Published in final edited form as:

J Pharm Sci. 2008 April ; 97(4): 1386–1398. doi:10.1002/jps.21102.

Phosphatidylserine Containing Liposomes Reduce Immunogenicity of Recombinant Human Factor VIII (rFVIII) in a Murine Model of Hemophilia A

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Abstract

Factor VIII (FVIII) is a multidomain protein that is deficient in hemophilia A, a clinically important bleeding disorder. Replacement therapy using recombinant human FVIII (rFVIII) is the main therapy. However, approximately 15-30% of patients develop inhibitory antibodies that neutralize rFVIII activity. Antibodies to epitopes in C2 domain, which is involved in FVIII binding to phospholipids, are highly prevalent. Here, we investigated the effect of phosphatidylserine (PS)-containing liposomes, which bind to C2 domain with high affinity and specificity, upon the immunogenicity of rFVIII. Circular dichroism studies showed that PS-containing liposomes interfered with aggregation of rFVIII. Immunogenicity of free- *versus* liposomal-rFVIII was evaluated in a murine model of hemophilia A. Animals treated with s.c. injections of liposomal-rFVIII had lower total- and inhibitory titers, compared to animals treated with rFVIII alone. Antigen processing by proteolytic enzymes was reduced in the presence of liposomes. Animals treated with s.c. injections of liposomal-rFVIII showed a significant increase in rFVIII plasma concentration compared to animals that received rFVIII alone. Based on these studies, we hypothesize that specific molecular interactions between PS-containing bilayers and rFVIII may provide a basis for designing lipidic complexes that improve the stability, reduce the immunogenicity of rFVIII formulations, and permit administration by s.c. route.

Keywords

hemophilia A; recombinant FVIII; immunogenicity; inhibitor antibodies; phosphatidylserine liposomes; protein delivery; protein formulation; lipids; immunology

INTRODUCTION

FVIII is a large multidomain glycoprotein consisting of domains A1, A2, B, A3, C1 and C2.^{1,2} It serves as a critical cofactor in the intrinsic pathway of the coagulation cascade. The deficiency or dysfunction of factor VIII (FVIII) causes hemophilia A, an inherited bleeding disorder.³ Replacement therapy with plasma-derived or recombinant human FVIII (rFVIII) is the most common therapy employed to control bleeding episodes.⁴ A major complication in

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therapy is the induction of neutralizing antibodies against the exogenously administered protein, which occurs in approximately 15-30% of patients.⁴⁻⁶ Although several approaches are employed clinically to manage patients manifesting inhibitor activity,^{7,8} FVIII-neutralizing antibodies represent a major challenge in the management of the disease.

The formation of antibodies to therapeutic proteins can have a profound impact on the pharmacology and efficacy of protein drugs.⁹ Factors that influence their immunogenicity include aggregation, protein sequence and frequency of administration.^{10,11} The presence of protein aggregates can affect not only the therapeutic activity of the protein, but also the intensity of the antibody-based immune response.^{11,12} Investigations of rFVIII have revealed that conformational changes in the lipid binding region (residues 2303-2332), which is localized to the C2 domain, may be involved in the initiation of the aggregation process.¹³

Based on systematic epitope mapping experiments, inhibitory anti-FVIII antibodies have been found to target defined regions in the A2 (heavy chain), A3 and C2 (light chain) domains of FVIII.^{14,15} Within the C2 domain, epitope regions have been mapped to residues 2181-2312^{16,17} which encompass the immunodominant, universal CD4+ epitopes 2191-2210, 2241-2290, 2291-2330.^{18,19} Inhibitory antibodies against the C2 domain have been shown to interfere with the binding of FVIII to platelet membrane domains rich in phosphatidylserine (PS), a critical amplification step in the coagulation cascade.⁷ It has been reported that the head group of PS, *O*-phospho-L-serine (OPLS) binds to the lipid binding region localized to the C2 domain of FVIII.²⁰ Despite the lower affinity of rFVIII to OPLS compared to its affinity to PS containing membranes,^{20,21} Purohit et al. showed that OPLS improved the physical stability and decreased the immunogenicity of rFVIII in a murine model of hemophilia A.²⁰ However, OPLS did not alter the pharmacokinetic parameters of rFVIII, such as extended half-life. It is possible that the lack of beneficial systemic effect was due to the low affinity constant of OPLS for rFVIII that could cause the dissociation of the complex following administration (Purohit, unpublished results).

Here, we have evaluated the effect of PS-containing liposomes, which appear to interact with the C2 domain of FVIII specifically and with higher affinity than OPLS, upon the conformation and stability of rFVIII. We sought to test the hypothesis that the interaction of liposomes containing PS with rFVIII could reduce immunogenicity of the protein in a murine model for hemophilia A, while preserving its activity. The use of PS for immunomodulation receives support from the observation that apoptotic cells inhibit both inflammation and the adaptive immune response to self antigens by the exposure of PS on the outer leaflet of the plasma membrane.²² Our data indicate that binding of PS containing liposomes to rFVIII results in reduced immunogenicity of the protein. Furthermore, an increase in plasma concentration following s.c. administration was observed in the case of animals treated with PS containing rFVIII, compared to animals that received free rFVIII alone, suggesting the potential feasibility to use this route of administration for rFVIII-PS formulations.

