Research Article

Phosphatidylinositol Containing Lipidic Particles Reduces Immunogenicity and Catabolism of Factor VIII in Hemophilia A Mice

Aaron Peng,¹ Robert M. Straubinger,² and Sathy V. Balu-Iyer^{3,4}

Received 28 January 2010; accepted 18 May 2010; published online 2 June 2010

Abstract. Factor VIII (FVIII) is an important cofactor in blood coagulation cascade. It is a multidomain protein that consists of six domains, NH₂-A1-A2-B-A3-C1-C2-COOH. The deficiency or dysfunction of FVIII causes hemophilia A, a life-threatening bleeding disorder. Replacement therapy using recombinant FVIII (rFVIII) is the first line of therapy, but a major clinical complication is the development of inhibitory antibodies that abrogate the pharmacological activity of the administered protein. FVIII binds to anionic phospholipids (PL), such as phosphatidylinositol (PI), via lipid binding region within the C2 domain of FVIII. This lipid binding site not only consists of immunodominant epitopes but is also involved in von Willebrand factor binding that protects FVIII from degradation *in vivo*. Thus, we hypothesize that FVIII-PL complex will influence immunogenicity and catabolism of FVIII. The biophysical studies showed that PI binding did not alter conformation of the protein but improved intrinsic stability as measured by thermal denaturation studies. ELISA studies confirmed the involvement of the C2 domain in binding to PI containing lipid particles. PI binding prolonged the *in vivo* circulation time and reduced catabolism of FVIII in hemophilia A mice. FVIII-PI complex reduced inhibitor development in hemophilia A mice following intravenous and subcutaneous administration. The data suggest that PI binding reduces catabolism and immunogenicity of FVIII and has potential to be a useful therapeutic approach for hemophilia A.

KEY WORDS: factor VIII; hemophilia A; inhibitor development; immunogenicity; phosphatidylinositol.

INTRODUCTION

Factor VIII (FVIII) is a large glycoprotein containing six domains, NH₂-A₁-A₂-B-A₃-C₁-C₂-COOH (1,2). It is a critical cofactor in the blood coagulation cascade that circulates as a heterodimer, consisting of a heavy chain (A₁-A₂-B) and a light chain (A₃-C₁-C₂) (3). *In vivo*, catabolism of FVIII is mediated by its binding to von Willebrand factor (vWF) and low-density lipoprotein receptor-related protein (LRP), a

multi-ligand endocytic receptor (4,5). The binding of FVIII to vWF is important for the survival of FVIII as the plasma concentration of FVIII is considerably reduced in the absence of vWF (6–8). FVIII binds to vWF involving A₃ and C₂ domains of the light chain (9–13). It has been shown that the A₂, A₃, and C₂ domains of FVIII are involved in the interaction with LRP (14,15). Saenko *et al.* have shown that high-affinity binding sites are localized in A₂ and A₃ domains and a low-affinity binding site in C₂ domain (14). Lenting *et al.* reported that the monoclonal antibody ESH4 directed against epitope 2303–2332, a site within the C₂ domain known to be involved in vWF binding, interfered with the binding of the light chain of FVIII to LRP (15). This suggests that within the light chain, vWF and LRP binding sites are in close proximity and/or overlap. The C₂ domain also contains phospholipid binding epitope that mediates interaction of FVIII with phospholipids on platelet membrane (16–20). In the coagulation cascade, FVIII binds to activated surfaces of platelet membrane via interaction between phosphatidylserine (PS) and C₂ domain. It has been shown that FVIII binds to other anionic lipids including phosphatidylinositol (PI), but the specificity and affinity is higher for PS compared to other anionic lipids (21).

Hemophilia A is a bleeding disorder caused by the deficiency or dysfunction of FVIII. Administration of exogenous FVIII is currently the most common therapy. Unfortunately, 15–35% of patients develop FVIII neutralizing antibodies that abrogate the activity of the protein, thus complicating FVIII replacement therapy and clinical manage-

¹ Department of Pharmaceutical Sciences, University at Buffalo, State University of New York, 526 Hochstetter Hall, Amherst, New York 14260, USA.

² Department of Pharmaceutical Sciences, University at Buffalo, State University of New York, 539 Cooke Hall, Amherst, New York 14260, USA.

³ Department of Pharmaceutical Sciences, University at Buffalo, State University of New York, 521 Hochstetter Hall, Amherst, New York 14260, USA.

⁴ To whom correspondence should be addressed. (e-mail: svb@buffalo.edu)

ABBREVIATIONS: aPTT, activated partial thromboplastin time; CD, circular dichroism; Con A, concanavalin A; DMPC, dimyristoylphosphatidylcholine; FVIII, Factor VIII; i.v., intravenous; LRP, low-density receptor-related protein; PI, phosphatidylinositol; PL, phospholipids; PS, phosphatidylserine; RES, reticuloendothelial system; rFVIII, recombinant factor VIII; SI, stimulation index; s.c., subcutaneous; *t*_{1/2}, circulation half-life; *T*_m, transition temperature; Trp, tryptophan; vWF, von Willebrand factor.

ment of the disease (22,23). Based on systematic epitope mapping experiments, the anti-FVIII antibodies have been found to mainly target defined regions in the A2 (heavy chain) and A3 and C2 domains (light chain) of FVIII (24–26). Thus, macromolecular interactions of FVIII involve A2, A3, and C2 domains, and these domains also contain epitope regions that can lead to inhibitor development in FVIII replacement therapy. Therefore, it is important to investigate the role of macromolecular interactions on catabolism and immunogenicity of FVIII. Our previous results showed that FVIII–PS complex reduced inhibitor development (27,28), but molecular details of catabolism of FVIII *in vivo* could not be clearly understood due to rapid uptake of PS by reticuloendothelial system (RES) (29,30). Here, we have replaced PS with another anionic phospholipid, PI, which resists RES uptake (31), and investigated its effect on the immunogenicity and catabolism of FVIII. FVIII–PI reduced inhibitor development and prolonged the circulation half-life ($t_{1/2}$) of FVIII in hemophilia A mice, suggesting that FVIII interaction with macromolecules is important for its catabolism and immunogenicity. Detailed understanding of these macromolecular interactions is potentially useful as a therapeutic option to overcome inhibitor development in hemophilia A patients. A less immunogenic FVIII would improve the safety and efficacy of replacement therapy.

