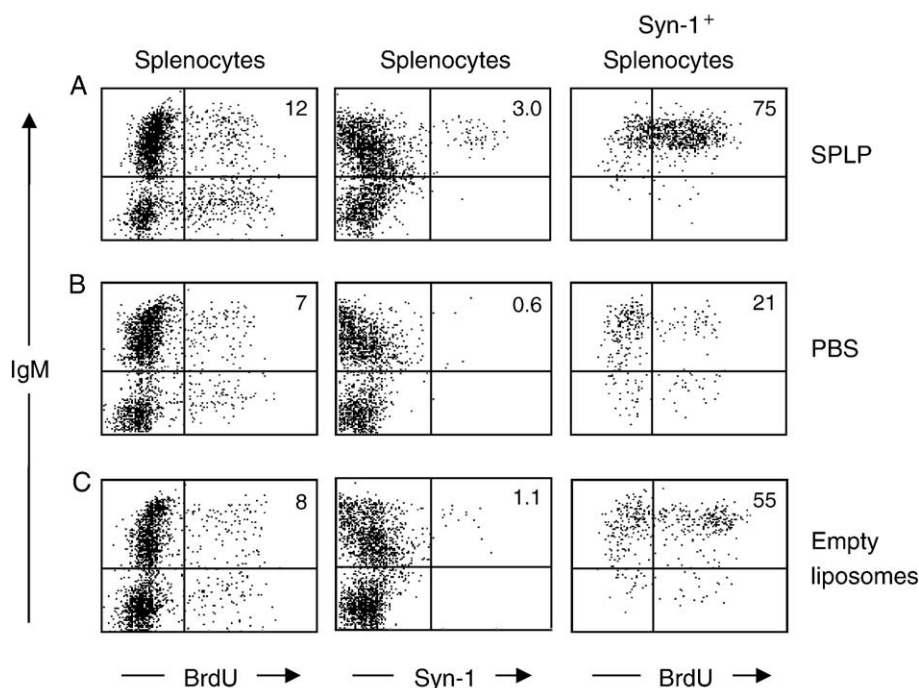


FIG. 3. B cell proliferation and differentiation in response to PEGylated liposomes. Mice were treated with (A) SPLP, (B) PBS, or (C) empty PEGylated liposomes and fed drinking water containing bromodeoxyuridine (BrdU) continually for 3 days to label proliferating cells. Four-color staining of spleen cell suspensions for flow cytometry identified IgM⁺ B cells incorporating BrdU (left) or expressing the plasma cell marker syndecan-1 (Syn-1; middle) and gated Syn-1⁺, IgM⁺ cells that had incorporated BrdU (right). The majority of IgM⁺, Syn-1⁺, BrdU⁺ plasmablasts in SPLP-treated animals had down-regulated the B cell marker B220 (not shown). Plots are representative of spleens from three to six mice in each group assayed in two separate experiments. Values in upper right quadrant represent percentage IgM⁺ splenocytes.

Analysis of the early B cell response indicates that the generation of Abs against PEG likely reflects the selective activation and differentiation of a small subset of B cells rather than polyclonal B cell activation triggered by the nonspecific uptake of pDNA. We therefore envisage a multistep model leading to the production of anti-PEG Ab: first, liposome binding and crosslinking of surface immunoglobulin (sIg) on PEG-reactive B cells; second, internalization of the payload and activation of B-cell-stimulatory pathways such as TLR9 by the pDNA [9]; and third, the induction of cytokines from accessory cells that support maturation of the nascent Ab response. These signals have been shown to act in concert on B cells to generate Ab responses that are independent of T cell help [9,34,35]. Although not observed with our formulations, we do not exclude the possibility that PEGylated liposomes containing nonstimulatory payloads may also be weakly immunogenic. This may become apparent if the liposomes can more effectively crosslink sIg on B cells, a scenario analogous to thymus-independent type II Ab responses characterized by polymeric antigens with multiple repeating epitopes [36]. The efficiency of sIg crosslinking will likely be influenced by liposome size, composition, bilayer fluidity, and epitope density on the liposomal surface. Several groups have demonstrated the accelerated blood clearance of empty PEGylated liposomes in both rodents [37–40] and primates [37] following repeated administration. These phenomena have been attributed to unidentified soluble serum factors [37,40] and enhanced phagocytic activity of the RES [38]. However, it would be interesting to reexamine such models using appropriate assays to determine if Ab responses against the PEG or lipid components may be responsible.

