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Investigation of antibody-coated liposomes as a new treatment for immune thrombocytopenia

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Abstract

Immune thrombocytopenia (ITP) is an autoimmune disease that is mediated by anti-platelet antibodies. It is believed that anti-platelet antibody-opsonized platelets are eliminated through Fcγ receptor-mediated and complement-mediated phagocytosis by macrophages of the reticuloendothelial system (RES). Polyclonal pooled immunoglobulin with high titer for the D-antigen of erythrocytes (i.e., anti-D) has been successfully used to ameliorate ITP. Based on the pathogenesis of ITP and based on the successful application of anti-D for the treatment of ITP, we hypothesized that antibody-coated liposomes may be used to inhibit Fcγ receptor-mediated and complement-mediated phagocytosis, thereby increasing platelet counts in ITP. To test this hypothesis, we have developed a liposome preparation that is coated with a model monoclonal IgG1 antibody. Antibody-coated liposomes were found to inhibit complement deposition and macrophage phagocytosis in vitro. Furthermore, antibody-coated liposomes were also found to attenuate thrombocytopenia in a rat model of ITP, in a dose-dependent manner. The results suggest that antibody-coated liposomes may be used as 'decoy particles' to competitively inhibit the destruction of antibody-coated platelets; thus, antibody-coated liposomes may have value in the treatment of ITP.
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Keywords: Antibody-coated liposome; Immune thrombocytopenia

1. Introduction

Immune thrombocytopenia (ITP), a common autoimmune disease, is characterized by low platelet counts and by high rates of platelet elimination (George et al., 1996; Cines and Blanchette, 2002). ITP patients develop autoantibodies that bind to platelet antigens (such as glycoprotein IIb/IIIa (GPIIb/IIa) or GPIb/IX) and mediate platelet destruction via the reticuloendothelial system (RES) (McMillan, 2000). Immune-mediated clearance of particles is a function of immunoglobulin class, complement activation, and specific effector cells within the RES (Coetzee et al., 2000). Three discrete pathways may result in the destruction of antibody-coated platelets. In one path-

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way, the direct activation of the complement cascade results in the formation of a membrane attack complex that produces pores in the platelet membrane and subsequent platelet lysis. Platelet destruction may also occur via engagement of complement receptors (CR1), Fcγ receptors (FcγR), or both (Prodinger et al., 1999).

The role of FcγR for eliminating platelets in ITP patients has been well documented (Bussel, 2000). Fcγ-chain deficient mice cannot develop ITP (Clynes and Ravetch, 1995), and ITP is palliated by therapy with anti-FcγR antibodies (Clarkson et al., 1986) and Fc fragments of intravenous immunoglobulin (IVIG) (Debre et al., 1993). However, the role of complement in pathogenesis of ITP is not yet clear (Hed, 1998). Several studies have shown that ITP patients demonstrate elevated levels of platelet-associated C3, C4, and C9, suggesting in vivo complement activation (Kurata et al., 1985), and some of the effects of IVIG may occur by reducing C3 and C4 deposition on platelets (Nomura et al., 1993). Additionally, it has been suggested that FcγR-mediated platelet elimination most likely occurs in the spleen and that complement-mediated platelet elimination most likely occurs in the liver; as such, complement-mediated platelet elimination may be of particular importance in splenectomized ITP patients.

Traditional treatments for ITP include the administration of immunosuppressants (e.g., corticosteroids) and splenectomy (George et al., 1996; Cines and Blanchette, 2002). Approximately 25–30% of chronic ITP patients are refractory to available therapies, and these patients are at high risk for fatal hemorrhage (George et al., 1996; Cines and Blanchette, 2002). The American Society of Hematology published guidelines for the management of ITP in 1996; however, there is no consensus for the management of refractory ITP (George et al., 1996), and there is substantial need for the development of new strategies to treat refractory patients.

Imbach et al. (1981) described the successful use of high dose intravenous immunoglobulin to treat ITP. Shortly thereafter, Salama et al. (1986, 1983) proposed that the increase in platelet counts after IVIG infusion is due to competitive inhibition of the macrophage binding of platelets by preferential sequestration of antibody-coated RBCs by RES. Based on this hypothesis, anti-D was used to treat ITP. Anti-D, a polyclonal antibody preparation containing high titers of antibodies against the Rho (D) red blood cell antigen, has been well documented to increase platelet counts in both children and adults with ITP (Scaradavou et al., 1997; Blanchette et al., 1998). It is believed that anti-D primarily achieves its effects via the binding of anti-D sensitized RBCs to macrophage FcγR, leading to RES blockade. This competitive blockade of FcγR reduces FcγR-mediated elimination of platelets, thereby increasing platelet counts in ITP (Ware and Zimmerman, 1998).

In March 1995, anti-D was approved by the U.S. Food and Drug Administration for the treatment of ITP. However, anti-D is associated with hemolysis and bone-pain (Bussel et al., 1991), and anti-D is not effective in splenectomized ITP patients (perhaps due to an inability to inhibit complement-mediated platelet destruction) (Bussel et al., 1991). Based on the successful application of anti-D in ITP patients, we hypothesize that antibody-coated liposomes may be used to compete with antibody-coated platelets for FcγR in the RES. The antibody density on the surface of liposomes may be engineered to allow complement activation, potentially allowing competitive inhibition of complement-mediated platelet elimination in ITP. Fig. 1 is the proposed mechanism of action of antibody-coated liposomes in ITP.