MATERIALS AND METHODS

Materials

Albumin-free recombinant full-length FVIII (Baxter Health Care, Glendale, California, USA) was used for the studies. Advate was provided as a gift from the Western New York Hemophilia foundation. Dimyristoylphosphatidylcholine (DMPC) and soybean PI were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA). Cholesterol was purchased either from Avanti Polar Lipids or from Sigma (St. Louis, Missouri, USA). IgG-free bovine serum albumin (BSA) and diethanolamine were purchased from Sigma (St. Louis, Missouri, USA). Alkaline phosphatase conjugates of goat anti-mouse Ig and anti-rat Ig were obtained from Southern Biotechnology Associates, Inc. (Birmingham, Alabama, USA). *p*-Nitrophenyl phosphate disodium salt was purchased from Pierce (Rockford, Illinois, USA). Monoclonal antibodies ESH4, ESH5, and ESH8 were purchased from American Diagnostica Inc. (Greenwich, Connecticut, USA). Monoclonal antibody N77210M was purchased from Biodesign International (Saco, Maine, USA). Normal coagulation control plasma and FVIII-deficient plasma were purchased from Trinity Biotech (County Wicklow, Ireland). The Coamatic FVIII kit from DiaPharma Group (West Chester, Ohio, USA) was used to determine FVIII activity in plasma samples. Dynal magnetic beads T cell isolation kits were purchased from Invitrogen Inc. (Carlsbad, California, USA).

Preparation of FVIII–PI Lipidic Particle

A thin lipid film consisting of DMPC/PI/cholesterol (50:50:5 molar ratio) was rehydrated with Tris buffer (25 mM Tris, and 150 or 300 mM NaCl, pH=7.0). These

multilamellar vesicles were extruded through double polycarbonate membranes of 80-nm pore size (GE Osmonics Labstore, Minnetonka, Minnesota, USA) using a high-pressure extruder (Northern Lipids Inc., Burnaby, British Columbia, Canada) and then sterile-filtered through a 0.22- μ m MillexTM-GP filter unit (Millipore Corporation, Bedford, Massachusetts, USA) to yield small unilamellar vesicles. The concentration of phospholipid and its recovery was determined by inorganic phosphorous assay (32). Particle size was determined using a Nicomp Model CW 380 particle size analyzer (Particle Sizing Systems, Santa Barbara, California, USA). The protein was added to vesicles at 37°C at a ratio of 1:10,000 for all experiments, unless stated otherwise. A discontinuous dextran density gradient flotation technique was used to separate free protein from particle-associated protein and permit estimation of the amount of protein associated with the particle (33).

In Vitro Characterization of FVIII–PI

Activity. Activity of the protein associated with PI was determined using one-stage activated partial thromboplastin time (aPTT) assay and by chromogenic assay. For aPTT assay, the samples were mixed with an equal volume of FVIII-deficient plasma and incubated at 37°C. Following addition of activator (platelin-L reagent) and CaCl_2 , the clotting time was measured using a Coag-A-Mate XM coagulometer (Organon Teknica Corporation, Durham, North Carolina, USA). Activity of FVIII samples was also determined using Coamatic FVIII kit according to manufacturer instructions. For both assays, the activities of FVIII and FVIII–PI samples were estimated from a calibration curve constructed using the clotting times or the optical densities values determined from various dilutions of a FVIII concentrate of known activity.

Conformational studies. The effect of PI binding on the tertiary structure of FVIII was determined by fluorescence spectroscopy. The samples (5 $\mu\text{g/mL}$) were either excited at 280 or at 265 nm, and the emission spectra were obtained in the wavelength range of 300–400 nm. Slit widths were set at 4 nm for both the excitation and emission paths. The spectra were acquired on a PTI-Quantamaster fluorescence spectrophotometer (Photon Technology International, Lawrenceville, New Jersey, USA). The contribution of PI vesicles on the emission spectra of the protein was corrected by subtracting the spectra acquired for the vesicles alone and by using a long pass filter on emission path.

Circular dichroism (CD) spectra were acquired on a JASCO-715 spectropolarimeter calibrated with d-10 camphor sulfonic acid. Far-UV CD spectra of FVIII and FVIII–PI were obtained over the range of 255 to 208 nm for secondary structural analysis using a 10-mm quartz cuvette. The protein concentration used in this experiment was 20 $\mu\text{g/mL}$, and the protein/lipid ratio was maintained at 1:2,500. Multiple scans were obtained and averaged to improve the signal quality. FVIII CD spectra were corrected by subtracting the baseline of the Tris buffer whereas FVIII–PI spectra were corrected by subtracting the baseline of PI particles. Thermal denaturation of the FVIII and FVIII–PI was determined by monitoring the ellipticity at 215 nm from 20°C to 80°C using

a heating rate of 60°C/h with a 2-min holding time at every 5°C controlled by a Peltier 300 RTS unit. The cuvette was sealed with Teflon tape in order to minimize sample loss, and volume of the sample was monitored before and after each thermal stress experiment. The temperature of the sample compartment was determined using a temperature probe that was inserted in the sample cell holder adjacent to the cuvette, as recommended by the manufacturer. The transition temperatures (T_m) for the unfolding profiles were determined by fitting the data to a sigmoid function using WinNonlin (Pharsight Corporation, Mountainview, California, USA):

$$Y_{\text{observed}} = Y_{\text{native}} - \frac{\Delta m \times T^\gamma}{T_m^\gamma + T^\gamma} \quad (1)$$

where Y_{observed} is the ellipticity at 215 nm at any given temperature T , Y_{native} is the ellipticity value for the native state (at 20°C), T_m is the transition temperature, gamma (γ) is the hill coefficient, and Δm denotes the magnitude of the ellipticity change defined as ($Y_{\text{native}} - Y_{\text{unfolded}}$), where Y_{unfolded} is the ellipticity value of the unfolded state.