MATERIALS AND METHODS

Materials

Purified full-length rFVIII (Baxter Biosciences, Carlsbad, CA) was used as antigen. Advate, commercially available albumin-free full length rFVIII—was obtained as a gift from Dr. Bernstein from the Hemophilia Center of Western New York. Normal (control) plasma and FVIII-deficient plasma was purchased from Trinity Biotech (Co Wicklow, Ireland). Brain phosphatidylserine (BPS), dimyristoylphosphatidylcholine (DMPC), and distearoylphosphatidylcholine (DSPC) were obtained from Avanti Polar Lipids (Alabaster, AL), stored in chloroform at -80°C, and used without further purification. Sterile, pyrogen free water was purchased from Henry Schein Inc. (Melville, NY). Goat antimouse

immunoglobulins conjugated to alkaline phosphatase were from Southern Biotechnology Associates, Inc. (Birmingham, AL). The monoclonal antibodies ESH8 and ESH4 were purchased from American Diagnostica Inc. (Greenwich, CT). *O*-phospho-L-serine (OPLS), IgG-free bovine serum albumin (BSA), cathepsin-B and diethanolamine were obtained from Sigma (Saint Louis, MO). *p*-Nitrophenyl phosphate disodium salt was purchased from Pierce (Rockford, IL). Phosphate buffered saline (PBS), was obtained from Invitrogen Corp., (Carlsbad, CA). 4-15% Tris-HCl precast acrylamide gels were obtained from BioRad (Hercules, CA). All other buffer salts used in the study were purchased from Fisher Scientific (Fairlawn, NJ).

Preparation of Liposomal-rFVIII

Liposomes were prepared from DSPC (phase transition temperature, $T_c \sim 55^\circ\text{C}$), DMPC ($T_c \sim 23^\circ\text{C}$), or DMPC and BPS ($T_c \sim 6-8^\circ\text{C}$) in 70:30 molar ratio. The required amounts of phospholipid were dissolved in chloroform and the solvent was evaporated using a rotary evaporator (Buchi- R200, Fisher Scientific) to form a thin lipid film. Liposomes were formed by rehydrating the film in Tris buffer (300 mM NaCl, 25 mM Tris, 5 mM CaCl_2 pH 7.0, prepared in sterile pyrogen free water) at $60-65^\circ\text{C}$ (DSPC) or 37°C (DMPC and DMPC/BPS). The liposome formulations were extruded multiple times through double-stacked polycarbonate membranes of progressively smaller pore size (400, 200, and 80 nm) using a high-pressure extruder (Mico, Inc., Middleton, WI) operated at a pressure of ~ 250 psi. In some cases, 80 nm extruded liposomes were further subjected to sonication for 45 min (Laboratory Supplies Co., Inc., Hicksville, NY). Liposome preparations that were either sonicated or extruded were sterilized by filtration through a $0.22 \mu\text{m}$ MillexTM-GP filter unit (Millipore Corporation, Bedford, MA). Lipid recovery was estimated by assay of inorganic phosphorous using the method of Bartlett.²³ The size distribution of the liposomes was determined using a Nicomp Model CW 380 particle size analyzer (Particle Sizing Systems, Santa Barbara, CA) as described previously.²⁴

Liposomes were associated with the appropriate amount of rFVIII by incubating at 37°C for ~ 30 min with gentle swirling. The protein to lipid molar ratio was maintained at 1:10000 for all the experiments, unless stated otherwise. Preparations were endotoxin negative by Limulus amoebocyte assay (Charles River Laboratories, Inc., Wilmington, MA) and were used immediately after preparation.

Separation of Free Protein from Liposome Associated Protein

To estimate the amount of protein associated with liposomes, free protein was separated from liposome-associated protein by flotation on a discontinuous dextran density gradient as described previously.²⁵ The liposomes and their associated protein floated to the interface of the buffer/10% dextran bands, and the unassociated protein remained at the bottom. The amount of protein associated with the liposomes was estimated by activity assay. For formulations containing 80 nm liposomes, the protein associated with the lipid particles was also estimated by absorbance at 280 nm. The absorbance readings were corrected by subtracting the scattering contribution of the protein-free liposomes.