The objectives of this study were to test the hypotheses that antibody-coated liposomes could be used as decoy particles to: (a) inhibit complement deposition in vitro, (b) inhibit macrophage phagocytosis in vitro, and (c) attenuate thrombocytopenia in a rat model of ITP. We have found that antibody-coated liposomes can inhibit complement deposition and macrophage phagocytosis in vitro and attenuate thrombocytopenia in a rat model of ITP.

2. Materials and methods

2.1. Animals

Female Sprague–Dawley rats, 200–224 g, were used for the in vivo analyses. Rats were kept under a natural light dark cycle, maintained at $22 \pm 4\,^{\circ}\mathrm{C}$, and fed with standard rat chow and water, ad libitum. All animal experiments were performed according to the regulations set forth by the Institutional Animal Care and Use Committee (IACUC) at the University at Buffalo.

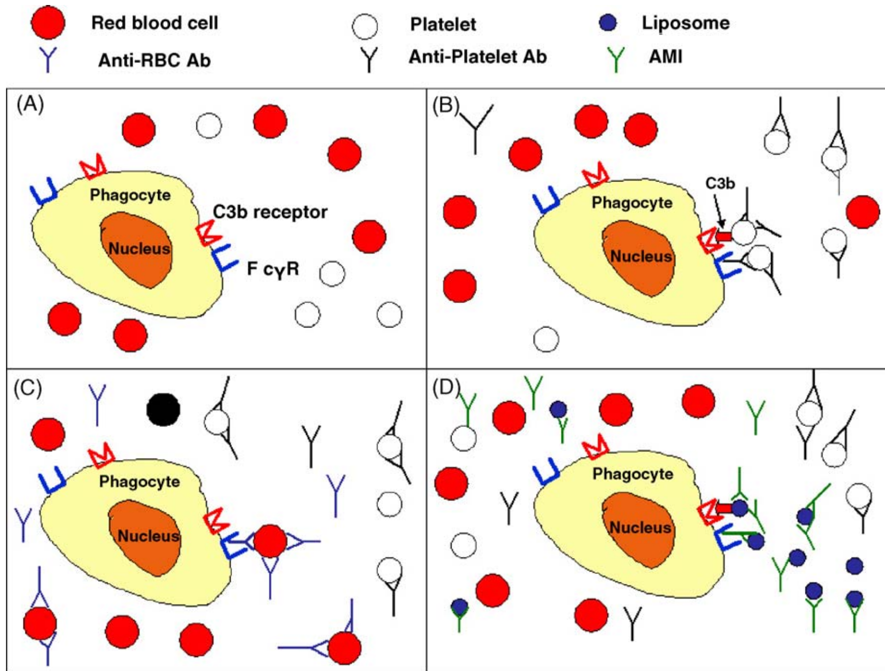

Fig. 1. Proposed mechanism of action of antibody-coated liposomes in ITP. (A) Platelets and RBCs in healthy individuals. (B) Anti-platelet antibody-coated platelets are destroyed by FcγR-mediated and complement-mediated phagocytosis. (C) Antibody-coated RBCs compete with antibody-coated platelets for FcγR after anti-D treatment. (D) Antibody-coated liposomes compete with antibody-coated platelets for FcγRs and complement receptors (CR1).

2.2. Reagents

7E3, a murine anti-GPIIb/IIIa monoclonal antibody, was produced and purified as previously reported (Hansen and Balthasar, 2001). A murine anti-methotrexate IgG1 monoclonal antibody (AMI) was generated and purified in our laboratory (Lobo et al., 2003). Rat alveolar macrophages, NR8383, were obtained from the American Type Culture Collection (ATCC Number: CRL-2192, Manassas, VA), and cultured in F-12K medium with 15% heat inactivated FBS (Gibco, Great Island, NY). Rabbit IgG and Fab specific goat anti-mouse antibody–alkaline phosphatase conjugate were from Sigma (St. Louis, MO). Murine anti-rat complement C3 monoclonal antibody (YSRT-MCA733) and goat anti-rat complement antibody were from Accurate Chemical (Westbury, NY). Donkey anti-goat antibody–alkaline phosphatase conjugate was obtained from Rockland (Gilbertsville, PA). Dimyristoylphosphatidylethanolamine (DMPE), polyethylene glycol-2000 conjugated to dimyristoylphosphatidylethanolamine (PEG_{2000}–PE), cholesterol, and dimyristoylphosphatidylcholine (DMPC) were from Avanti (Alabaster, FL). Methotrexate (MTX), 1-ethyl-3-(3-(dimethylamino)propyl) carbodimide hydrochloride (EDC), and bovine serum albumin (BSA) were obtained from Sigma. *p*-Nitrophenyl phosphate (pNPP) was from Pierce (Rockford, IL). Buffers were phosphate-buffered saline (PBS), 20 mM Na_2HPO_4 (PB), and PB plus 0.05% Tween-20 (PB–Tween).