Protein-lipid interaction studies. In order to determine the FVIII epitopes that participate in the association with PI, sandwich ELISA studies were performed (34). Briefly, Nunc-Maxisorb 96-well plates were coated with capture monoclonal antibodies in carbonate buffer (0.2 M, pH 9.6) overnight at 4°C. Plates were washed with Tween-PBS (2.7 mM KCl, 140 mM NaCl, 1.8 mM KH_2PO_4 , 10 mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.05% w/v Tween 20, pH 7.4) and then blocked in 1% BSA (prepared in PBS) for 2 h at room temperature. One hundred microliters of 0.5 µg/mL of FVIII-PI at various protein/lipid ratios (1:5,000, 10,000, and 50,000) or PI particles in blocking buffer was incubated at 37°C for 1 h. Plates were washed and then incubated with 100 µL of a 1:500 dilution of rat polyclonal antibody containing a 1:1,000 dilution of goat anti-rat Ig alkaline phosphatase conjugate in blocking buffer at room temperature for 1 h. Plates were washed again and 200 µL of a 1-mg/mL *p*-nitrophenyl phosphate solution in diethanolamine buffer (1 M diethanolamine, 0.5 mM MgCl_2) was added and incubated for 30 min at room temperature in dark for color development. One hundred microliters of 3 N NaOH was added to stop the reaction. A plate reader was used to measure the optical density at 405 nm. The ELISA was standardized previously to determine the linear range of response for each individual antibody in the concentration of FVIII used (34).

In vitro release kinetics. FVIII-PI complexes were prepared in Tris buffer as described above and incubated at 37°C in the presence or absence of 10% FVIII-deficient plasma. One of each of the tubes was taken from each treatment group at 0, 2, 4, 8, 12, 24, 36, and 48 h, and FVIII released at these time intervals were determined by discontinuous dextran density gradient flotation technique and aPTT assay. The association efficiency of FVIII to PI at time 0 h was determined as initial FVIII retention and was normalized to 100%. Samples analyzed at subsequent time points were compared to the initial FVIII retention.

In Vivo Characterization of FVIII-PI

Animal experiments and ethics policy. All animal experiments were approved and performed according to the guidelines of Institutional Animal Care and Use Committee of the University at Buffalo.

Animals. Breeding pairs of hemophilia A mice (C57BL/6J) with a target deletion in exon 16 of the FVIII gene were provided by Dr. Kazazian and Dr. Sarkar from University of Pennsylvania, Philadelphia, Pennsylvania, USA (35). A colony was established and animals of age 8–12 weeks were used for the *in vivo* studies. Since the sex of the mice has no impact on the immune response, both male and female mice were used for the immune response studies (36).

In vivo activity of FVIII-PI. The biological activity of FVIII-PI *in vivo* was investigated in hemophilia A mice by tail clip method and chromogenic activity assay (37,38). Briefly, 400 IU/kg of FVIII-PI was administered to male mice ($n=3$) via penile vein injection. The bleeding time was monitored in these mice after 2-cm segment of the tail was cut at 48 h post-injection. Male mice ($n=3$) that received Tris buffer alone were treated as controls.

Catabolism studies. FVIII and FVIII-PI (400 IU/kg) were administered to groups of male hemophilia A mice as a single intravenous (i.v.) bolus injection via the penile vein. Blood samples were collected from at least three mice/time point by cardiac puncture using syringes containing acid citrate dextrose (ACD; 85 mM sodium citrate, 110 mM D-glucose, and 71 mM citric acid) buffer (10:1 v/v blood/ACD) at 0.08, 0.5, 1, 2, 4, 8, 16, 24, 30, 36, 42, and 48 h after administration. Plasma was collected immediately by centrifugation (5,000×g at 4°C) and stored at -80°C until analysis. The plasma concentration of FVIII was followed for 48 h (39), and chromogenic assay was used to measure the activity of FVIII in plasma samples. The average values of FVIII activity at each time point were used to compute drug elimination rate constant and elimination half-life ($t_{1/2}$). The dose-independent elimination rate constant (λ_z) was estimated by log-linear regression of the terminal phase concentration, and the elimination $t_{1/2}$ was calculated as $\ln 2/\lambda_z$ using a non-compartmental analysis in WinNonlin (Pharsight Corporation, Mountainview, California, USA) (39,40).

Immunogenicity studies. The relative immunogenicity of free FVIII and FVIII-PI was determined in hemophilia A mice. The preparations were confirmed endotoxin negative by limulus amebocyte assay (Charles River Laboratories, Inc., Wilmington, Massachusetts, USA) and were injected immediately after preparation. Groups of mice received four weekly i.v. (via penile vein, $n=8$) or subcutaneous (s.c.) injections ($n=10$) of FVIII or FVIII-PI (10 IU/injection). Two weeks after the last injection, blood samples were collected by cardiac puncture in ACD buffer at a 10:1 (v/v) blood/ACD ratio. Plasma was separated by centrifugation at 5,000×g at 4°C for 5 min. Previous studies have shown that 6 weeks is an appropriate time to compare antibody titers between various treatment groups (27). Samples were stored at -80°C immediately after centrifugation until analysis.

Measurement of inhibitory anti-FVIII antibody titers.

Inhibitory antibody titers were measured by the Nijmegen modification of the Bethesda assay (27,41). This assay is a modification of the one-stage aPTT assay which measures the ability of FVIII antibodies to inhibit the biological activity of FVIII. Briefly, a calibration curve was constructed using clotting times determined from various dilutions (1:2 to 1:1,024) of normal coagulation control plasma. Serial dilutions (1:8 to 1:16,000) of the mouse plasma samples were prepared in human FVIII-deficient plasma. One hundred microliters of each diluted plasma sample was mixed with an equal volume of normal human plasma and incubated at 37°C for 2 h. FVIII residual activity was determined at the end of the incubation using aPTT assay. Residual activity at various dilutions was plotted against log of dilution, and a linear regression was performed on the linear part of the curve. Inhibitory titers, the dilution at which 50% of FVIII activity is reduced, were expressed in Bethesda Units.

T cell proliferation studies. In order to understand the immunological significance of the reduction in anti-FVIII antibody development, stimulation of FVIII specific T cells *in vivo* following immunization with FVIII or FVIII-PI were carried out. *In vitro* T cell proliferation response to FVIII challenge was conducted with splenocytes isolated from immunized animals as described previously (34). Two weekly s.c. injections of FVIII or FVIII-PI (10 IU/injection) were administered to female hemophilia A mice ($n=6$ per treatment group). Three days after the second injection, animals were sacrificed and the spleens were harvested as a source of T cells. To enrich CD4⁺ T cells, a CD8⁺ T cell depletion kit was used. CD4⁺ T cells (2×10^5 cells/200 μ L/well) were cultured in 96-well plates with FVIII (100 ng/well) in complete RPMI-1640 culture medium containing 10,000 U/mL penicillin, 10 mg/mL streptomycin, 2.5 mM sodium pyruvate, 4 mM L-glutamine, 0.05 mM 2-mercaptoethanol, 2 mg/mL polymyxin B, and 0.5% heat inactivated hemophilic mouse serum for 72 h. Plain cells in culture medium were treated as negative control whereas cells treated with concanavalin A were used as positive control. T cells that have been challenged with antigen were then incubated with ³H-thymidine (1 μ Ci/well) and harvested at the end of 16 h using a Micromate Harvester (Packard, Meriden, Connecticut, USA). ³H-thymidine incorporation to T cells was measured using a TopCountTM microplate scintillation and luminescence counter (Packard Instrument Company, Meriden, Connecticut, USA). The data were reported as stimulation index (SI), which is the ratio of ³H-thymidine incorporation in the presence of antigen to incorporation in the absence of antigen. Representing the results as SI normalized the data of each experiment and allowed comparison between experiments conducted at different times with different animals.

