Exposure of FVIII in the Presence of Phosphatidyl Serine Reduces Generation of Memory B-Cells and Induces Regulatory T-Cell-Mediated Hyporesponsiveness in Hemophilia A Mice

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Received 30 December 2014; revised 1 May 2015; accepted 4 May 2015

Published online 2 June 2015 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jps.24513

ABSTRACT: A major complication of replacement therapy with Factor VIII (FVIII) for hemophilia A (HA) is the development of unwanted immune responses. Previous studies showed that administration of FVIII in the presence of phosphatidyl serine (PS) reduced the development of anti-FVIII antibodies in HA mice. However, the impact of PS-mediated effects on immunological memory, such as generation of memory B-cells, is not clear. The effect of PS on memory B-cells was therefore investigated using adoptive transfer approach in FVIII$^{-/-}$ HA mice. Adoptive transfer of memory B-cells from a PS-FVIII-treated group to naïve mice followed by challenge of the recipient mice with FVIII showed a significantly reduced anti-FVIII antibody response in the recipient mice, compared with animals that received memory B-cells from free FVIII and FVIII-charge matched phosphatidyl glycerol (PG) group. The decrease in memory B-cell response is accompanied by an increase in FoxP3 expressing regulatory T-cells (Tregs). Flow cytometry studies showed that the generation of Tregs is higher in PS-treated animals as compared with FVIII and FVIII-PG treated animals. The PS-mediated hyporesponsiveness was found to be antigen-specific. The PS-FVIII immunization showed hyporesponsiveness toward FVIII rechallenge but not against ovalbumin (OVA) rechallenge, an unrelated antigen. This demonstrates that PS reduces immunologic memory of FVIII and induces antigen-specific peripheral tolerance in HA mice.
 J Pharm Sci 104:2451–2456, 2015
Keywords: protein delivery; lipids; phosphatidyl serine; immunology; regulatory T cells; memory B cells

INTRODUCTION

Hemophilia A (HA) is a bleeding disorder characterized by a deficiency or dysfunction of the clotting factor, Factor VIII (FVIII). The use of recombinant FVIII is the first line of therapy for the disease. A major clinical complication of the therapy is the development of anti-FVIII neutralizing antibodies (Nabs) observed in about 15%–30% of the patients.[1] Nabs abrogate the activity of the protein, rendering it less efficacious. Our previous studies have shown that FVIII-associated with phosphatidyl serine (PS) significantly reduced anti-FVIII antibody responses in HA mice.[2] *In vitro* studies aimed at understanding the mechanism of this reduction showed that PS-FVIII downregulated the expression of costimulatory molecule CD40 in bone-marrow-derived dendritic cells (DCs). The coculture of FVIII and PS-FVIII primed DCs with FVIII-specific splenic CD4+ T-cells showed significant reduction in the ability of PS primed DCs to activate T-cells. This was accompanied by an increase in the secretion of the immune regulatory cytokines, TGF beta, IL-10, and a concomitant decrease in proinflammatory IL-6 and IL-17 cytokine levels.[3] These observations were in agreement with the notion that PS-FVIII is the tolerogenic form of the protein that converts an immunogenic protein to a tolerogen.[4–6] This is also consistent with the observation that pre-exposure of the FVIII in the presence of PS led to hyporesponsiveness upon subsequent rechallenge with the FVIII alone.[7] Although PS reduces B-cell effector functions, as measured by the antibody secretion, the impact of the PS-mediated effects on immunologic memory is not yet clear. Here, we investigated the role of memory B-cells in PS-mediated hyporesponsiveness. Adoptive transfer of memory B-cells from PS-FVIII immunized animals to naïve recipient mice resulted in hyporesponsiveness upon FVIII rechallenge, confirming that PS reduces B-cell memory. This is accompanied by an increase in generation of FoxP3 expressing regulatory T-cells. Thus, these PS-mediated effects on immune cells result in antigen-specific hyporesponsiveness.

MATERIALS AND METHODS

Materials

Full-length recombinant human FVIII was a generous gift from the Western New York Hemophilia Foundation (Buffalo, New York). Endograde ovalbumin (OVA) was purchased from BioVendor LLC (Asheville, North Carolina). Brain PS, dimyristoyl phosphatidylcholine (DMPC), and dimyristoyl phosphatidyl glycerol (PG) were purchased from Avanti Lipids (Alabaster, Alaska). All solvents and buffer salts were obtained from Fisher Scientific (Fairlawn, New Jersey) or from Sigma (St. Louis, Missouri). Anti-FVIII monoclonal antibody ESH8 was obtained from American Diagnostica Inc. (Greenwich, Connecticut). Endosafe Endochrome-K® kit was purchased from Charles River Laboratories (Charleston, South Carolina). NUNC MaxiSorp 96 well plates were obtained from Thermoscientific (Waltham, Massachusetts). Activated partial

Abbreviations used: FVIII, Factor VIII; HA, hemophilia A; PS, phosphatidyl serine; PG, Dimyristoyl phosphatidylglycerol; OVA, ovalbumin; Tregs, regulatory T-cells; GFP, green fluorescent protein; Nabs, anti-FVIII neutralizing antibodies.