2.3. Liposomes and AMI-coated liposomes

A methothexate dimyristoylphosphatidylethanolamine (MTX–PE) conjugate was formed by a method described previously (Balthasar and Fung, 1996). Briefly, 54 mg of MTX, 40 mg of EDC, and 25 mg of PE were added into 5 mL of 1:4 (v/v) pyridine/distilled water solution. After stirring at room temperature for 1 h, the solution was dialyzed against 3×1 L of distilled water for 3 days. The conjugate crystallized to

a yellow precipitate, which was collected and stored at −20 °C. This conjugate was characterized by thin layer chromatography (TLC) and by high performance liquid chromatography/mass spectrometry (LC/MS).

Liposomes were prepared by the thin-film method (Berger et al., 2001). Briefly, MTX–PE, PEG_{2000}–PE, cholesterol, and DMPC were combined in chloroform with the final lipid concentration as 15 mg/mL. The molar ratio of MTX–PE:PEG_{2000}–PE:cholesterol:PC is 1:10:50:90. Once lipids were thoroughly mixed in chloroform, the chloroform was evaporated using dry nitrogen gas in a fume hood to form a thin lipid film. Sterilized saline was then added to the thin film. The preparation was incubated for 2 h at 65 °C with agitation every 15 min, and then the suspension was extruded twice through two polycarbonate filters (100 nm, GE Osmonics Labstore, Minnetonka, MN) under nitrogen gas pressure at 65 °C. The liposome suspensions were then incubated with AMI at 37 °C for 2 h (AMI:lipid molar ratio, 1:3000). Following incubation, the liposome mixture was sterilized through a 0.22 μm sterile filter, and stored under nitrogen at 4 °C. Gradient ultracentrifugation was used to separate free AMI from AMI-coated liposomes (Heath et al., 1981). Lipid concentration was determined by the modified Bartlett method described by Rouser et al. (1970). AMI concentration was determined by modified Lowry method (Rodriguez-Vico et al., 1989). The size distribution of liposomes was determined by dynamic light scattering (DLS) (Submicron Particle Sizer, NICOMP™ 380, San Barbra, CA).

2.4. Effects on complement deposition in vitro

An enzyme-linked immunsorbent (ELISA) method, which was modified from the method described by Rieben et al. (1999), was developed to examine the effects of uncoated liposomes and antibody-coated liposomes on the complement deposition in vitro. Briefly, heat aggregated rabbit IgG was prepared by incubating rabbit IgG, 10 mg/mL, for 20 min at 63 °C; insoluble aggregates were removed by centrifugation for 10 min at $3000 \times g$. Aggregated IgG was used to coat Nunc Maxisorp 96-well microplates (Nunc model #4-42404, Roskilde, Denmark) at 10 μg/mL in PB overnight at 4 °C. After incubation, plates were washed with PB–Tween, and then blocked with 1% BSA in PB–Tween (50 μL/well) at room temperature for 1 h. After incubation, plates were washed with PB–Tween. Fresh rat plasma (1:10 dilution in 1 mM Ca^{2+}, 1 mM Mg^{2+} PBS buffer, and 50 μL/well) plus AMI-coated liposomes, uncoated liposomes, or IVIG were applied to the plates and incubated at room temperature for 2 h. Following incubation, the wells were washed with PB–Tween, and a murine anti-rat complement C3 monoclonal antibody (1:500 dilution in PB–Tween, 50 μL/well) was added. After a 2 h incubation at room temperature, the plates were washed with PB–Tween and goat anti-mouse antibody–alkaline phosphatase conjugate was applied (50 μL/well, 1:500 dilution in PB–Tween). After a 1 h incubation at room temperature, plates were washed with PB–Tween, and rinsed with distilled water. Finally, pNPP (4 mg/mL in 10% diethanolamine buffer, 200 μL/well) was added to the washed plates, and the change in absorbance with time (over 10 min) was monitored via a microplate reader at 405 nm (Spectra Max 250, Molecular Devices, Sunnyvale, CA). Both the intra-assay and inter-assay variability of this assay were less than 15%. AMI in the immunoliposome was found to cause some interference with the above procedure. As such, goat anti-rat complement antibody was used as primary antibody and donkey anti-goat antibody–alkaline phosphatase conjugate was used as the secondary antibody when AMI-coated liposomes were applied to assay (thus preventing interference associated with AMI).

2.5. Effects on macrophage phagocytosis in vitro

Anti-D-coated RBCs were used as target cells to examine the effects of antibody-coated liposomes on FcγR-mediated phagocytosis. Rat alveolar macrophages, NR8383, were used in this experiment. This macrophage cell line is an immortalized rat alveolar macrophage line, which has been used to assess the phagocytosis of antibody-coated particles (Helmke et al., 1989; Gebran et al., 1992). The effects of antibody-coated liposomes on macrophage phagocytosis of anti-D-coated RBCs were assessed via a previously reported colorimetric assay using 2,7-diaminofluorene (Gebran et al., 1992). The 2,7-diaminofluorene assay is based on the specific oxidation of 2,7-diaminofluorene to fluorene blue by the pseudoperoxdiase activity of hemoglobin. Briefly, macrophages in F-12K medium (15% heat inactivated FBS) were seeded into flat-bottomed