Anti-PEG Antibodies Trigger Platelet-Activating Factor (PAF)-Dependent Hypersensitive Reactions

A single treatment with SPLP containing PEG-S-DSG was sufficient to induce hypersensitivity in mice that manifested as acute toxicity upon subsequent treatment. Symptoms typically developed 5–10 min after re-administration and included lethargy, facial puffing, vasodilation, labored respiration, and significant mortality rates at higher challenge doses. These symptoms appeared typical of an Ab-mediated anaphylactic reaction [41]. Establishment of hypersensitivity required dosing intervals of at least 6 days and was achieved at SPLP doses as low as 2 µg pDNA (~30 µg total lipid), correlating with the development of anti-PEG Ab response in SPLP-treated animals. As such, we still observed hypersensitivity at dosing intervals greater than 28 days in animals with established anti-PEG IgG responses (Fig. 2). In contrast, priming mice with empty liposomes did not induce anti-PEG Ab (Fig. 2B) or hypersensitivity to subsequent SPLP treatment, although empty liposomes could trigger the anaphylactic reaction in animals presensitized with SPLP. Features of the

anaphylactic response to PEGylated liposomes are summarized in Table 1.

In the mouse, one mechanism of anaphylaxis involves PAF [42]. This reaction can be initiated by the formation of IgG containing immune complexes in the blood that trigger excess PAF release from Fcγ receptor-expressing cells [42]. To test if this mechanism was responsible for the anaphylactic reaction to PEGylated liposomes, we treated SPLP-sensitized mice with PAF receptor antagonists immediately prior to a second administration of SPLP. Prophylactic treatment with PAF antagonist CV6209 or CV3988 inhibited the anaphylactic reaction to PEGylated liposomes at challenge doses that otherwise proved fatal in control mice. This suggests that the acute toxicities following repeat administration of PEGylated liposomes are due to the systemic release of PAF, triggered by immune complex formation involving anti-PEG Ab. Hypersensitivity in this model was not associated with excessive systemic cytokine release or significant elevations in plasma histamine or complement activation products (not shown).

Although PEGylated liposomes have not been reported to be immunogenic in humans, their iv administration is associated with hypersensitive infusion reactions in a substantial number of patients [43,44]. Unlike the reactions described here, these clinical events occur upon first exposure to the liposome and have been correlated with the activation of complement [45,46] and a rapid redistribution of the liposomes from the blood to the liver and spleen [43]. Naturally occurring Abs against lipid components have been implicated in this hypersensitive response [46] and it cannot be discounted that this may include preexisting anti-PEG Ab in some patients. In this regard, preliminary analyses of sera from healthy volunteers have identified low levels of anti-PEG reactivity in certain donors (A.J., unpublished data), a finding consistent with previous reports of naturally occurring anti-PEG IgM in a proportion of human subjects [47,48]. It appears therefore that the human B cell repertoire can generate anti-PEG Ab and suggests that the immunogenicity of PEGylated liposomes may become a clinically relevant

TABLE 1: Characteristics of the hypersensitive response following repeat challenge with SPLP containing PEG-S-DSG

First dose (Day 0)	Second dose (Day 7)	Reaction
SPLP 100 µg	SPLP 100 µg	Moderate–severe
SPLP 20 µg	SPLP 100 µg	Moderate–severe
SPLP 2 µg	SPLP 100 µg	Moderate–severe
SPLP 100 µg	SPLP 20 µg	Mild
Empty liposomes	SPLP 100 µg	No reaction
SPLP 100 µg	Empty liposomes	Moderate–severe
SPLP 100 µg	SPLP + PAF antagonists	No reaction

Mice were treated on day 0 (first dose) and day 7 (second dose) with DSG-SPLP or empty liposomes at 100, 20, or 2 µg pDNA or the equivalent lipid dose. Hypersensitive reactions were scored according to the severity of symptoms 10–60 min after the second dose as described under Materials and Methods.

concern as these nucleic acid drug delivery vehicles are advanced into human studies.