Statistical analysis. Statistical differences ($p < 0.05$) were tested using the Student independent *t* test and one-way ANOVA followed by Dunnet's post hoc multiple comparison test. For catabolism studies, repeated-measures ANOVA was used to compare the profiles generated by the two treatments.

RESULTS

Association efficiency and *in vitro* FVIII activity of FVIII-PI. The association efficiency of FVIII with PI containing lipidic particles was found to be $72 \pm 9\%$. Both aPTT and chromogenic assays showed that the protein retained activity upon association with PI, but only a small delay in clotting time in the aPTT assay was observed for FVIII bound to PI vesicles (data not shown). Furthermore, FVIII activity after spiking of PI particles into FVIII-deficient plasma showed full recovery of protein activity suggesting that PI particles did not interfere with aPTT measurement (data not shown). In order to gain further insight into the molecular nature of the protein-PI interaction, a variety of biophysical and biochemical studies were carried out.

Conformation of FVIII associated with PI. The secondary structural features of FVIII bound to PI containing vesicles were monitored by far-UV CD (255–208 nm) spectrum. At 20°C, the free protein showed a broad negative band around 215 nm consistent with the β -sheet structure of the protein. Association with PI did not alter the far-UV CD spectrum of FVIII, suggesting that lipid binding does not result in significant changes in protein secondary structure (Fig. 1a). The tertiary structural change in FVIII upon association with PI was investigated using fluorescence spectroscopy (Fig. 1b). For FVIII, a peak with emission maxima at 335 nm was observed. However, in the presence of PI, a small blue shift in fluorescence emission maximum that is accompanied by an increase in intensity (data not shown) was observed, indicating a small tertiary structural change that is possibly due to the involvement of hydrophobic interaction between protein and PI particle. The intrinsic stability of the protein in the presence and in the absence of PI was monitored by thermal denaturation studies (Fig. 1c). For FVIII, a gradual increase in temperature to 50°C did not show any change in spectral characteristics suggesting that the protein undergoes little alteration in the secondary structure. Further increase in temperature resulted in an increase in negative ellipticity. In the presence of PI, a small shift in T_m to higher temperature was observed indicating that association of FVIII with PI improved intrinsic stability of the protein. Based on biophysical and size exclusion chromatography studies, it has been shown that the onset of transition coincides with the formation of aggregates and the presence of aggregates impacts equilibrium unfolding and T_m measurements (42,43). This is consistent with our observation that T_m is dependent on heating rate, suggesting aggregation kinetics of the protein (42,43). Since the onset of unfolding transition coincides with the formation of aggregates, the effect of PI on stability of FVIII was inferred from the onset of unfolding transition. As is clear from Fig. 1c, the onset of transition was observed at 50°C for free FVIII, but it increases to 55°C for FVIII-PI. This delay suggests that PI improves the stability by delaying formation of aggregates of the protein.

FVIII-PI interaction. Sandwich ELISA studies were carried out to investigate domains of FVIII involved in binding with PI containing vesicles (Fig. 2). Monoclonal antibodies that recognize different epitope regions of the protein were coated on the plates as stationary antibody, and

Fig. 1. Conformation and stability of FVIII in the presence and in the absence of PI. **a** Far-UV CD spectra of FVIII in the presence (*empty circles*) and in the absence (*filled circles*) of PI acquired at 20°C. The concentration of the protein used was 20 $\mu\text{g/mL}$, and the protein/lipid ratio was maintained at 1:2,500. The path length of the quartz cuvette was 10 mm. **b** Normalized fluorescence emission spectra of free FVIII (*solid line*) and FVIII-PI (*dashed line*) acquired in the range of 300–400 nm. The excitation monochromator was set at 265 nm. The protein concentration used was 5 $\mu\text{g/mL}$. **c** Intrinsic stability of FVIII in the presence (*empty circles*) and in the absence (*filled circles*) of PI as studied by changes in ellipticity as a function of temperature. Secondary structural transition of FVIII was monitored from 20°C to 80°C at a heating rate of 60°C/h. The concentration of the protein used was 20 $\mu\text{g/mL}$ and the protein/lipid ratio was maintained at 1:2,500. The path length of the quartz cuvette was 10 mm. The CD and fluorescence spectra were acquired multiple times with multiple samples and representative spectra are shown

recognized C2 domain (2302–2332, lipid binding domain), C2 domain (2248–2285), a1 acidic region, and A2 domain, respectively, were used as stationary antibodies (44). The binding of free FVIII to each antibody was normalized to 100% to account for differences in binding affinity. In order to rule out possible interference from lipid on antibody binding, a control experiment was carried out in which lipid alone was incubated with stationary antibody. The results demonstrated that antibody binding was decreased to a small extent, but no lipid concentration-dependent changes were observed, indicating non-specific interaction between lipid and antibodies used. As is clear from Fig. 2, FVIII binding to ESH4 was decreased with increasing concentration of PI suggesting the involvement of lipid binding domain in PI binding. In the presence of 178 μM lipid, the ESH4 binding is about that observed for lipid background alone which further indicates participation of the C2 domain (2302–2332) in PI binding. A lipid concentration-dependent reduction in binding of other antibodies, though to a much smaller extent, was also observed for other stationary antibodies. The results suggest that epitope regions recognized by ESH8 (C2 domain 2248–2285), ESH5 (a1 acidic region), and N77210M (A2 domain) may also contribute to the binding of FVIII to PI containing membranes. The participation of FVIII domains

Fig. 2. FVIII interaction with PI as studied by sandwich ELISA; the binding of monoclonal antibodies to FVIII-PI at various lipid concentrations. Protein-free vesicles were used to evaluate the effect of lipid on the coating of the plate

rat polyclonal antibody against FVIII was used as probe antibody. If PI bind to a FVIII epitope region that is recognized by the stationary antibody, a reduction in FVIII binding to that stationary antibody due to competitive binding should be expected. Thus, sandwich ELISA can provide qualitative details of lipid-protein interaction. Monoclonal antibodies, ESH4, ESH8, ESH5, and N77210M, which

with PI was consistent with biophysical studies of tryptophan (Trp) fluorescence that showed interaction of FVIII with the PI rendered a significant fraction of Trp residues inaccessible to quenching by acrylamide (data not shown).