microplate wells at a density of 4×10^4 cells/well, and allowed to adhere for 24 h at 37 °C (within a 5% CO_2 incubator). Wells were washed once with pre-warmed medium to remove non-adherent cells. Macrophage survival was determined by trypan blue exclusion. One milliliter 10^6 Rho (D)-positive expired human RBCs (American Red Cross, Buffalo, NY) were incubated for 45 min with 2.5 μg/mL of anti-D. Anti-D-coated RBCs were washed by PBS, and suspended to a final concentration of 2×10^5 in culture media. The adherent macrophages were washed twice with pre-warmed medium and exposed to anti-D-coated RBCs, or RBCs with IVIG, uncoated liposomes, or AMI-coated liposomes. After incubation for 150 min, extracellular RBCs were lysed and washed by 0.2% NaCl. Phagocytosed RBCs and macrophages were lysed by 0.2 M Tris–HCl with 6 M urea. Samples were combined with 2,7-diaminofluorene (10 mg/mL) to determine the absorbance at 620 nm according to the method as previously reported (Gebran et al., 1992).

2.6. *Effects on 7E3-induced thrombocytopenia*

Rats were instrumented with jugular vein cannulas under ketamine/xylazine (75:15 mg/kg) anesthesia. Two days after the surgery, rats were dosed with uncoated liposomes (30 μmol lipid/kg) or AMI-coated liposomes (15, 30, 60, or 120 μmol lipid/kg) via the jugular vein catheter. Following liposome dosing, a blood sample (150 μL) was withdrawn for a baseline measurement of platelet counts. Rats were then dosed with 7E3 (8 mg/kg), and platelet counts were taken over 24 h using a Cell-Dyn 1700 multi-parameter hematology analyzer (Abbott Laboratories, Abbott Park, IL). Control animals were dosed with saline, followed by 7E3. Platelet counts data were normalized by the initial platelet counts, as previously reported, due to the large inter-animal variability in initial platelet counts (Hansen and Balthasar, 2001, 2002). Catheters were kept patent by flushing with 0.1 mL heparin (10 U/mL) after each draw. Each group consisted of four rats.

2.7. *Effects on 7E3 pharmacokinetics*

Rats were instrumented with jugular vein cannulas under ketamine/xylazine (75:15 mg/kg) anesthesia. Two days after the surgery, rats were pre-treated with 30 μmol lipid/kg uncoated liposomes or AMI-coated liposomes via the jugular vein catheter. Rats were then dosed 8 mg/kg 7E3, and control animals were dosed with saline, followed by 7E3. Blood samples (150 μL) were taken for 7E3 pharmacokinetic analysis at 1, 3, 6, 12, 24, 48, 72, 96, and 168 h after 7E3 dosing. 7E3 concentrations were obtained using ELISA as previously reported (Hansen and Balthasar, 2001). Non-compartmental pharmacokinetic analysis was used to analyze 7E3 concentration versus time data and obtained the area under 7E3 plasma concentration time curve (AUC), clearance (CL), and volume of distribution at steady state (V_{ss}). Three rats were used for each dose, and the values for the pharmacokinetic parameters are reported as mean ± standard deviation (S.D.).

2.8. *Statistical analysis*

Student's *t*-tests were used to compare the effects of uncoated liposomes and AMI-coated liposomes on complement deposition. One-way ANOVA with Tukey comparison was used to compare the effects of uncoated liposomes and AMI-coated liposomes on macrophage phagocytosis. Differences in the time course of platelet counts for treatment groups were tested for statistical significance using two-way, repeated measures ANOVA. Platelet nadir values and the area of thrombocytopenia were tested for statistical significance using one-way ANOVA, with a Dunnett multiple comparison test to compare treatment groups to the control group. One-way ANOVA with Dunnett multiple comparison was used to compare pharmacokinetic parameters. Statistical analyses were accomplished using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA) and the level of significance for each test was $\alpha = 0.05$.

3. Results

3.1. *Liposomes and AMI-coated liposomes*

TLC results showed that the final product from PE and MTX conjugation reaction was phospholipids with no free amine group. LC/MS results indicated that the molecular weight of MTX–PE is 1072, which is consistent with the MTX–PE chemical structure (Fig. 2).

Uncoated liposomes and AMI-coated liposomes were found to show a normal size distribution

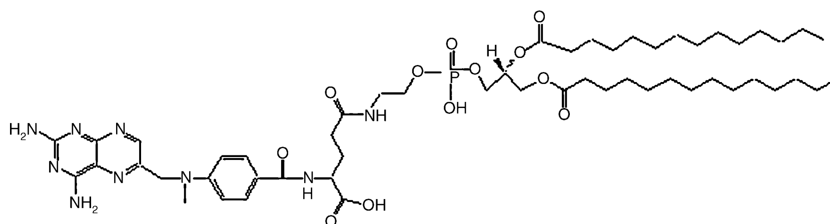

Fig. 2. Structure of methotrexate dimyristoylphosphatidylethanolamine conjugate (MTX–PE).

with mean particle size of 98.23 ± 1.31 nm ($n = 3$, uncoated liposomes) and 101.5 ± 2.76 nm ($n = 3$, AMI-coated liposomes). There are no significant differences between the size of uncoated liposomes and AMI-coated liposomes ($p > 0.05$), which suggests that antibody coating does not lead to liposome aggregation.