Circular Dichroism Experiments

Circular dichroism (CD) spectra were acquired with a JASCO J-715 spectropolarimeter calibrated with d10 camphor sulfonic acid and equipped with a Peltier 300 RTS temperature control unit. Because the CD spectra of samples containing liposomes may be distorted by light scattering, correction was applied as described earlier.²⁶ Unfolding of the protein was determined by monitoring the ellipticity at 215 nm over a temperature range of $20-80^\circ\text{C}$, using a heating rate of 60°C/h and a 2 min holding time at every 5°C increment. The thermal

denaturation studies were carried out in buffers with a low metal binding capacity (Tris buffer)²⁷ and with a low temperature coefficient (MOPS buffer).¹³

***In Vivo* Studies**

A colony of hemophilic mice, bearing a targeted deletion in exon 16 of the FVIII gene, was established using breeding pairs provided by Drs. Kazazian and Sarkar, University of Pennsylvania, PA.²⁸ Equal numbers of adult male and female mice, aged 8-12 weeks, were used for the studies; the characteristics of their immune response to rFVIII are comparable.^{29,30}

Blood samples obtained by cardiac puncture were added at a 10:1 (v/v) ratio to acid citrate dextrose (ACD) containing 85 mM sodium citrate, 110 mM D-glucose and 71 mM citric acid. Plasma was separated by centrifugation and stored at -80°C until analysis. All studies were performed in accordance with the guidelines of Institutional Animal Care and Use Committee (IACUC) of the University at Buffalo.

Immunization of hemophilic mice ($n = 12-16$) consisted of four subcutaneous (s.c.) injections of rFVIII or rFVIII-liposomes (containing 2 µg protein) at weekly intervals. A control group of male hemophilic mice ($n = 8$) received four intravenous (i.v.) injections of free rFVIII *via* the penile vein (containing 2 µg protein) at weekly intervals. Blood samples were obtained at the end of 6 weeks.

Detection of Total Anti-rFVIII Antibodies

Total anti-rFVIII antibody titers were determined by ELISA as described previously.²⁰ Antibody titers were expressed as follows: linear regression was performed on the absorbance values obtained with standard solutions of monoclonal ESH8 antibody over a range of 25-150 µg/mL. Half the difference between the maximum and minimum predicted absorbance was calculated as the plate specific factor (PSF), i.e., $PSF = (1/2)(\text{maximum} - \text{minimum})$ absorbance value. A linear regression of the plot of absorbance values *versus* log dilution (over the range of 1:100-1:40000) was used to calculate the dilution that gave an optical density equal to the PSF. The dilution so obtained was considered the antibody titer of the sample.

Detection of Inhibitory Anti-rFVIII Antibodies

Inhibitory (neutralizing) anti-rFVIII antibodies were detected using the Nijmegen modification of the Bethesda assay.³¹ Residual rFVIII activity was measured using the one stage aPTT assay.³² Each dilution (1:2-1:32000) was tested in duplicate. One Bethesda Unit (BU) is the inhibitory activity that produces 50% inhibition of rFVIII activity. The point of 50% inhibition was determined by linear regression of those data points falling within the range of approx. 20-80% inhibition.

***In Vitro* Cathepsin-B Digestion Studies**

rFVIII and liposomal-rFVIII at a concentration of 100 µg/mL in sodium acetate buffer (sodium acetate 40 mM, 1 mM EDTA and 1 mM dithiothreitol, pH 5.0) was incubated for 60 min with 0.05 U of cathepsin-B at 37°C. Fifteen microliters of the sample were mixed with an equal volume of Laemmli buffer (Biorad, Hercules, CA) and heated for 5 min at 90°C. Samples loaded onto the gels were subjected to electrophoresis under a constant voltage of 120 V for 65 min. The gel was stained with coomassie blue and densitometric scans were obtained using a Kodak Image Station (Rochester, NY).

***In Vitro* Determination of Liposomal-rFVIII Activity**

The activity of rFVIII associated with liposomes was determined using the one-stage activated partial thromboplastin time (aPTT) assay.³² Briefly, samples containing liposomal-rFVIII were mixed with an equal volume of FVIII-depleted human plasma and incubated at 37°C. Following addition of the activator (platelin-L reagent) and CaCl₂ the clotting time of the sample was measured using a Coag-A-Mate XM coagulometer (bioMérieux, Inc., Durham, NC).

***In Vivo* Determination of Liposomal-rFVIII Activity**

The *in vivo* activity of liposomal-rFVIII following s.c. administration was investigated using the tail clip method in hemophilia A mice.³³ Three animals per group were injected s.c. with free rFVIII (400 IU/kg), liposomal-rFVIII (400 IU/kg) or buffer (placebo). Two hours later, 1 cm of the tip of the tail was excised with a scalpel. Survival of the animals was monitored for 18 h.

In a separate *in vivo* experiment, the amount of rFVIII in plasma following s.c. administration was quantified using the chromogenic assay method (Coamatic FVIII, DiaPharma Group, West Chester, OH). Hemophilic mice received a dose of 4000 IU/kg of either free or liposomal-rFVIII (80 nm DMPC/BPS (70/30)). Blood samples were collected 1, 3, 5 and 7 h postdose using cardiac puncture and added to ACD. Following centrifugation of the blood, plasma samples were collected and stored at -80°C until analysis.

Statistical Analysis

Data was analyzed using Minitab (Minitab Inc., State College, PA). One-way ANOVA followed by Dunnett's post hoc multiple comparison test was used to detect significant differences ($p < 0.05$) unless stated otherwise.