Modified Liposomes Containing Diffusible PEG-Lipids Are Less Immunogenic

Liposomes can be engineered in which the PEG-lipid dissociates more rapidly from the lipid bilayer upon *in vivo* administration by using a PEG-lipid with shorter alkyl chain length [25,49]. Since the Ab response to SPLP was directed against the PEG component and required close association with the immunostimulatory pDNA, we speculated that the use of more rapidly diffusible PEG-lipids may limit the opportunity for anti-PEG Ab responses to develop. To test this, we constructed a series of SPLP with similar lipid composition containing one of PEG-S-DSG, PEG-S-DPG, or PEG-S-DMG (C18, C16, or C14 alkyl chain length, respectively). Treatment of mice with SPLP containing PEG-S-DMG (DMG-SPLP) induced peak anti-PEG IgM titers on day 7 that were approximately 10-fold lower than the response in DSG-SPLP-treated mice (Fig. 4A). Consistent with earlier results (Fig. 2C), the weak IgM response to DMG-SPLP was transient and had fully resolved within 14 days after treatment. Significantly, anti-PEG IgG could not be detected after treatment with DMG-SPLP at any time point (Fig. 4B). Antibody responses to the other lipid components in the formulation were also undetectable (not shown). In contrast, anti-PEG IgM and IgG responses were comparable in DSG-SPLP- and DPG-SPLP-treated mice (Figs. 4A and 4B), indicating that reducing the alkyl chain length of the PEG-lipid from C18 to C16 had minimal effect on the immunogenicity of the lipid vesicle.

Correlating with the significant reduction in immunogenicity, mice treated with DMG-SPLP showed no symptoms of anaphylaxis, or other overt signs of toxicity, when subsequently treated with PEGylated liposomes, even when the second dose comprised an immunogenic PEG-lipid formulation such as DSG-SPLP (Table 2). As predicted by the PEG Ab response, pretreatment with either DSG-SPLP or DPG-SPLP resulted in anaphylaxis when mice were rechallenged with any one of the PEGylated liposome formulations (Table 2).

Maintenance of Tumor Targeting When Multidosing with DMG-SPLP

To address whether DMG-SPLP was still able to deliver pDNA systemically to disease sites, we compared the ability of the modified liposomes to target the expression of pDNA to distal Neuro2a tumors. High-level transgene expression in the tumor was achieved after a single iv administration of DMG-SPLP (Fig. 4C), which was reduced approximately 2-fold compared to DSG-SPLP. This slight decrease in gene expression correlated with an equivalent reduction in DMG-SPLP accumulation at the tumor site (not shown). Strikingly, however, equivalent levels of tumor transgene expression were achieved following

repeat administration of the same DMG-SPLP formulation 7 days later without any changes to transgene expression in nontarget tissues, including the liver. This was in contrast to the properties of liposomes containing PEG-lipids with longer alkyl chains in which tumor transgene expression following repeat administration was reduced 70 to 85% in DPG-SPLP- and DSG-SPLP-treated mice, respectively. As in earlier studies, the loss of tumor transfection with immunogenic liposomes was coincident with increased gene expression in the liver (Fig. 4C). Successful re-administration of DMG-SPLP correlated with a 10-fold reduction in anti-PEG IgM titers, determined immediately prior to the second administration of SPLP (Fig. 4D). Unlike a number of other mouse strains examined, A/J mice used in this syngeneic tumor model failed to mount a significant IgG response to any of the SPLP formulations tested at this dose. This finding accounted for the observation that hypersensitivity and accelerated blood clearance were transient phenomena in this particular mouse strain and related to the duration of the anti-PEG IgM response (not shown).

Radiolabeled formulations were used to assess the blood clearance rates of the PEG-S-DMG-modified liposomes upon repeat administration. Equivalent amounts of DMG-SPLP were present in the blood 15 min after the first or second iv injection given 7 days apart (Fig. 4E). After 1 h, only a minor increase in the rate of clearance of the second dose was observed, possibly reflecting the low anti-PEG IgM titers that were detected in the serum 7 days after initial treatment (Figs. 4A and 4D). By contrast, a significant proportion of the second dose of either DPG-SPLP or DSG-SPLP was cleared from the blood within 15 min of administration (43 and 56%, respectively) and blood clearance of these immunogenic formulations was almost complete by 1 h (88 and 90% of first dose respectively; Fig. 4E).