The average AMI concentration before separation was 125.4 ± 11 μg/mL ($n = 3$), and the average AMI concentration after separation was 37 ± 4 μg/mL ($n = 3$). The protein:lipid ratio after separation was 67 ± 4 μg/μmol lipid ($n = 3$). Based on the method described by Sada et al. (1990), the number of vesicles per mole lipid was calculated as 4.32×10^{18} for AMI-coated liposomes (100 nm). As such, there were 62 ± 4 ($n = 3$) AMI molecules per liposome vesicle. The molar ratio of MTX–PE:PEG-PE:cholesterol:PC in our AMI-coated liposome preparation (1:10:50:90) provides an antigen density of about 1 MTX per 70 nm^2, which is total 442 MTX molecules on each liposome surface (based on an average molecule cross-sectional area of 0.47 nm^2, which assumes the average head group molecule weight is 200D, and molecule radius is estimated by Stoke's equation: r (nm) $= 0.066 \times MW^{1/3}$). Therefore, the antibody density in our AMI-coated liposome preparation is about 14% of the maximum achievable density for this liposome formulation.

3.2. *Effects on complement deposition in vitro*

Fresh rat plasma was used as a complement source. Results are expressed as inhibition percent (i.e., compared with the control). Since IVIG have the ability to inhibit complement deposition in vivo and in vitro (Larroche et al., 2002; Rieben et al., 1999), IVIG was used as a positive control. The effect of IVIG on complement deposition was concentration dependent with the maximum inhibition about 95% in our testing range (Fig. 3, inset). This result is consistent with the report of Rieben et al. (1999). Uncoated liposomes inhibited complement deposition with percent inhibition values of 4 ± 2%, 11 ± 2, and 32 ± 3% for the 0.129, 0.258, and 0.516 mM lipid concentrations, respectively. AMI-coated liposomes demonstrated percent inhibition values of 24 ± 7, 30 ± 6, and 37 ± 8%, over the same lipid concentration range. Comparing the liposome treatments, the AMI-coated liposomes demonstrated significantly greater inhibition than uncoated liposomes at lipid concentrations of 0.129 and 0.258 mM ($p < 0.01$); however, no significant difference was found at 0.516 mM (Fig. 3).

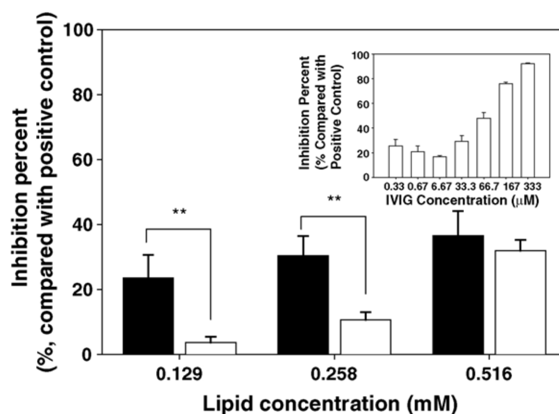

Fig. 3. Effects of uncoated liposomes (□) and AMI-coated liposomes (■) on complement deposition in vitro. The inset represents effects of IVIG on complement deposition in vitro. Rat plasma diluted 1:10 in 1 mM Ca^{2+} and 1 mM Mg^{2+} PBS buffer was incubated for 2 h at room temperature in aggregated rabbit IgG-coated wells together with IVIG, uncoated liposomes, and AMI-coated liposomes. The control group did not receive any treatment. Solid-phase bound C3 was then detected. Error bars represent the standard deviation of the mean of six replicates per treatment. Significance values are indicated ($^{**}p < 0.01$).

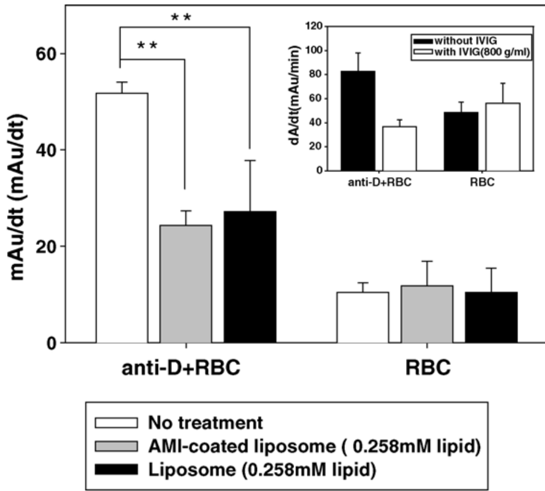

Fig. 4. Effects of uncoated liposomes and AMI-coated liposomes on Fcγ receptor-mediated phagocytosis of RBCs by macrophages in vitro. The change in absorbance with time (dA/dt) is indicative of the quantity of RBCs that were phagocytosed. The inset represents effects of IVIG on Fcγ receptor-mediated phagocytosis of RBCs by macrophages. Error bars represent the standard deviation of the mean of six replicates per treatment. Significance values are indicated ($^{**}p < 0.01$).

3.3. Effects on macrophage phagocytosis in vitro

RBC phagocytosis was substantially increased by opsonization with anti-D. High concentrations of IVIG (e.g., 800 μg/mL) did not alter the phagocytosis of control RBC, but did lead to a significant attenuation of the rate of phagocytosis of anti-D opsonized RBC (Fig. 4, inset). Application of AMI-coated liposomes and uncoated liposomes at a lipid concentration of 0.258 mM was found to decrease the rate of phagocytosis of anti-D-coated RBC by 54 and 48%, respectively, as the assay response was reduced from the control value of 52 ± 2 to 24 ± 3 and 27 ± 11 mAu/min ($p < 0.01$, Fig. 4).