In vivo activity and hemostatic efficacy of FVIII-PI. In order to investigate whether PI binding alter *in vivo* activity of FVIII-PI and also to investigate whether FVIII is released from PI particles to provide hemostatic efficacy, tail clip assay was performed in hemophilia A mice. Hemophilia A mice do not produce any endogenous active FVIII. Hence, bleeding caused by tail clipping will result in the death of the animal in about 17 h as observed for animals treated with Tris buffer alone, and the survival of the animal following a tail clip is an indicator of *in vivo* efficacy. Tails of three animals that received FVIII-PI were clipped 48 h post-injection, and the survival was monitored. This time point was chosen based on our catabolism studies (Fig. 3). At this time point, animals given FVIII alone did not show measurable plasma levels, but FVIII-PI treatment group did. All three animals survived and visual examination showed that, in two animals, blood clotting were observed within 2 h of tail clip and about 6 h in the third animal. The results clearly indicate that FVIII binding with PI particles retained FVIII activity and is efficacious. Furthermore, FVIII is released from the particle to impart biological activity. The release may be mediated by several mechanisms including plasma protein-mediated leakage, competitive binding between vWF and lipid, and degradation of the PI vesicles.

In vitro release kinetics. In order to evaluate the role of plasma components on the release of the protein from the particle, the release of protein was monitored at 37°C in the presence and absence of 10% plasma. As shown in Fig. 4, there is a gradual release of protein (about 20% of the associated protein) over 48 h at 37°C in the absence of plasma. However, in the presence of 10% FVIII-deficient plasma, a rapid release of FVIII (about 60%) was observed within 2 h, and this is followed by a slow, sustained release for about 24 h. The data indicate that plasma component is required for the rapid release of the protein. The release

Fig. 3. Influence of PI on catabolism of FVIII in hemophilia A mice (filled circles = FVIII, empty circles = FVIII-PI). The animals were given 400 IU/kg per animal via penile vein, and the plasma concentration of FVIII was followed for 48 h. Error bars represent standard error of the mean

Fig. 4. Release kinetics of FVIII from PI particles in the presence (squares) and in the absence (triangles) of 10% FVIII-deficient plasma at 37°C over 48 h. Values on y-axis represent percent of FVIII retention of the initial encapsulated FVIII. Error bars represent standard error of the mean

could be mediated by multiple mechanisms, including possible competitive binding between vWF and lipid for FVIII that induces the release of FVIII from PI particles and lipid transfer from lipid particles to plasma proteins such as lipoproteins (45), leading to destabilization and leakage of entrapped content. Further, degradation/destabilization of the particles due to the oxidation of phospholipids or the hydrolysis of the ester bonds that hold the lipid membrane together could also contribute to the release of the protein (46). After 24 h, no further significant release was observed, possibly due to the establishment of equilibrium in *in vitro* condition.

Catabolism of FVIII-PI. FVIII interaction with vWF is important for its plasma survival (6–8). As the binding sites of PI and vWF (also LRP) are in close proximity and/or overlap, we investigated the catabolism of FVIII-PI and compared to that of free FVIII in hemophilia A mice to determine the influence of lipid binding on vWF (LRP)-mediated catabolism of FVIII. Figure 3 showed the mean plasma activity profiles for free FVIII and FVIII-PI. In all cases, about 100% of FVIII activity was recovered at 5 min post-injection suggesting both FVIII and FVIII-PI have reached systemic circulation and that the plasma survival of the protein will be dependent on the *in vivo* behavior of the protein. In addition, it also confirmed that the binding of PI to FVIII does not have any impact on the recovery of FVIII activity. The most striking difference between the profiles of free FVIII and FVIII-PI is the terminal slope or the drug elimination rate constant; for FVIII, the observed elimination rate constant was ~ 0.38 and the association of FVIII with PI showed shallower slope (elimination rate constant ~ 0.09). Moreover, the activity was reproducibly detectable for only 36 h post-injection following administration with FVIII, whereas FVIII activity was detectable up to 48 h following administration with FVIII-PI complexes. Furthermore, circulation $t_{1/2}$ for free protein was 1.82 h in hemophilia A mice, but it was increased to 7.71 h for FVIII-PI. The data show that FVIII associated with PI prolonged the circulation time and improved plasma survival for FVIII.

Immunogenicity of FVIII-PI. The relative immunogenicity of FVIII and FVIII-PI was investigated in hemophilia A mice (Fig. 5). Murine models are valuable to measure relative immunogenicity of protein therapeutics (47,48). The sequence homology between human and murine FVIII is up to 84–93% in the conserved regions (49). Furthermore, hemophilia A mice lack endogenous FVIII and produce an anti-FVIII antibody response pattern similar to those observed in hemophilic patients, making them a suitable preclinical animal model to investigate immunogenicity (36).

The inhibitory titers in immunized mice were determined by the Nijmegen modification of the Bethesda assay (41). Hemophilia A mice immunized via s.c. route resulted in mean inhibitory titer of 690 ± 78 (S.E.; $n=15$) for free FVIII and 183 ± 57 (S.E.; $n=10$) for FVIII-PI (Fig. 5a). The titer was significantly reduced by more than 70% in the presence of PI ($p < 0.05$, Student's t test). In intravenously administered mice, FVIII inhibitory titers were 675 ± 71 (S.E.; $n=8$) for animals given free FVIII and were 385 ± 84 (S.E.; $n=8$) for animals receiving FVIII-PI (Fig. 5b). This difference was statistically significant ($p < 0.05$, Student's t test). Overall, association of FVIII with PI reduced FVIII antibody responses by either route suggesting that FVIII-PI complex has lower immunogenicity compared with free FVIII in hemophilia A mice. The effect of PI was also investigated upon sensitization of T cells *in vivo* following administration of FVIII-PI and *in vitro* T cell proliferation studies (Fig. 5c). For splenic T-lymphocytes harvested from animals administered with FVIII-PI, the mean SI was 1.87 ± 0.86 (S.E.; $n=6$), about 2-fold lower ($p < 0.05$, Student's t test) than that from animals treated with free FVIII (3.61 ± 0.99 , S.E.; $n=6$). The data suggest that both FVIII and FVIII-PI stimulate FVIII specific T cell response; however, a significant difference in their antigenic properties is possible.