Taken together, these results demonstrate that the immunogenicity of PEGylated liposomes containing pDNA can be greatly reduced by using alternative PEG-lipids that diffuse more readily from the lipid bilayer upon administration. By substantially eliminating the Ab response to PEG, these modified liposomes can be safely re-administered to mice while maintaining the effective delivery of the pDNA payload to distal tumor sites. Administration of these modified liposomes was still associated with substantial cytokine induction (not shown), indicating that the reduced immunogenicity was not due to abrogation of the immunostimulatory activity of the pDNA payload. Instead, these findings support our hypothesis that robust Ab responses to PEG require the close physical association of the PEG-lipid with the pDNA and are driven by the specific binding and internalization of the PEGylated liposome containing stimulatory pDNA by PEG-reactive B cells. An alternative approach therefore to reduce carrier immunogenicity may be the development of less immunostimulatory

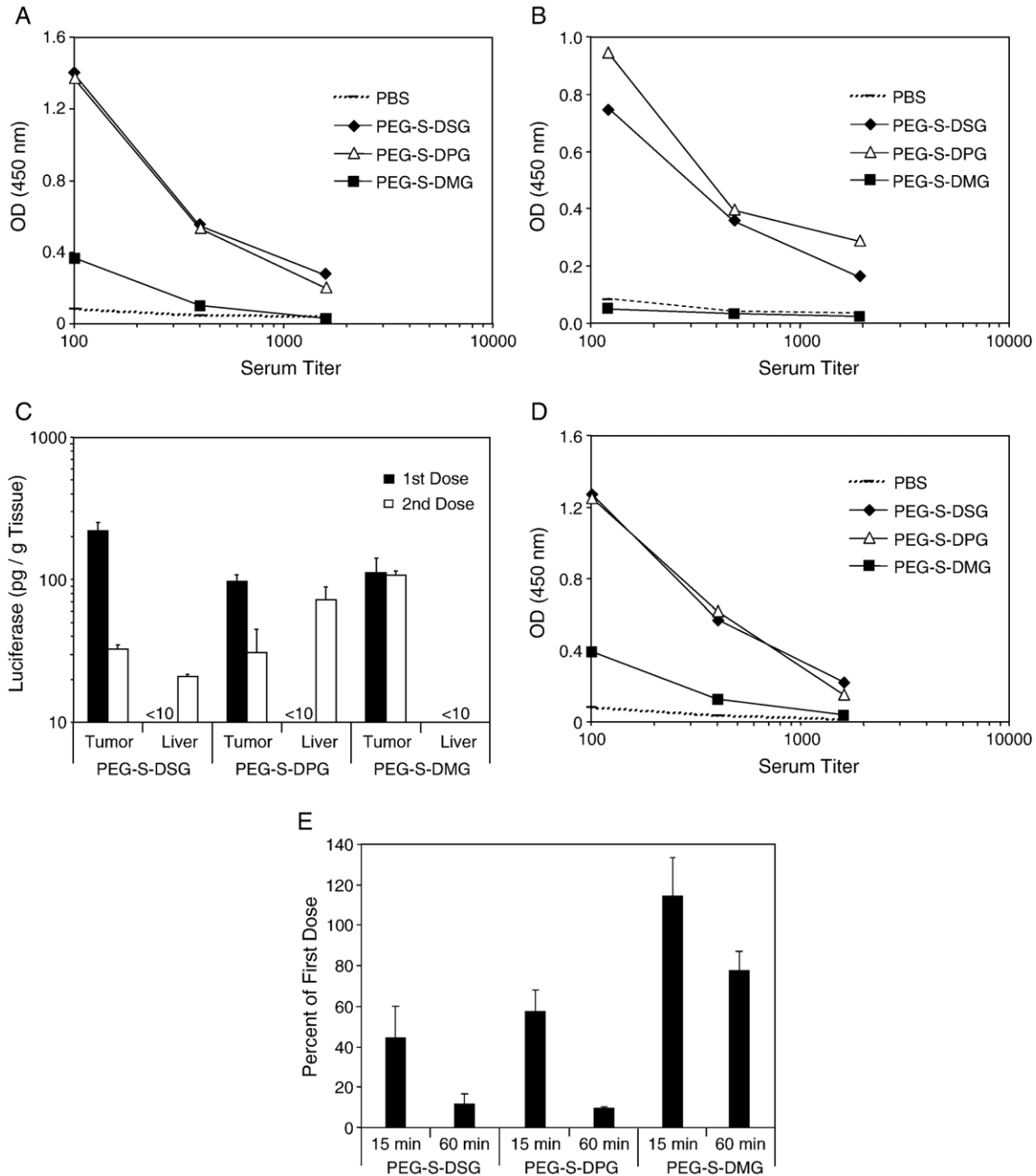


FIG. 4. PEGylated liposomes can be modified to be less immunogenic and maintain disease-site targeting after multiple administrations. (A and B) Anti-PEG antibody responses to SPLP containing PEG-lipids with shorter alkyl chain lengths. (A) Anti-PEG IgM and (B) IgG titers in mice 7 days after administration of PBS or SPLP (50 μ g) containing PEG-S-DSG (C18 alkyl chain length), PEG-S-DPG (C16), or PEG-S-DMG (C14); $n = 4$ mice per group assayed as pooled sera. Qualitatively similar results were obtained in three separate experiments. (C) Luciferase expression in the tumor and liver 48 h after administration of one or two doses (50 μ g pDNA) of modified SPLP (as in A and B). Neuro2a tumor-bearing animals were either naïve (first dose) or pretreated with the same SPLP formulation 7 days earlier (second dose). Transgene expression is expressed as pg luciferase/g tissue (means + SD; $n = 5$). (D) Anti-PEG IgM titers in A/J mice from (C) immediately prior to receiving a second treatment of SPLP. Values are mean ODs from pooled serum samples ($n = 5$ per group). (E) Blood clearance of a second treatment with SPLP containing PEG-S-DSG, DPG, or DMG administered 7 days after initial treatment. Mice were treated with unlabeled SPLP (100 μ g pDNA) 7 days prior to receiving 3 H-labeled SPLP. Values are expressed as the amount of radiolabel (% of single dose values) remaining in the blood 15 and 60 min after administration (means + SD; $n = 4$).

TABLE 2: Absence of a hypersensitive response following repeat challenge with SPLP containing PEG-S-DMG

First SPLP dose (Day 0)	Second SPLP dose (Day 7)	Reaction (n)
PEG-S-DSG	PEG-S-DSG	Moderate (4/9), severe (5/9)
PEG-S-DPG	PEG-S-DPG	Mild (1/9), moderate (4/9), severe (4/9)
PEG-S-DMG	PEG-S-DMG	No reaction (13/13)
PEG-S-DSG	PEG-S-DMG	Mild (1/4), moderate (3/4)
PEG-S-DMG	PEG-S-DSG	No reaction (13/13)

Mice were treated on day 0 (first dose) and day 7 (second dose) with 100 μ g pDNA in SPLP containing PEG-S-DSG, PEG-S-DPG, or PEG-S-DMG. Hypersensitive reactions were scored according to the severity of symptoms 10–60 min after the second dose as described under Materials and Methods. Parentheses indicate number of mice reacting per group. Data are from two separate experiments.

nucleic acids such as CpG-free pDNA and antisense oligos or synthetic siRNA with minimal capacity to activate cytokine responses [13].