3.4. Effects on 7E3-induced thrombocytopenia in rats

As found in previous work, administration of 8 mg/kg 7E3 to rats led to the rapid development of severe thrombocytopenia (Hansen and Balthasar, 2001). Nadir platelet counts were found to be 38 ± 7 k/μL, which was 11 ± 2% of the platelet counts observed prior to 7E3 administration. Pre-treatment of rats with 30 μmol lipid/kg of AMI-coated liposomes and uncoated liposomes led to an attenuation of 7E3-induced thrombocytopenia, increasing nadir platelet counts by 208 and 45%, respectively (e.g., 11 ± 2% of the baseline platelet count to 34 ± 8 and 16 ± 1% for AMI-coated and uncoated liposomes, $p < 0.001$). Pre-treatment of rats with AMI-coated liposomes was shown to alter the time course of thrombocytopenia induced by 7E3 in a dose-dependent manner ($p < 0.001$, Fig. 5). Pre-treatment with AMI-coated liposomes at doses of 15, 60, and 120 μmol lipid/kg increased nadir platelet counts by 17–208 to 12 ± 3, 20 ± 4, and 32 ± 3%, respectively ($p < 0.01$ for the 60 and 120 μmol lipid/kg doses of AMI-coated liposomes versus the control, Fig. 6). The extent of thrombocytopenia was also characterized by determining the area under of the time course of thrombocytopenia. Following 7E3 administration to control animals, the area of thrombocytopenia was found to be 2005 ± 11% × h; pre-treatment with AMI-coated liposomes decreased the area of thrombocytopenia in a dose-dependent manner, with area decreasing by 70 ± 15 and 64 ± 3% for animals treated with 60 and 120 μmol lipid/kg, respectively ($p < 0.01$ versus control).

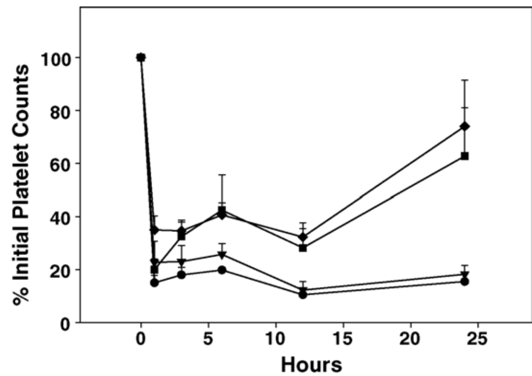

Fig. 5. Effects of AMI-coated liposomes on the time course of 7E3-induced thrombocytopenia in rats. Rats (four per group) received AMI-coated liposomes (or saline) followed by 8 mg/kg 7E3.Treatment groups are designated as follows: saline (●), 15 μmol lipid/kg (▼), 60 μmol lipid/kg (■), and 120 μmol lipid/kg (♦). AMI-coated liposomes and 7E3 were given intravenously, and platelet counts were obtained using Cell-Dyn 1700 multi-parameter hematology analyzer. Error bars represent the standard deviation of the mean. AMI-coated liposomes attenuated thrombocytopenia in a dose-dependent manner. Treatment differences were statistically significant ($p < 0.001$).

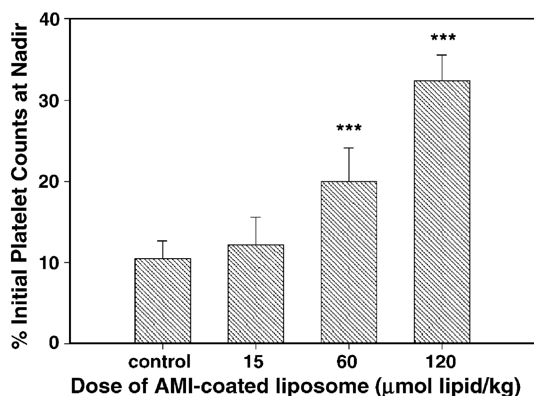

Fig. 6. Percent of initial platelet counts at nadir obtained after AMI-coated liposomes (15, 60, and 120 μmol lipid/kg) administration followed by 8 mg/kg 7E3. Control animals were pre-treated with saline. Each group consisted of four rats. Error bars represent the standard deviation of the mean value. Platelet counts were determined 1, 3, 6, 12, and 24 h after administration of 7E3. Nadir counts were observed 12 h after 7E3 administration for control animals, 12 h after 7E3 administration for animals treated with 15 and 120 μmol lipid/kg, and 1 h after 7E3 administration for animal treated with 60 μmol lipid/kg. The 60 and 120 μmol lipid/kg treatment groups demonstrated significant increases in platelet count at nadir relative to control ($^{***}p < 0.001$).