DISCUSSION

The A2, A3, and C2 domains of FVIII participate in vWF (9–13), LRP (14,15), and phospholipid binding (16–20) that contributes to catabolism of FVIII, and these domains also contain epitopes that can lead to inhibitor development (24–26). Therefore, it will be of interest to investigate the influence of macromolecular interactions on catabolism and immunogenicity. Incubation of FVIII with PI particles at 37°C promotes interaction and formation of FVIII-PI complex. As the phospholipid binding region of the FVIII C2 domain contains two to four hydrophobic loops and other charged residues that promote lipid binding, this association would involve both hydrophobic and electrostatic interactions (19,50). The biophysical characterization of FVIII-PI showed that lipid binding did not lead to major changes in the secondary structures but may involve small tertiary structural changes of the protein (Fig. 1). Both the *in vitro* activity assay and the *in vivo* efficacy study confirmed that lipid binding did not result in neither loss of FVIII activity nor its haemostatic efficacy. Thermal denaturation studies (CD) suggested that PI binding improved the intrinsic stability of the protein. A small tertiary structural change in the lipid binding region of the C2 domain has been shown to promote aggregation of the protein (43,51), and PI binding to this domain may interfere

Fig. 5. Influence of PI on immunogenicity of FVIII. **a** The mean of inhibitory antibody titers (horizontal bars) and individual (open circles) inhibitory titers was determined following four weekly s.c. administrations of FVIII or FVIII-PI. **b** The mean of inhibitory titers (horizontal bars) and individual (open circles) inhibitory titers was determined following four weekly i.v. administrations of FVIII or FVIII-PI. **c** T cell clonal expansion represented as the SI (individual = open circles, mean = horizontal bars). Hemophilia A mice were given two weekly s.c. injections of FVIII or FVIII-PI (10 IU/injection). CD4⁺ T cells harvested from these animals were challenged with intact FVIII (100 ng/well) in order to proliferate.

with these tertiary structural changes to promote stability and reduce aggregation.

The sandwich ELISA assay confirmed the participation of lipid binding region of C2 domain. The study also showed that in addition to the C2 domain, PI binding also involves other domains, possibly A2, though to a much lesser extent compared to the C2 domain (Fig. 2). In contrast, model membranes containing PS only bind to the previously characterized lipid binding region (2303–2332) of FVIII (19,50,52). One hypothesis to explain this may be differences in lipid organization between PS and PI containing lipidic vesicles. PI contains –OH groups that can bind to water molecules leading to large surface area of the head group which may result in a distorted bilayer organization. This could increase the depth of protein penetration into membrane bilayer and modulate the surface of FVIII molecule that comes in contact with PI membranes. This is consistent with a small blue shift observed for FVIII–PI by fluorescence spectroscopy. Further, this association can prevent binding of antibody to other domains due to steric hindrance.

As the lipid binding shields domains in FVIII that participate in vWF and LRP binding, this could influence plasma survival of the protein. Our present studies showed ~4-fold increase in the circulation $t_{1/2}$ of FVIII upon association with PI particles in hemophilia A mice suggesting that lipid binding reduced catabolism of FVIII (Fig. 3). vWF is required for plasma survival of FVIII as it prevents binding of FVIII to LRP. The binding affinities between FVIII–vWF (53) and FVIII–LRP (15) have been shown to be in the nano-molar range with a stronger binding for vWF interaction. As the lipid (PS) binds to same binding sites (C2 domain) on the protein with affinities in nano-molar range, lipid binding could also provide some protective effect against LRP-mediated clearance. However, due to rapid uptake of PS by RES system (29,30), FVIII–PS complex did not show improved plasma survival of FVIII following i.v. administration. On the other hand, FVIII–PI complex showed increased plasma survival of FVIII, and this could be due to stealth like properties of PI that resist RES uptake (31) and also due to the molecular configuration of FVIII–PI complex. Due to deeper bilayer penetration of the protein, molecular topology of FVIII–PI complex involves not only the C2 domain but also other domains (A2 domain), leading to further reduction in LRP-mediated clearance as both A2 and C2 domains are involved in the LRP binding (14,15). It is appropriate to mention here that further characterization of FVIII–PI interaction using methods such as isothermal titration calorimetry and surface plasmon resonance studies are certainly necessary to assess binding affinities between FVIII and PI.

The precise mechanism by which FVIII–PI reduces FVIII antibody responses *in vivo* is complex and not clear at this time. However, we speculate that the PI reduces FVIII immunogenicity by multiple mechanisms. Anti-FVIII antibodies targeted against epitopes in A2, A3, and C2 domains of FVIII (24–26). As the lipid binding involve these domains, these immunogenic epitopes were shielded resulting in reduction of antibody titer levels by immune ignorance. In addition, the immunomodulatory effects of PI could also likely to play a significant role. In terms of effects upon immune cells, PI has been shown to suppress phagocytic function in macrophages (54) and could exert an inhibitory effect on lymphocyte activity (55). However, PI effect on

dendritic cells, a primary initiator of T cell responses, is not clearly understood. Our preliminary cell culture studies showed PI reduced upregulation of co-stimulatory signals such as CD40, a phenotypic marker for dendritic cell maturation upon exposure to antigens, and increased secretion of TGF- β , an important cytokine in the regulation of T cell responses (to be published elsewhere). These effects of PI could lead to suppression of T cell responses.

In conclusion, PI binding reduces catabolism and immunogenicity of FVIII, and this may be mediated by possible influence on macromolecular interactions and components of immune system. These interactions could be useful as therapeutic option to prolong FVIII survival *in vivo* and overcome inhibitor development in hemophilia A patients.