The potential for a drug or its excipient to be immunogenic is a serious concern in drug development since the establishment of an Ab response can severely compromise both the safety and the efficacy of a drug. This has hampered the development of certain drug classes, including protein-based therapeutics such as monoclonal Abs and viral vectors that contain immunogenic components. The present study highlights that the potential for nonviral vectors to be immunogenic also becomes a concern when these systems are used for the delivery of immunostimulatory agents, such as pDNA, that can act as potent immune adjuvants. We have recently demonstrated that certain synthetic siRNAs can induce potent immune stimulation *in vivo* [13] and have found that this can also drive the production of a strong anti-PEG Ab response when these molecules are encapsulated in PEGylated liposomes containing C18 PEG-lipids (A.J., unpublished data). The phenomenon of anti-PEG Ab responses to PEGylated liposomes is therefore not unique to pDNA formulations. Given the diverse array of RNA and DNA species that stimulate innate cytokine responses and B cell activation [7,10–14], vehicle immunogenicity may prove to be problematic for a range of nucleic acid-based therapeutics. Antibody responses against surface components, including targeting ligands, of nonviral delivery vehicles should therefore be closely monitored. The ability to abrogate the immunogenicity of PEGylated liposomes by simple modification of their lipid composition without significantly compromising *in vivo* performance has important implications for the design and clinical development of these promising drug delivery systems.

MATERIALS AND METHODS

Lipids and plasmid DNA. The phospholipid distearoylphosphatidylcholine (DSPC) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Synthetic cholesterol (CHOL) was obtained from Sigma–Aldrich Co.

(Oakville, ON, Canada). Tritium-labeled cholesteryl hexadecyl ether (3 H-CHE) was obtained from Mandel NEN Products (Guelph, ON, Canada). The cationic lipid 1,2-dioleoyloxy-*N,N*-dimethylaminopropane (DODMA) was synthesized using methodology detailed in Jeffs *et al.* [24]. The PEG–lipid 3-*O*-(2'-(ω -methoxypolyethyleneglycol) 2000)-1,2-distearoyl-*sn*-glycerol (PEG-S-DSG) and the corresponding C16 and C14 diacylglycerol conjugates PEG-S-DPG and PEG-S-DMG, respectively, were synthesized as previously described [24,25].

The pCMVluc plasmid, encoding the luciferase reporter gene under the control of the cytomegalovirus promoter, was propagated in *Escherichia coli* strain DH5 α and purified by alkaline lysis and 2 \times cesium chloride density gradient centrifugation. Endotoxin levels in pDNA batches purified by this method were very low, ranging from 0.78 to 3.8 EU/mg by LAL assay. Testing was performed by IG Micromed Environmental, Inc. (Richmond, BC, Canada). Experiments in endotoxin-insensitive C3H/HeJ mice confirmed that immune stimulation by liposomal pDNA formulations in these studies was not due to contaminating endotoxin (not shown).

Liposomal encapsulation of pDNA. PEGylated liposomes encapsulating CMV-Luc pDNA (SPLP) were prepared by spontaneous vesicle formation as described previously [24]. The SPLP used in this study contained CHOL:DSPC:DODMA:PEG-S-DAG at molar ratios of 55:20:15:10. Empty vesicles of similar lipid composition, but lacking DNA, were prepared in a manner similar to that for SPLP, with the exception that an equivalent volume of 10 mM TE buffer was added to the initial citrate buffer solution instead of DNA. Radiolabeled SPLP were prepared by incorporating 3 H-CHE at 0.5 μ Ci/mg lipid into the SPLP formulation. Final SPLP typically contained greater than 98% of the pDNA encapsulated within the PEGylated liposomes and were dialyzed in PBS prior to *in vivo* use.

Mice. All animal studies were conducted in accordance with Canadian council on animal care guidelines and protocols approved by the institutional animal care committee. Six- to 8-week-old CD1 ICR and A/J mice were obtained from Harlan and subject to a 3-week quarantine and acclimation period prior to use. SPLP and empty liposomes were administered by standard iv injection into the lateral tail vein in 0.2 ml PBS. For Ab analysis, blood was collected for serum by tail nicks during long-term studies and by cardiac puncture at time of sacrifice. In experiments to examine the role of PAF in the hypersensitive response, mice were administered PAF antagonist CV6209 or CV3988 (BioMol International, Plymouth Meeting, PA, USA) by intraperitoneal injection (50 μ g/mouse) 5 min prior to receiving a second administration of SPLP.

Hypersensitive reactions in mice were scored by two individual people, based on a severity of symptoms scale developed for recording anaphylaxis in the mouse [41]. Symptoms were scored as follows: mild—scratching around face, lethargy, increased respiration rate; moderate—labored respiration, swelling around eyes, cyanosis; severe—no activity, convulsions, morbidity.