3.5. *Effects on 7E3 pharmacokinetics in rats*

Effects on 7E3 pharmacokinetics were determined by measuring 7E3 plasma concentrations following pre-treatment of rats with uncoated liposomes and AMI-coated liposomes, respectively. Pharmacokinetic data were analyzed by WinNonlin software (Pharsight Corporation, Mountain View, CA). As can be seen in Table 1, pre-treatment of uncoated liposomes and AMI-coated liposomes did not alter 7E3 pharmacokinetics. No significant differences were found between groups with regard to 7E3 clearance, volume of distribution at steady state, or cumulative plasma exposure ($p > 0.05$, for each metric).

4. Discussion

We investigated a new strategy for ITP treatment in vitro and in vivo in this paper. In vitro results indicated that AMI-coated liposomes inhibited the deposition of rat complement on heat-aggregated rabbit IgG and also inhibited the phagocytosis of anti-D sensitized RBCs by rat macrophages. Rats were pre-treated with AMI-coated liposomes, 15–120 μmol lipid/kg, and then treated with 7E3, an anti-platelet antibody. As shown in Fig. 5, pre-treatment of rats with AMI-coated liposomes led to a significant attenuation of 7E3-induced thrombocytopenia.

Table 1
Effects of uncoated liposomes and AMI-coated liposomes on pharmacokinetic parameters of 7E3

	CL (mL/(h kg))	$AUC_{0-168\,h}$ (μg/(mL h))	V_{ss} (mL/kg)
7E3 + saline	0.79 ± 0.09	8073 ± 619	83 ± 7
7E3 + uncoated liposomes	0.94 ± 0.09[†]	7724 ± 839[†]	61 ± 15[†]
7E3 + AMI-coated liposomes	1.64 ± 1.28[†]	7252 ± 5755[†]	62 ± 28[†]

Rats (three per group) received 30 μmol lipid/kg AMI-coated liposomes, uncoated liposome, or saline followed by 8 mg/kg 7E3. 7E3 concentrations were obtained using ELISA. Non-compartmental pharmacokinetic analysis (WinNonlin software) was used to analyze 7E3 concentration vs. time data and obtained clearance (CL), the area under 7E3 plasma concentration time curve (AUC), and volume of distribution at steady state (V_{ss}). The results are expressed as mean ± S.D. ($n = 3$).

[†] One-way ANOVA with Dunnett comparison test; $p > 0.05$.

Liposomes were first described by Bangham et al. (1965) as a model for studying biological membranes, and liposomes were applied to the field of drug delivery in the 1970s. The behavior of liposomes in vivo is dependent upon dose, morphology (size and lamellarity), lipid composition, bilayer packing structure, and surface characteristics (charge, hydrophobicity/hydrophilicity, presence of polymers and ligands) and the route of administration (Allen and Stuart, 1999). Most liposomes administrated intravenously are eliminated by the phagocytic cells of the RES. The clearance of liposomes by the RES is a major obstacle in the use of liposomes to achieve drug delivery to non-RES cells; however, this clearance pathway allows enhanced drug delivery to phagocytic cells (i.e., "passive targeting"). Alves-Rosa et al. (2000) have used liposome-encapsulated clodronate and the passive targeting strategy as a "suicide approach" to deplete macrophages selectively in the treatment of experimental thrombocytopenia in a mouse model. Additionally, liposome-encapsulated doxorubin has also been shown to attenuate thrombocytopenia (Cosgriff et al., 1998); however, the mechanism of this effect is still under investigation. Although the macrophage

suicide approach has some promise, the depletion of macrophages may be associated with substantial risks. The antibody-coated liposome strategy that we have proposed may be more selective than the suicide approaches, as we anticipate that antibody-coated liposomes will inhibit CR1 receptor-mediated phagocytosis and Fcγ receptor-mediated phagocytosis, while not altering other macrophage functions.

Our prototype liposome preparation is expected to provide approximately 4.32×10^{18} vesicles per mole of lipid. Therefore, after administration of AMI-coated liposomes, AMI-coated liposomes far outnumber the other blood cells (such as antibody-coated platelets) and may be preferentially sequestered by the RES. Hence, treatment with antibody-coated liposomes may saturate elimination pathways within the RES, allowing prolonged survival of antibody-coated platelets. Several antibody–liposome conjugates have been described in the literature, most of which used conjugation methods to covalently coat liposomes with antibodies (Hansen et al., 1995). The reversible binding strategy that we have used was based on our intent to mimic anti-D-coated RBC and antibody-coated platelets; however, it is likely that covalent binding strategies may lead to the generation of antibody-coated liposomes that are equally effective to our prototype formulation.

Liposomes can activate the complement system, and the extent of this activation has been shown to vary with lipid dose, lipid saturation, cholesterol content, the presence of charged lipids, and liposome size (Bradley and Devine, 1998). Liposomal activation of the classical pathway may occur when antibodies to liposomal phospholipids and cholesterol bind to the vesicles. Liposomes can also activate complement through non-antibody-mediated mechanisms via the alternative pathway (e.g., via activation of C1q through electrostatic interaction). We have observed that our liposome preparations inhibit complement deposition in vitro (Fig. 3), and we have found that AMI-coated liposomes were more potent than uncoated liposomes in achieving this effect. It is plausible that antibody on the surface of liposomes allows a more efficient activation of the complement pathway, but we have not yet attempted to test this hypothesis directly. At least four different mechanisms of complement modulation by IVIG have been postulated: (a) binding of activated complement components, particularly C3b and C4b, thus functioning as a "scavenger" (Basta, 1996); (b) binding of C1q to IgG, consuming C1q in plasma and diverting C1 attack opsonized cells (i.e., "depletion") (Mollnes et al., 1995); (c) enhanced inactivation of C3b bound to immune complexes; (d) blockade of the C1R receptor (Mollnes et al., 1997). It is possible that antibody-coated liposomes may inhibit complement-mediated platelet elimination by all of the above mechanisms; future studies will attempt to examine the significance of these possible effects.