RESULTS AND DISCUSSION

Interaction of rFVIII with PS-Containing Liposomes

A gentle and controlled thermal unfolding procedure (described in Materials and Methods Section) was employed to promote interaction of rFVIII with PS-containing liposomes, and the fraction of protein associated with liposomes of varying composition was investigated. Free (unassociated) protein was eliminated from the formulation by flotation of the liposomes on a polymer density gradient.²⁵ Approximately 47% of the added rFVIII was associated with liposomes composed of DMPC/BPS, whereas only approximately 10% was associated with liposomes composed of DMPC alone. These results were consistent with previous reports that showed that the relative affinity of FVIII for binding PS was much greater than for PC,³⁴ and suggested that the interaction of FVIII with PS-containing bilayers was stable and not readily reversible. The clotting times of the protein in the presence and absence of PS-containing liposomes were comparable (data not shown), showing that the rFVIII associated with PS-containing liposomes was as active as free rFVIII.

The C2 domain of FVIII has been shown to be important in the interaction of FVIII with platelet membranes.^{35,36} Previously we reported on the conformation and topology of rFVIII bound to liposomes composed of DMPC/BPS.²⁴ The data indicated that the lipid binding epitope consisting of residues 2303-2332, localized in the C2 domain of rFVIII, was involved in specific interactions of the protein with DMPC/BPS liposomes. To investigate whether a lack of specific interactions between the C2 domain and PC membrane bilayers was the cause of lower association of rFVIII with DMPC liposomes, a sandwich ELISA was employed.²⁴ In this system, we evaluated the ability of the liposomes to compete with the binding of monoclonal antibody ESH4, which is directed against the phospholipid binding epitope

2303-2332.^{37,38} ESH4 was used as the stationary antibody to capture rFVIII or liposomal-rFVIII, and a rat polyclonal antibody was used as the probe antibody. The rationale for this assay design was that if liposomes interact with rFVIII *via* the epitope region 2303-2332, the liposomes would be expected to compete with ESH4 binding to that domain.

Liposomes composed of DMPC alone did not reduce significantly the binding of ESH4 to rFVIII (Fig. 1), suggesting little interaction of the epitope region 2303-2332 with DMPC bilayers. In contrast, a dose dependent reduction in ESH4 binding was observed for PS-containing liposomes (Fig. 1). Thus, the ELISA studies, consistent with the gradient flotation data, suggested that the increased association of rFVIII with PS-containing liposomes resulted from a specific interaction between rFVIII domains and PS-containing bilayers, and that this interaction involved the participation of the epitope region 2303-2332. Within the three-dimensional structure of the C2 domain, this membrane binding epitope was located in a region having a high degree of solvent exposure.¹⁸ Furthermore, it has been proposed that this peptide has unstructured regions forming universal CD4+ epitopes that are immunodominant.¹⁸

Effect of PS-Containing Liposomes on the Aggregation of rFVIII

The aggregation of rFVIII, which represents a serious pharmaceutical problem may involve a complex unfolding process, in which conformational changes in the lipid-binding C2 domain contributed to the initiation of aggregation.¹³ Therefore, to investigate whether the interaction of rFVIII with PS-containing bilayers can affect the aggregation of rFVIII, thermal denaturation studies were carried out in the presence and absence of PS-containing liposomes. Thermal stress was chosen as an unfolding method because it is used frequently to probe the relationship between protein folding and stability, and to gain insight into aggregation mechanisms.^{26,39}

To investigate whether rFVIII interactions with PS-containing bilayers alters the overall conformation of FVIII during thermal unfolding, far-UV CD spectra were acquired at various temperatures. At 20°C, the far-UV CD spectrum of rFVIII showed a broad negative band at 215 nm (Fig. 2A), indicating that the protein existed predominantly in a β -sheet conformation. The spectrum was similar to that observed for rFVIII in the presence of PS-containing liposomes, suggesting that the association of the protein with liposomes did not result in substantial secondary structural changes.

The unfolding of rFVIII in the presence or absence of liposomes was monitored by acquiring far-UV CD spectra over the temperature range of 20-80°C (Fig. 2B). Samples were heated at a rate of 60°C/h. For free rFVIII, there was no significant change in ellipticity at 215 nm over the temperature range of 20-45°C (Fig. 2B). At temperatures greater than 50°C, the negative ellipticity at 215 nm decreased sharply, and the midpoint of the transition (T_m) was ~61°C. For liposomal-rFVIII, no significant change was observed in the ellipticity at 215 nm below 45°C (Fig. 2B). At temperatures greater than 50°C, the ellipticity decreased, but less sharply than for free rFVIII. However, the midpoint of the transition (T_m) was also ~61°C, suggesting that the intrinsic stability of rFVIII was not decreased by the presence of liposomes.

The aggregation behavior of rFVIII was investigated previously using CD spectroscopy.¹³ At elevated temperatures, spectra of free rFVIII showed a red shift in the negative band at 215 nm, as well as a positive band below 210 nm.⁴⁰ These spectral features suggested the formation of FVIII aggregates. A representative CD spectrum acquired at 75°C is shown in Figure 2A. The spectral features observed at elevated temperatures were consistent with observations by Fourier transform infrared (FTIR) spectroscopy⁴¹ that indicate the formation of intermolecular β -strands during aggregation.¹³ For rFVIII associated with PS-containing liposomes, no evidence of intermolecular β -strands were observed (Fig. 2A).