ACKNOWLEDGMENTS

This work was supported by a grant from the National Institutes of Health (R01 HL-70227) to SVB. The authors thank Pharmaceutical Sciences Instrumentation facility, University at Buffalo, State University of New York, for the use of CD and fluorescence instruments, which were obtained by Shared Instrumentation Grants S10-RR013665 and S10-RR15877 from the National Center for Research Resources, National Institute of Health. We are grateful to Drs. Kazazian and Sarkar of the University of Pennsylvania for providing the initial FVIII knockout mice model and to Dr. Bernstein of Western New York Hemophilia Foundation for providing albumin-free recombinant Factor VIII (Advate).

REFERENCES

1. Foster PA, Zimmerman TS. Factor VIII structure and function. *Blood Rev.* 1989;3(3):180–91.
2. Fay PJ. Factor VIII structure and function. *Thromb Haemost.* 1993;70(1):63–7.
3. Kaufman RJ, Wasley LC, Dorner AJ. Synthesis, processing, and secretion of recombinant human factor VIII expressed in mammalian cells. *J Biol Chem.* 1988;263(13):6352–62.
4. Lenting PJ, VANS CJ, Denis CV. Clearance mechanisms of von Willebrand factor and factor VIII. *J Thromb Haemost.* 2007;5(7):1353–60.
5. Saenko EL, Ananyeva NM. Receptor-mediated clearance of factor VIII: implications for pharmacokinetic studies in individuals with haemophilia. *Haemophilia.* 2006;12:15–22.
6. Nishino M, Girma JP, Rothschild C, Fressinaud E, Meyer D. New variant of von Willebrand disease with defective binding to factor VIII. *Blood.* 1989;74(5):1591–9.
7. Morfini M, Mannucci PM, Tenconi PM, Longo G, Mazzucconi MG, Rodeghiero F, *et al.* Pharmacokinetics of monoclonally-purified and recombinant factor VIII in patients with severe von Willebrand disease. *Thromb Haemost.* 1993;70(2):270–2.
8. Tuddenham EG, Lane RS, Rotblat F, Johnson AJ, Snape TJ, Middleton S, *et al.* Response to infusions of polyelectrolyte fractionated human factor VIII concentrate in human haemophilia A and von Willebrand's disease. *Br J Haematol.* 1982;52(2):259–67.
9. Saenko EL, Scandella D. The acidic region of the factor VIII light chain and the C2 domain together form the high affinity binding site for von willebrand factor. *J Biol Chem.* 1997;272(29):18007–14.
10. Hamer RJ, Koedam JA, Beeser-Visser NH, Bertina RM, Van Mourik JA, Sixma JJ. Factor VIII binds to von Willebrand factor via its Mr-80, 000 light chain. *Eur J Biochem.* 1987;166(1):37–43.
11. Lollar P, Hill-Eubanks DC, Parker CG. Association of the factor VIII light chain with von Willebrand factor. *J Biol Chem.* 1988;263(21):10451–5.