Neuro2A tumor transfection model in A/J mice. Ten to 14 days prior to SPLP treatment, 8-week-old male A/J mice were seeded subcutaneously in the hind flank with 1.5×10^6 cells of the Neuro2A murine neuroblastoma cell line (ATCC, Manassas, VA, USA). For luciferase expression studies, established tumors and other tissues were collected 48 h after final treatment with Luc SPLP and snap frozen. Tissues were then homogenized by mechanical disruption in 750 μ l of cell culture lysis reagent (Promega, Madison, WI, USA), and 20 μ l of lysate was assayed for luciferase activity in a 96-well microplate luminometer (Bernholdt Technologies, Oakridge, TN, USA) using the Promega Luciferase Assay reagent kit (Promega) according to the manufacturer's instructions. Recombinant luciferase from the American firefly, *Photinus pyralis* (Roche, PQ, Canada) was used to calibrate luminescence readings.

In vivo BrdU incorporation and flow cytometry. To assess *in vivo* B cell proliferation by BrdU incorporation, mice were injected with 2 mg BrdU (Sigma–Aldrich) in PBS at the time of SPLP administration and fed BrdU in drinking water (0.8 mg/ml) as described [32]. Spleens were harvested 3 days after liposome administration and prepared as single-cell suspensions. Using standard protocols, cells were stained for flow

cytometry in PBS containing 1% FCS, 5% rat serum, and 0.09% sodium azide with the following Abs: phycoerythrin-conjugated anti-CD138 (Syndecan-1, 281-2), Cy5-conjugated anti-CD45R (B220, RA3 6B2) from eBioscience (San Diego, CA, USA), and biotin-conjugated anti-IgM/streptavidin-PE Cy5 (Jackson ImmunoResearch, West Grove, PA, USA). After being stained for surface markers, cells were fixed and permeabilized and then stained with FITC-conjugated anti-BrdU mAb according to the manufacturer's instructions (BD Bioscience, San Diego, CA, USA). Cells were analyzed by a FACSCalibur flow cytometer (BD Bioscience) and CELLQuest software.

Biodistribution and clearance of SPLP. For plasma clearance and biodistribution studies, SPLP incorporating ^3H -CHE were used at a dose of 5 mg pDNA/kg. At appropriate time points, mice were anesthetized and blood was collected by cardiac puncture into EDTA microtainer tubes. Plasma was separated via centrifugation and analyzed for ^3H -CHE by liquid scintillation counting using Picofluor 20 (Perkin-Elmer, Boston, MA, USA) and a Beckman LS6500 (Beckman Instruments, Fullerton, CA, USA). For biodistribution studies, organs were harvested at the specified time points and homogenized in lysing matrix tubes containing 500 μl of distilled water. Tissue homogenates were assayed for radioactivity by liquid scintillation counting with Picofluor 40 (Perkin-Elmer).

Anti-PEG antibody ELISA. An ELISA was developed to detect IgM and IgG antibodies against the PEG-lipid and other lipid components of SPLP based on a method originally described for the detection of anti-cholesterol Ab [27]. All incubations were carried out at room temperature. Ten micrograms of PEG-S-DSG was added in 20 μl 100% ethanol to 96-well plates containing PVDF membranes (Immobilon-P; Millipore Corp., Bedford, MA, USA). PEG-S-DSG-coated membranes were allowed to air dry completely for 2 h before being blocked for 1 h with 10% FBS in PBS. One hundred microliters of serially diluted serum samples in blocking buffer was then applied in duplicate wells for 1 h and washed four times with 1% FBS in PBS. Plate-bound antibodies were detected with HRP-conjugated goat anti-IgM Fc μ , IgG Fc γ , or IgG(H+L chains) to detect total immunoglobulins. After being washed five times, PVDF membranes were developed with TMB substrate for approximately 15 min and stopped with 2 N sulfuric acid, and then 150 μl of the developed substrate was transferred to a clear-walled 96-well plate for reading in a spectrophotometer at 450 nm (minus 570 nm). Cholesterol, DSPC, and DODMA were also used as the coating lipid in this assay in attempts to detect antibodies against the other lipid components of SPLP.

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