The ellipticity monitored at 215 nm during heating and cooling of rFVIII suggested that the folding mechanism of rFVIII was an irreversible process¹³. Interestingly, the unfolding of liposome associated rFVIII was reversible, resulting in the recovery of several native-like features in CD spectra, such as the negative band at 215 nm (data not shown). Taken together, these results suggested that the interaction of the C2 domain of rFVIII with PS-containing bilayers, mediated by the epitope region (2303-2332), interfered with the formation of rFVIII aggregates by inhibiting the transition to a conformation that permitted formation of intermolecular β -strands.

Effect of PS-Containing Liposomes Upon the Induction of Antibody Response

It has been shown that the C2 domain had a critical role in the interaction between FVIII and the PS-rich domains on the platelet surface.^{35,36} In addition, studies by Reding et al.¹⁸ indicated the presence of multiple immunodominant epitopes within the C2 domain that contribute to inhibitory antibody formation. Taking into account the effect of OPLS on the immunogenicity of rFVIII,⁴² and the roles of the C2 domain in membrane binding and immunogenicity, we investigated whether the interaction of rFVIII with PS-containing liposomes could shield these epitopes and further reduce the immunogenicity of rFVIII. Subcutaneous administration, in a murine model for hemophilia A, was selected to maximize the immune response and to enable a more complete characterization of the immune response. In this model system, the antibody response patterns against rFVIII were similar to those observed in hemophilic patients.^{30,43}

Animals were injected s.c. with free rFVIII or rFVIII associated with DMPC/BPS liposomes, and the development of humoral immunity was investigated following repeated administration. A range of liposome diameters was tested, owing to the dependence of liposome biodistribution and pharmacokinetics upon particle size following s.c. administration.⁴⁴ Animals treated with rFVIII associated with 80 nm DMPC/BPS liposomes displayed significantly lower antibody titers (Fig. 3A, Tab. 1) compared to animals treated with rFVIII alone. Titers were 7057 ± 807 ($\pm$ SEM; $n = 12$ animals) for liposome-treated animals, compared to 13167 ± 2042 ($n = 15$ animals) for animals treated with rFVIII alone. These differences were significant at $p < 0.05$. Animals treated with rFVIII associated with 200 nm liposomes had antibody titers of 5700 ± 1255 ($n = 15$ animals) and those treated with rFVIII associated with liposomes of 400 nm had titers of 4030 ± 496 ($n = 12$ animals). These differences were significantly lower than for animals administered rFVIII in the absence of liposomes ($p < 0.05$). However, differences among rFVIII liposome preparations of different size were not significant ($p > 0.05$).

For the smallest liposomes, which should have the greatest ability to enter the systemic circulation *via* the lymphatics,⁴⁵ the liposome-mediated reduction in rFVIII immunogenicity was dependent upon the presence of PS in the bilayer. Antibody titers in animals administered rFVIII associated with 80 nm liposomes composed of DMPC alone were 9681 ± 2586 (1), ($n = 12$ animals) (Tab. 1), and were not significantly different from titers in control animals that received free rFVIII ($p > 0.05$). Compared to controls receiving free rFVIII, total antibody titers were significantly lower ($p < 0.05$) in animals treated with rFVIII associated with 200 nm DMPC liposomes (6075 ± 1114 ; $n = 15$ animals) and 400 nm DMPC liposomes (6403 ± 1730 ; $n = 12$ animals) (Tab. 1). However, the difference between these two titers was not significant ($p > 0.05$).

The generation of FVIII neutralizing antibodies was monitored using the Bethesda assay.³¹ Figure 3B shows the inhibitory antibody titers at the end of 6 weeks, expressed in Bethesda Units (BU), for animals receiving either free rFVIII or rFVIII associated with PS-containing liposomes of varying sizes. Compared to titers in animals administered free rFVIII (690 ± 78 BU/mL, $\pm$ S.E.M., $n = 13$ animals), the production of neutralizing antibodies was ~60-80% lower in animals given liposome-associated rFVIII (Tab. 2). Interestingly, inhibitory antibody

titers observed for liposomal-rFVIII given subcutaneously were ~60-80% lower than for free rFVIII given by the IV route (675 ± 71 BU/mL, $\pm$ SEM, $n = 8$) (data not shown). Together, these results indicate that PS-containing liposomes not only reduced overall anti-rFVIII antibody titers, but also lowered the titer of antibodies that abrogate the pharmacological activity of the protein.

