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Effect of Biophysical Properties of Phosphatidylserine Particle on Immune Tolerance Induction Toward Factor VIII in a Hemophilia A Mouse Model

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ABSTRACT

A major complication in the replacement therapy of Factor VIII (FVIII) for Hemophilia A is the development of unwanted immune responses. Previous studies from our laboratory have shown that pretreatment of FVIII in the presence of phosphatidylserine (PS) resulted in hyporesponsiveness to subsequent administration of FVIII alone, due to the ability of PS to convert an immunogen to a tolerogen. We investigated the importance of biophysical properties of PS liposomes on its ability to convert an immunogen to a tolerogen. PS particles were prepared differing in size, protein-lipid topology, lamellarity, and % association to FVIII keeping the composition of the particle same. PS particles were prepared in 2 different sizes with differing biophysical properties: smaller particles in the nanometer range (200 nm) and larger size particles in the micron range (2 μm). Hemophilia A animals treated with both the nanometer and micron size PS particles showed a significant reduction in anti-FVIII antibody titers when compared to animals receiving free FVIII alone. Upon rechallenge with free FVIII animals that received FVIII along with the nanometer size particle continued to show reduced antibody responses. Animals receiving the micron size particle showed a slight increase in titers although they remained significantly lower than the free FVIII treated group. Upon culture with bone marrow derived dendritic cells, the nanometer size particle showed a reduction in CD40 expression and an increase in transforming growth factor-β cytokine production, which was not observed with the micron size particle. These results show that biophysical properties of PS play an important role in tolerance.

Introduction

Factor VIII (FVIII) is an important clotting factor, which plays a central role in the blood coagulation cascade. Hemophilia A (HA) is a bleeding disorder characterized by a genetic deficiency or dysfunction of FVIII. Replacement therapy with recombinant FVIII is the first line of therapy for this disorder. However, one of the major drawbacks of this therapy is the development of unwanted immune responses that renders the therapy less efficacious. These immune responses include anti-FVIII neutralizing antibodies (Nabs) also known as inhibitors observed in about 30% of patients, as well as binding antibodies. Nabs bind to FVIII and abrogate its activity, while binding antibodies increase its clearance affecting its pharmacokinetics.[1] Any approach to reduce immunogenicity and induce tolerance to FVIII would improve safety and efficacy of replacement therapy for HA.

Our approach to this problem is to administer FVIII in the presence of phosphatidylserine (PS) liposomes. Our previous studies have shown that pre-exposure of FVIII in the presence of PS led to hyporesponsiveness toward FVIII upon subsequent rechallenges with the free FVIII alone in a HA mouse model.[2] This effect was found to be antigen specific. Bone marrow derived dendritic cells (BMDCs) upon exposure of FVIII in the presence of PS showed a downregulation of the costimulatory marker CD40 as well as an increase in the regulatory cytokine transforming growth factor (TGF)-β.[3] Further mechanistic studies showed that PS acts by increasing the regulatory T cells (Tregs) production as well as reducing the memory B cell development *in vivo*.[4] These results put together indicate that PS converts an immunogen to a tolerogen.

Although we have an insight into the mechanisms by which PS induces tolerance, it is not clear whether the biophysical properties

Abbreviations used: BMDCs, bone marrow derived dendritic cells; FVIII, factor VIII; HA, hemophilia A; Nabs, anti-FVIII neutralizing antibodies; PS, phosphatidylserine.

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of PS liposomes play a role in this novel regulatory property. Hence, here we investigated the biophysical properties of PS liposomes as a critical requirement for tolerance. PS particles were prepared differing in size, protein-lipid topology, lamellarity, and % association to FVIII keeping the composition of the particle same. The studies show that the smaller size particles, in the nanometer range, are more effective in inducing tolerance to FVIII.

Materials and Methods

Materials

Full-length recombinant human FVIII was a generous gift from the Hemophilia Center of Western New York, Buffalo, NY. Brain PS and dimyristoyl phosphatidylcholine (DMPC) were purchased from Avanti Lipids (Alabaster, AL). All solvents and buffer salts were obtained from Fisher Scientific (Fairlawn, NJ) or from Sigma (St. Louis, MO). Anti-FVIII monoclonal antibody ESH8 was obtained from American Diagnostica, Inc. (Greenwich, CT). Endosafe Endochrome-K® kit was purchased from Charles River Laboratories (Charleston, SC). NUNC MaxiSorp 96-well plates were from Thermoscientific (Waltham, MA). Activated partial thromboplastin time (aPTT) reagents were purchased from T-coag Ireland Ltd. (Ireland). Normal pooled and deficient human plasma were obtained from Precision Biologic (Canada). Antibodies for flow cytometry were purchased from BD Biosciences (San Jose, CA). TGF-β cytokine enzyme-linked immunosorbent assay (ELISA) kit was purchased from R&D System, Inc. (DuoSet) (Minneapolis, MN).