The role of complement-mediated platelet elimination in ITP is controversial. It is believed that C1q fixation requires a high density of available Fc domains (e.g., $\sim$1 IgG/800 nm^2) (Prodinger et al., 1999). Platelets (1–3 μm) would require approximately 3900–35,000 IgG/PLT in order to activate C1q (assuming uniform IgG surface distribution). About 80% ITP patients have autoantibodies directly against GPIIb/IIIa which has a density of about 40,000 GPIIb/IIIa per human platelet (McMillan, 2000; Wagner et al., 1996). Therefore, it is clearly possible that antibody density on the platelet surface may be high enough to activate complement, and antibody-coated platelets may be eliminated by complement-mediated destruction. Erythrocytes (5–10 μm) would require about 98,000–390,000 IgG/RBC to activate C1q (assuming uniform IgG surface distribution). However, the D-antigen density on the RBC surface only ranges from 10,000 to 30,000 (Rochna and Hughes-Jones, 1965). So, anti-D-coated RBCs cannot bind and activate C1q. It is well accepted that the anti-D preparation does not lead to complement fixation and that anti-D-coated RBCs are eliminated primarily by FcγR-mediated phagocytosis (Meryhew et al., 1985). Based on the size of our liposome particles, approximately 40 IgGs will be required for complement fixation. Our protein/lipid analyses suggest that there are approximately 60 antibody molecules associated with the surface of each liposome particle (on average) in our prototype formulation (assuming the homogeneous distribution of MTX–PE on the surface of liposomes). As such, the present AMI-coated liposome formulation may be expected to activate complement, potentially allowing blockade of complement-mediated platelet elimination. Additionally, the molar ratio of AMI and MTX–PE may be modulated to activate complement pathway more efficiently.

Several groups have reported strategies to increase Fcγ receptors-mediated targeting of liposomes to the RES. Macrophage uptake of liposomes has been increased tremendously by coating liposomes with antibody (via covalent and non-covalent strategies) (Betageri et al., 1993; Ahsan et al., 2002; Derksen et al., 1988). Petty et al. (1980) observed that the specific antibody-dependent phagocytosis of lipid vesicles resulted in a loss of Fc surface receptor activity from RAW264 macrophages. In another study, formation of an IgG–antigen complex on the liposome resulted in as much as 100-fold enhancement of uptake by mice peritoneal macrophages, presumably via the macrophage Fcγ receptor system (Hsu and Juliano, 1982). In this study, anti-dinitrophenyl (DNP) IgG was used to coat liposomes containing 1–2% DNP–PE, which is very similar to the approach that we have taken. In our initial work, we attempted to use DNP–DMPE as the target hapten in our liposome formulation; however, the affinity of prototype anti-TNP antibodies to DNP was too low for efficient formulation of liposomes in our hands (data not shown). The AMI antibody that we have used was developed in this laboratory, and it has shown high affinity ($K_A = 5.6 \times 10^8\,M^{-1}$) (Lobo et al., 2003). We found that it was quite easy to synthesize the MTX–PE conjugate and, thus, we selected AMI as a model antibody to coat liposomes. It is likely that any high-affinity IgG antibody could be used to produce qualitatively similar effects to those that we have reported here; however, engineered antibodies with improved affinity for Fcγ or complement may provide some unique advantages.

Interestingly, as we were performing experiments to test our hypothesis, Song et al. (2003) reported that anti-RBC monoclonal antibodies can mimic anti-D effects in a murine ITP model. Recently, the same group also found that IgG antibodies against soluble antigens can attenuate thrombocytopenia in the same murine ITP model (Siragam et al., 2005). However, the monoclonal anti-RBC treatment was associated with severe hemylosis (Song et al., 2003), and there is some concern that soluble immune complexes will be eliminated too quickly for feasible use in human ITP patients. The antibody-coated liposome formulations do not alter RBC count or hemoglobin levels (data not shown), and previous literature demonstrates that liposomes may be engineered to allow very long circulation times. As such, the shortcomings of other similar therapies likely do not apply to our proposed liposomal therapy.

The effects of the antibody-coated liposomes on 7E3-induced thrombocytopenia may be dependent on many factors, including liposome size, liposome surface antibody density, lipid composition, and the time interval between liposome pre-treatment and anti-platelet antibody treatment. Each of these factors will be investigated in future work. Additionally, it is recognized that the 7E3 model is a passive, acute model of ITP, which is substantially different from ITP in humans. Current work in the laboratory is evaluating the effects of the liposome strategy in chronic, active models of ITP, which may be more clinically relevant.

To our knowledge, this is the first report demonstrating that antibody-coated liposomes can attenuate thrombocytopenia in ITP. Thus, this work presents a new treatment for ITP, which may provide benefit to ITP patients that are refractory to the existing therapies.

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