To investigate whether the reduction in inhibitory antibody titers in the presence of liposomes was a PS specific effect, mice were immunized with rFVIII in the presence of liposomes containing phosphatidylglycerol (PG) another negatively charged phospholipid. Animals treated with rFVIII associated with 200 nm PG-containing liposomes had inhibitory antibody titers of 299 ± 88 BU/mL ($\pm$ SEM, $n = 14$). The reduction in antibody titers in the presence of PS liposomes was the lowest (215 ± 71 , mean $\pm$ SEM, $n = 14$) (Tab. 2), but liposomes composed of PG also lowered the titers significantly and the difference between liposome treatment groups were not statistically significant.

Overall, the immunogenicity studies described here showed that the total and inhibitory titers reduced in the presence of liposomes containing either PS or DMPC or PG with lowest being for animals that are given PS containing liposomes. It is appropriate to mention here that the statistical significance could not be established between liposome treatment groups. Based on these results, it appears that liposomal formulations containing PS or DMPC or PG could be potentially used with FVIII for therapeutic interventions of hemophilia A. At this time, the underlying mechanism of liposome mediated reduction in antibody development is not clear, but we speculate that the particulate nature of liposomes may influence the antigen uptake and processing. We pursued further development of PS containing liposomal-rFVIII, considering that the observed titers are lowest for PS formulations and are likely to be immunologically significant as PS liposomes have been shown to negatively modulate the immunogenicity of antigens.²² This is in line with our preliminary experiments aimed at determining the mechanism of reduction in immune response. The studies showed that T-cell proliferation and pro inflammatory cytokine levels were lower for PS treatment group (data not shown). This was further supported by the observation of Hoffmann et al.²³ that PS could promote secretion of transforming growth factor beta (TGF- β), an antiinflammatory cytokine that could inhibit antigen specific CD4+ T-cells and B-cells, thereby reducing the immunogenicity of the antigen.

Further, use of PS liposomes offers an opportunity for sc routes of administration. It has been shown that smaller liposomes containing PS improved the bioavailability of its cargo following s.c. administration.⁴⁵ In addition, several investigators established that binding of FVIII to PS has a high affinity and specificity, compared to other phospholipids.^{21,46,47} This was reflected in the ~threefold increase in association efficiency of FVIII to PS liposomes compared to FVIII PC liposomes (or FVIII PG liposomes) and in the better shielding of the C2 domain by PS liposomes than by PC liposomes (Fig. 1).

To investigate the stability of PS liposomal-rFVIII in biological matrices and to understand the immunological significance of the PS-mediated reduction in anti-rFVIII antibody titers, we addressed the role of liposomes in processing of rFVIII by proteolytic enzymes. If liposomes interfere with the proteolysis of the endocytosed antigen it is likely that the repertoire of peptides displayed on the surface of antigen presenting cells might be altered and could be responsible for observed differences in immune response.

Cathepsin-B has been proposed to be involved in processing of exogenous antigens.⁴⁸⁻⁵⁰ Thus, in an *in vitro* study, we used cathepsin-B as a model protease to compare the proteolytic patterns produced by enzymatic digestion of either free or liposomal-rFVIII. As expected, proteolytic cleavage of rFVIII leads to the formation of smaller polypeptides, ranging in size from 10 to 70 kDa (Fig. 4). Following 60 min incubation of rFVIII with cathepsin-B, two

prominent bands were observed, one at 40.5 kDa and the other at 29.5 kDa. Neither intact rFVIII heavy chain nor intact light chain can be identified, suggesting that both chains are efficiently digested by the enzyme. Digestion of the rFVIII-liposomal complex by cathepsin-B led to a different proteolytic pattern (major bands at 62, 42 and 29.5 kDa). Moreover, a significant amount of intact light chain peptide could be identified on the gel (indicated by the arrow in Fig. 4). The differences in the proteolytic patterns could be explained on the basis of altered enzyme accessibility to the rFVIII polypeptide backbone. The observation that the light chain was processed at a lesser extent was also in line with our observation that the C2 domain was involved in membrane binding²⁴ and thus the steric hindrance of the liposomes was more prominent at this position.

The results indicated that the rFVIII associated with PS-containing liposomes underwent altered proteolysis under these experimental conditions compared to rFVIII. The significance of the distinct proteolytic digestion patterns of rFVIII and liposomal-rFVIII cannot be ascertained at this time. However, the observation that there exist differences in proteolysis implied that processing of rFVIII and liposomal-rFVIII may also differ, leading to the generation of distinct immunogenic peptides which, in turn, can lead to disparate immune responses.