Animals

A colony of hemophilic mice with targeted deletion in the exon 16 of FVIII gene (termed HA mice) was maintained on site in accordance with the Institutional Animal Care and Use Committee of the University at Buffalo. The animals were handled and surgical procedures performed as per the protocol approved by the Institutional Animal Care and Use Committee.

Preparation and Characterization of Protein Lipid Complex

Preparation of PS-FVIII (200 nm)

PS liposomes were prepared at a 30:70 molar ratio of PS to DMPC as previously described.[5] To get the appropriate size, PS liposomes were extruded multiple times through double stacked polycarbonate membrane of pore size 200 nm using high pressure extruder. The size of the liposomes was measured using a NICOMP Model CW380 particle size analyzer from Particle Sizing Systems (Port Richley, FL). This procedure results in small unilamellar vesicles. The size of the PS liposomes was found to be 194 ± 61.2 nm (mean ± standard deviation [SD]). The lipid content was assayed by a phosphate assay.[6] The protein to lipid molar ratio used was at 1:10,000. FVIII was associated with liposomes by a trigger-loading mechanism by incubating the preparation at 37°C for 30 min. The trigger-loading mechanism is a gentle and controlled thermal unfolding procedure employed to promote interaction between FVIII and PS liposomes. The procedure results in approximately 47% of FVIII to be associated with PS liposomes. The remaining FVIII remains unbound to the PS and is left in the preparation prior to injection. The interaction of FVIII with PS liposomes is mainly through the C2 domain of FVIII[5] and in this configuration the protein is surface bound to the liposome. The FVIII is bound either on the surface of the PS liposomes or is embedded in the inner bilayer of liposome.[7]

Preparation of PS-FVIII (2 μm)

PS liposomes were prepared at a 30:70 molar ratio of PS to DMPC. The appropriate amounts of lipids were dissolved in chloroform and the solvent was evaporated using a rotary evaporator (Buchi R200; Fisher Scientific) to form a thin lipid film. The FVIII in Tris buffer (300 mM sodium chloride, 25 mM Tris, pH 7.0) was added slowly to the film (at a 1:10,000 protein to lipid molar ratio) and gently mixed. This mixture was then placed at 37°C for 2 min and then gently mixed by rotating in hand. This cycle was repeated for a total of 3 times. This procedure results in the generation of multilamellar vesicles. At the end, this PS-FVIII complex was spun at 13,000 g for 60 min. This is to remove any free FVIII unbound to the PS liposomes. The supernatant is removed and assayed for free FVIII by an activity assay using an aPTT assay. This procedure results in about 85% of FVIII to be associated with the PS liposomes. The remaining 15% of the FVIII is free and is removed in the supernatant. This method results in all the FVIII to be associated with PS. All the FVIII is embedded in the inner bilayer of the liposomes. The size of the liposomes was measured using a NICOMP Model CW380 particle size analyzer from Particle Sizing Systems. The size of the PS particle was found to be 2320 ± 271 nm (mean ± SD). The pellet is resuspended in Tris buffer and the appropriate amount used for studies.

It is important to note that in both preparations the ratio of protein:lipid and the amount of protein administered is the same. The PS-FVIII complexes in both the cases above were tested for endotoxin level by using Endosafe Endochrome-K endotoxin assay kit (Charles River, Inc., Wilmington, MA) and endotoxin negative samples were used for all studies.

Biophysical properties of the 2 different size PS particles are summarized in Table 1.

Tolerance Induction Studies

Animal studies were conducted in HA mice. Animals ($n = 14$) were immunized with 4 weekly subcutaneous injections of FVIII (1 μg/injection) alone or in the presence of PS (200 nm) or PS (2 μm). This was followed by a 2-week washout period. At the end of the sixth week, half the animals in each group were sacrificed and the plasma samples collected as baseline samples for relative immunogenicity analysis of antibody titers. The remaining animals were then aggressively rechallenged with 2 weekly doses of just the free FVIII alone (0.5 μg/injection) via the subcutaneous route to determine tolerance. Two weeks after the last dose, all animals were sacrificed and their plasma samples collected via cardiac puncture into acid citrate dextrose buffer. The plasma samples were stored at −80°C until further analysis.

Generation of BMDCs

Bone marrow was isolated from naïve HA mice as reported in Lutz et al.[9] Briefly, bone marrow was cultured in Roswell Park

Table 1
Biophysical Characterization of PS Preparations

Biophysical Property	PS-FVIII (200 nm)	PS-FVIII (2 μm)
Protein:lipid ratio	1:10,000	1:10,000
Size (mean ± SD)	194 ± 61.2 nm	2320 ± 271 nm
% Association[5]	47%	100%
Lamellarity	Small unilamellar vesicles	Multilamellar vesicles
Protein-lipid topology[7,8]	Surface association via C2 domain	Surface association and incorporation into bilayer as a result of multilamellarity

This table summarizes the biophysical properties of PS liposomes including protein:lipid ratio, size, %association, lamellarity, and protein:lipid topology.