12. Leyte A, Verbeet MP, Brodniewicz-Proba T, Van Mourik JA, Mertens K. The interaction between human blood-coagulation factor VIII and von Willebrand factor. Characterization of a high-affinity binding site on factor VIII. *Biochem J*. 1989;257(3):679–83.
13. Saenko EL, Shima M, Rajalakshmi KJ, Scandella D. A role for the C2 domain of factor VIII in binding to von Willebrand factor. *J Biol Chem*. 1994;269(15):11601–5.
14. Saenko EL, Yakhyayev AV, Mikhailenko I, Strickland DK, Sarafanov AG. Role of the low density lipoprotein-related protein receptor in mediation of factor VIII catabolism. *J Biol Chem*. 1999;274(53):37685–92.
15. Lenting PJ, Neels JG, van den Berg BM, Clijsters PP, Meijerman DW, Pannekoek H, *et al*. The light chain of factor VIII comprises a binding site for low density lipoprotein receptor-related protein. *J Biol Chem*. 1999;274(34):23734–9.
16. Saenko EL, Scandella D, Yakhyayev AV, Greco NJ. Activation of factor VIII by thrombin increases its affinity for binding to synthetic phospholipid membranes and activated platelets. *J Biol Chem*. 1998;273(43):27918–26.
17. Gilbert GE, Drinkwater D. Specific membrane binding of factor VIII is mediated by O-phospho-L-serine, a moiety of phosphatidylserine. *Biochemistry*. 1993;32(37):9577–85.
18. Gilbert GE, Kaufman RJ, Arena AA, Miao H, Pipe SW. Four hydrophobic amino acids of the factor VIII C2 domain are constituents of both the membrane-binding and von Willebrand factor-binding motifs. *J Biol Chem*. 2002;277(8):6374–81.
19. Pratt KP, Shen BW, Takeshima K, Davie EW, Fujikawa K, Stoddard BL. Structure of the C2 domain of human factor VIII at 1.5 Å resolution. *Nature*. 1999;402(6760):439–42.
20. Saenko EL, Scandella D. A mechanism for inhibition of factor VIII binding to phospholipid by von Willebrand factor. *J Biol Chem*. 1995;270(23):13826–33.
21. Kembell-Cook G, Barrowcliffe TW. Interaction of factor VIII with phospholipids: role of composition and negative charge. *Thromb Res*. 1992;67(1):57–71.
22. Jacquemin MG, Saint-Remy JM. Factor VIII immunogenicity. *Haemophilia*. 1998;4(4):552–7.
23. Lollar P, Healey JF, Barrow RT, Parker ET. Factor VIII inhibitors. *Adv Exp Med Biol*. 2001;489:65–73.
24. Reding MT, Okita DK, Diethelm-Okita BM, Anderson TA, Conti-Fine BM. Human CD4+ T-cell epitope repertoire on the C2 domain of coagulation factor VIII. *J Thromb Haemost*. 2003;1(8):1777–84.
25. Reding MT, Okita DK, Diethelm-Okita BM, Anderson TA, Conti-Fine BM. Epitope repertoire of human CD4(+) T cells on the A3 domain of coagulation factor VIII. *J Thromb Haemost*. 2004;2(8):1385–94.
26. Hu GL, Okita DK, Conti-Fine BM. T cell recognition of the A2 domain of coagulation factor VIII in hemophilia patients and healthy subjects. *J Thromb Haemost*. 2004;2(11):1908–17.
27. Purohit VS, Ramani K, Sarkar R, Kazazian Jr HH, Balasubramanian SV. Lower inhibitor development in hemophilia A mice following administration of recombinant factor VIII-O-phospho-L-serine complex. *J Biol Chem*. 2005;280(18):17593–600.
28. Ramani K, Miclea RD, Purohit VS, Mager DE, Straubinger RM, Balu-Iyer SV. Phosphatidylserine containing liposomes reduce immunogenicity of recombinant human factor VIII (rFVIII) in a murine model of hemophilia A. *J Pharm Sci*. 2008;97(4):1386–98.
29. Kamps JA, Morselt HW, Scherphof GL. Uptake of liposomes containing phosphatidylserine by liver cells *in vivo* and by sinusoidal liver cells in primary culture: *in vivo-in vitro* differences. *Biochem Biophys Res Commun*. 1999;256(1):57–62.
30. Kamps JA, Scherphof GL. Receptor *versus* non-receptor mediated clearance of liposomes. *Adv Drug Deliv Rev*. 1998;32(1–2):81–97.
31. Gabizon A, Papahadjopoulos D. Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors. *Proc Natl Acad Sci USA*. 1988;85(18):6949–53.
32. Bartlett GR. Phosphorus assay in column chromatography. *J Biol Chem*. 1959;234(3):466–8.
33. Heath TD, Macher BA, Papahadjopoulos D. Covalent attachment of immunoglobulins to liposomes via glycosphingolipids. *Biochim Biophys Acta*. 1981;640(1):66–81.
34. Purohit VS, Middaugh CR, Balasubramanian SV. Influence of aggregation on immunogenicity of recombinant human factor VIII in hemophilia A mice. *J Pharm Sci*. 2006;95(2):358–71.
35. Bi L, Lawler AM, Antonarakis SE, High KA, Gearhart JD, Kazazian Jr HH. Targeted disruption of the mouse factor VIII gene produces a model of haemophilia A. *Nat Genet*. 1995;10(1):119–21.
36. Qian J, Borovok M, Bi L, Kazazian Jr HH, Hoyer LW. Inhibitor antibody development and T cell response to human factor VIII in murine hemophilia A. *Thromb Haemost*. 1999;81(2):240–4.
37. Denis C, Methia N, Frenette PS, Rayburn H, Ullman-Cullere M, Hynes RO, *et al*. A mouse model of severe von Willebrand disease: defects in hemostasis and thrombosis. *Proc Natl Acad Sci USA*. 1998;95(16):9524–9.
38. Sarkar R, Xiao W, Kazazian Jr HH. A single adeno-associated virus (AAV)-murine factor VIII vector partially corrects the hemophilia A phenotype. *J Thromb Haemost*. 2003;1(2):220–6.
39. Berntorp E, Bjorkman S. The pharmacokinetics of clotting factor therapy. *Haemophilia*. 2003;9(4):353–9.
40. Messori A, Longo G, Matucci M, Morfini M, Ferrini PL. Clinical pharmacokinetics of factor VIII in patients with classic haemophilia. *Clin Pharmacokinet*. 1987;13(6):365–80.
41. Verbruggen B, Novakova I, Wessels H, Boezeman J, van den Berg M, Mauser-Bunschoten E. The Nijmegen modification of the Bethesda assay for factor VIII:C inhibitors: improved specificity and reliability. *Thromb Haemost*. 1995;73(2):247–51.
42. Ramani K, Purohit V, Middaugh CR, Balasubramanian SV. Aggregation kinetics of recombinant human FVIII (rFVIII). *J Pharm Sci*. 2005;94(9):2023–9.
43. Ramani K, Purohit VS, Miclea RD, Middaugh CR, Balasubramanian SV. Lipid binding region (2303–2332) is involved in aggregation of recombinant human FVIII (rFVIII). *J Pharm Sci*. 2005;94(6):1288–99.
44. Griffin BD, Micklem LR, McCann MC, James K, Pepper DS. The production and characterisation of a panel of ten murine monoclonal antibodies to human procoagulant factor VIII. *Thromb Haemost*. 1986;55(1):40–6.
45. Comiskey SJ, Heath TD. Serum-induced leakage of negatively charged liposomes at nanomolar lipid concentrations. *Biochemistry*. 1990;29(15):3626–31.
46. Grit M, Crommelin DJ. Chemical stability of liposomes: implications for their physical stability. *Chem Phys Lipids*. 1993;64(1–3):3–18.
47. Braun A, Kwee L, Labow MA, Alsenz J. Protein aggregates seem to play a key role among the parameters influencing the antigenicity of interferon alpha (IFN-alpha) in normal and transgenic mice. *Pharm Res*. 1997;14(10):1472–8.
48. Chirino AJ, Ary ML, Marshall SA. Minimizing the immunogenicity of protein therapeutics. *Drug Discov Today*. 2004;9(2):82–90.
49. Elder B, Lakich D, Gitschier J. Sequence of the murine factor VIII cDNA. *Genomics*. 1993;16(2):374–9.
50. Stoilova-McPhie S, Villoutreix BO, Mertens K, Kembell-Cook G, Holzenburg A. 3-Dimensional structure of membrane-bound coagulation factor VIII: modeling of the factor VIII heterodimer within a 3-dimensional density map derived by electron crystallography. *Blood*. 2002;99(4):1215–23.
51. Grillo AO, Edwards KL, Kashi RS, Shipley KM, Hu L, Besman MJ, *et al*. Conformational origin of the aggregation of recombinant human factor VIII. *Biochemistry*. 2001;40(2):586–95.
52. Purohit VS, Ramani K, Kashi RS, Durrani MJ, Kreiger TJ, Balasubramanian SV. Topology of factor VIII bound to phosphatidylserine-containing model membranes. *Biochim Biophys Acta*. 2003;1617(1–2):31–8.
53. Vlot AJ, Koppelman SJ, van den Berg MH, Bouma BN, Sixma JJ. The affinity and stoichiometry of binding of human factor VIII to von Willebrand factor. *Blood*. 1995;85(11):3150–7.
54. Wassef NM, Roerdink F, Richardson EC, Alving CR. Suppression of phagocytic function and phospholipid metabolism in macrophages by phosphatidylinositol liposomes. *Proc Natl Acad Sci USA*. 1984;81(9):2655–9.
55. Caselli E, Baricordi OR, Melchiorri L, Bellini F, Ponzin D, Bruni A. Inhibition of DNA synthesis in peripheral blood mononuclear cells treated with phosphatidylserines containing unsaturated acyl chains. *Immunopharmacology*. 1992;23(3):205–13.