The inclusion of PS in liposomes not only promoted specific interaction with the protein *via* domains that contain the immunodominant epitopes, but might also contribute to immunomodulation by the FVIII/PS complex. It has been reported that PS can interact directly with lymphocytes, negatively modulate their activity, and prevent antibody response to T-cell-dependent and -independent antigens.^{51,52} Furthermore, studies by Caselli et al.,⁵² have shown that of the negatively charged phospholipids, only PS, and to a lesser extent phosphatidylinositol (PI), is capable of exerting an inhibitory effect on lymphocyte activity; other common negatively charged phospholipids, such as phosphatidic acid (PA) and phosphatidylglycerol (PG), are ineffective.⁵² It is possible that the observed reduction in immunogenicity of rFVIII in the presence of PS liposomes would result from repression of rFVIII specific T-cell clones *in vivo*. Further T-cell proliferation assays are warranted to test this hypothesis. Nonetheless, Hoffmann et al. have reported that PS-containing liposomes can inhibit adaptive immune responses against an antigen.²²

Association of rFVIII with PS-containing liposomes not only reduces immunogenicity, but may also provide an opportunity for s.c. delivery of rFVIII. This route of administration is more desirable than i.v. because of the convenience and patient comfort, and can possibly increase the circulation half-life of the therapeutic agent. As an example, the half-life of tumor necrosis factor inhibitor (Etanercept) is 30-fold higher following s.c. injection than after i.v. administration, possibly due to slow absorption from the injection site.⁵³

However, the s.c. administration of therapeutic biomolecules is challenging as a result of incomplete bioavailability.⁵⁴ This route of administration has also been shown to be highly immunogenic¹². The results presented here suggested that liposomal-rFVIII was less immunogenic following s.c. administration compared to free rFVIII. Furthermore, liposomal properties such as size and surface characteristics (lipid composition) may increase the stability of rFVIII by reducing the protein degradation at the injection site through a mechanism similar to the observed inhibition of proteolysis by cathepsin-B, as shown in Figure 4. Although the formulations employing liposomal particles with a diameter of 200 nm or more had lower antibody titers compared to the formulations using 80 nm liposomes, the former particles will reside at the injection site and will only exert a local protective effect.⁴⁵ This will be detrimental for the use FVIII as a therapeutic agent for hemophilia A. In contrast, smaller lipidic nanoparticles (e.g. 80 nm particles) will be efficiently taken up by the lymphatic system, so delivery to the systemic circulation of the protein is possible. Furthermore, the presence of PS

in the liposome composition has been shown to improve the migration of liposomes to the lymphatic system by increasing the lymph node uptake.⁴⁵ Thus, by altering liposomal properties, the lymphatic uptake and the subsequent distribution of the delivery vehicle and its cargo into the systemic circulation can be achieved.

In order to investigate the activity and systemic effect of rFVIII and liposomal-rFVIII formulations upon s.c. administration, survival studies were carried out in hemophilia A mice. These mice do not produce any active endogenous FVIII. Therefore, the bleeding resulting from tail clipping will result in the death of the animal in about 17 h as observed for animals treated with Tris buffer alone.²⁰ In this model, survival of the animal is a true and clinically relevant pharmacodynamic endpoint. All animals given rFVIII or liposomal-rFVIII *via* the s.c. route ceased to bleed within 2 h of the tail clip and survived beyond 18 h. Thus, liposomal-rFVIII and rFVIII were equally effective in promoting coagulation and survival of the animals after tail clipping. This bioassay suggested that sufficient protein reached the systemic circulation after s.c. administration, possibly *via* the lymphatics, to exert the pharmacological effect.

To further investigate the effect of liposomes on the bioavailability of rFVIII following s.c. administration, a preliminary experiment was conducted to determine the concentration of rFVIII in the systemic circulation. As shown in Figure 5, detectable amounts of rFVIII were found in the blood following s.c. administration of free rFVIII. However, the association of the protein with PS-containing lipidic nanoparticles significantly increased the plasma concentrations of rFVIII measured at 3 and 5 h postinjection, suggesting the feasibility for development of s.c. formulations of rFVIII based on this approach. In the presence of PC liposomes, two out of three animals did not show any detectable levels of FVIII activity in the blood, 5 h post-s.c. administration (data not shown). Thus, the net advantage of PS lipidic particles to migrate into the lymphatic system, taken together with the lower association efficiency of pure PC liposomes, may explain the lower bioavailability of rFVIII—PC liposomes compared to that of the r-FVIII-PS complexes. Detailed PK studies are in progress. Preliminary studies, carried out by measuring blood concentration of rFVIII at different time points, indicate that the area under the curve (AUC) for animals treated with PS-containing liposomal-FVIII is approximately seven times higher than observed for animal given rFVIII alone. Incorporation of polyethylene glycol and cholesterol has also been shown to increase the stability and bioavailability of liposomes following s.c. administration.⁵⁵ Those strategies, as well as an investigation of the effect of PS-containing nanocochleates upon the biodistribution and immunogenicity of rFVIII, will be the subject of future investigations.

In conclusion, the findings presented here suggest that formulations containing liposomal-rFVIII may be exploited to create formulations that are less immunogenic and can potentially be delivered through the s.c. route of administration. The replacement of BPS with synthetic lipids such as dioleoyl PS or other unsaturated lipids is possible to avoid lipids from animal source.

ACKNOWLEDGMENTS

We wish to dedicate this paper to the memory of Prof. Demetrios Papahadjopoulos, who has published more than 200 research and review articles in the field of liposomes. His pioneering research provided an inspiration for this work. We are grateful to Drs. Kazazian and Sarkar of the University of Pennsylvania, PA for providing the FVIII knockout mice. The CD spectropolarimeter was obtained by a grant (S10-013665) from the National Center for Research Resources, National Institutes of Health. This work was supported by NHLBI, National Institute of Health grant (#R01 HL-70227) to SVB.

Abbreviations

ACD, acid citrate dextrose
 aPTT, activated partial thromboplastin time
 BPS, brain phosphatidylserine
 BSA, bovine serum albumin
 DMPC, dimyristoylphosphatidylcholine
 DSPC, distearoylphosphatidylcholine
 ELISA, enzyme-linked immunosorbent assay
 FVIII, factor VIII
 Ig, immunoglobulin
 MHC-II, major histocompatibility complex class II
 OPLS, *O*-phospho-L-serine
 PB, phosphate buffer
 PBT, phosphate buffer containing tween
 PA, phosphatidic acid
 PC, phosphatidylcholine
 PG, phosphatidylglycerol
 PI, phosphatidylinositol
 PS, phosphatidylserine
 rFVIII, recombinant human factor VIII
 RES, reticuloendothelial system
 T_c, phase transition temperature
 TGF- β , transforming growth factor β

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Figure 1.

Relative % binding of monoclonal ESH4 antibody to liposomal-rFVIII as a function of lipid concentration and composition (DMPC/BPS (70/30)—open circles and DMPC—open triangles). ESH4 was used as the stationary antibody. A rat polyclonal antihuman rFVIII antibody was used as the probe antibody. In all preparations, rFVIII concentration was maintained constant (150 ng/mL). The error bars represent the standard deviation ($\pm$ SD, $n = 3$).

Figure 2.

(A) Far-UV CD spectra of rFVIII (in 300 mM NaCl, 25 mM Tris, 5 mM CaCl₂, pH 7.0) before (dotted line) and after (solid line) heating to 80°C in the absence (gray line) and presence (black line) of liposomes. The protein concentration was ~20 µg/mL, and the protein to lipid ratio was ~1:2500. All spectra were corrected by subtracting the spectrum of the buffer alone. (B) Temperature dependent changes in ellipticity ($\delta\theta$) at 215 nm for rFVIII (solid line) and rFVIII associated with liposomes (DMPC/BPS, 70/30) (dotted line) during the unfolding process. The transition temperature (T_m) was determined by fitting the thermal unfolding profile to a sigmoid function using WinNonlin (Pharsight Corp., Mountainview, CA).

Figure 3.

(A) Total and (B) inhibitory anti-rFVIII antibody titers in hemophilic mice following administration of rFVIII in the absence or presence of liposomes of DMPC/BPS (70/30) of varying sizes at the end of 6 weeks. Treatment was administered *via* s.c. route. Each point represents values from individual animals and the horizontal bar depicts the mean titer. Blood samples were obtained 2 weeks after the fourth injection.

Figure 4. SDS PAGE analysis of proteolytic (cathepsin-B) pattern of rFVIII and rFVIII associated with liposomes. Position of the light chain is indicated by the arrow.

Figure 5. rFVIII activity levels in the absence (dark bars) and presence (gray bars) of 80 nm DMPC/BPS liposomes following s.c. administration (4000 IU/kg) of the preparations. The data points collected at 3 and 5 h are averages of three animals. Error bars represent standard deviation; * denotes $p < 0.05$.

Table 1

Mean Anti-rFVIII Total Titers After the End of 6 Weeks Following Immunization of Hemophilic Mice with rFVIII in the Absence and Presence of Liposomes of Varying Size and Composition

Liposome Size (nm) and Composition	Total Antibody Titers ($\pm$ SEM)	Reduction in Total Titers (Relative to Control) (%)
Control (free rFVIII)	13167 $\pm$ 2042 ($n=12$)	—
80 nm DMPC/BPS (70:30)	7057 $\pm$ 807* ($n=15$)	~46
200 nm DMPC/BPS (70:30)	5700 $\pm$ 1255* ($n=15$)	~57
400 nm DMPC/BPS (70:30)	4030 $\pm$ 496* ($n=12$)	~69
80 nm DMPC	9681 $\pm$ 2586 ($n=12$)	~26
200 nm DMPC	6075 $\pm$ 1114* ($n=15$)	~54
400 nm DMPC	6403 $\pm$ 1730* ($n=12$)	~51

* $p<0.05$, statistical analysis was carried out as described under Materials and Methods Section.

Table 2

Mean Anti-rFVIII Inhibitory Titers after the End of 6 Weeks Following Immunization of Hemophilic Mice with rFVIII in the Absence and Presence of PS-Containing Liposomes of Varying Size

Liposome Size (nm)	Inhibitory Titers (BU/mL, Mean $\pm$ SEM)	Reduction in Inhibitory Titers (Relative to Control) (%)
Control (rFVIII)	690 $\pm$ 78 (n=13)	—
80	258 [*] $\pm$ 35 (n=12)	~63
200	215 [*] $\pm$ 71 (n=14)	~69
400	161 [*] $\pm$ 27 (n=12)	~77

* $p < 0.05$, statistical analysis was carried out as described under Materials and Methods Section.