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Journal of Pharmaceutical Sciences, Vol. 104, 2451–2456 (2015)

thromboplastin time (aPTT) reagents were purchased from T-coag Ireland Ltd. (Parsipanny, New Jersey). Normal pooled and deficient human plasma were obtained from PrecisionBiologic (Dartmouth, Nova Scotia).

Animals

Two different animal models were used as mentioned in different sections. HA mice model that has a targeted deletion in the exon 16 of FVIII gene (termed HA mice). The other mice model in addition to having the targeted deletion in the exon 16 of FVIII gene has a green fluorescent protein (GFP)-tagged FoxP3 knocked-in and is termed GFP-FoxP3 HA mice. The animals were handled and surgical procedures performed as per the protocol approved by the Institutional Animal Care and Use Committee (IACUC) of the University at Buffalo.

Preparation of Protein Lipid Complex

PS and PG liposomes were prepared at a 30:70 molar ratio of PS or PG to DMPC as previously described.[2] The protein to lipid molar ratio used was at 1:10,000 and the lipid content assayed by a phosphate assay.[8] The size of the liposomes was monitored using a NICOMP Model CW380 particle size analyzer from Particle Sizing Systems (Port Richley, Florida). 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 complexes were tested for endotoxin level by using Endosafe Endochrome-K endotoxin assay kit (Charles River Inc., Massachusetts) and endotoxin negative samples were used for *in vivo* studies.

Role of Memory B-Cell in PS-Mediated Hyporesponsiveness

Adoptive transfer studies were carried out in GFP-FoxP3 HA mice model as a proof-of-concept to determine the role of memory B-cells. Adoptive transfer involves transferring cells of interest from a treated animal to an untreated naïve animal and the function mediated by the immune cell will transfer to the naïve mice. This allows determining the role of that immune cell. For example, if memory B-cells are involved in PS-mediated effect, adoptive transfer of memory B-cells from immunized mice to recipient mice; the recipient will acquire the tolerogenic capability. The animals ($n = 7$ or 8/treatment group) received s.c. injections of either free FVIII or PS-FVIII or PG-FVIII (2 μg of FVIII) every week for 12 weeks. One group of animals ($n = 5$) was left untreated and served as the naïve control. Two weeks after the last injection, all animals were sacrificed and their spleens collected. The memory B-cells were isolated from the spleens using a memory B-cell isolation kit, mouse (Miltenyi Biotec, Auburn, California) as per the manufacturer's instructions. The cell count was determined by the BC 2800 Vet auto hematology analyzer (Mindray, Mahwah, New Jersey).

Approximately 0.1×10^6 memory B-cells from all mice were adoptively transferred via penile vein to corresponding individual naïve GFP-FoxP3 HA mice. After a 48-h wait period, all the recipient animals were immunized aggressively with four weekly s.c. injections of 1 μg of free FVIII per injection. Two weeks after the last injection, all animals were sacrificed and their plasma samples collected for analysis of antibody titer levels.

Role of Regulatory T-Cell in PS-Mediated Memory B-Cell Reduction

The animals used as donors in the above study (described in "*Role of Memory B-Cell in PS-Mediated Hyporesponsiveness*") were used for analysis of regulatory T-cell (Treg) expression. These animals have the GFP knocked-in to the FoxP3 gene allowing for the GFP expression to be used as a read-out for FoxP3 expression and hence Treg. The inguinal lymph nodes were isolated from these animals and prepared for analysis using flow cytometry. The dot plots were analyzed by the Cell Quest software provided by the manufacturer. Further, total lymphocytes were selected based on side scatter versus forward scatter criteria. A histogram analysis was used to analyze GFP expression and the data expressed as the percentage of GFP-$FoxP3^+$ cells in the total lymphocytes region.

Antigen Specificity of PS-Mediated Hyporesponsiveness

Naive HA mice were used for this study. The animals ($n = 10$ per treatment group) received four weekly s.c. injections of either free FVIII or PS-FVIII or PG-FVIII (1 μg of FVIII) and then given a 2-week washout. After this period, half of the animals in each group were rechallenged with free FVIII and the other half of the animals were rechallenged with free OVA weekly for 4 weeks with 1 μg protein given s.c. Two weeks after the last injection, all animals were sacrificed and their plasma samples were collected for the analysis of antibody titer levels.

Effect of PS on Pre-Existing Anti-FVIII Antibody Levels

Naive HA mice were used for this study. Animals were immunized with four weekly s.c. injections of 1 μg of FVIII to establish titers. Two weeks after the last injection, all animals were rechallenged with four weekly s.c. injections of 1 μg of FVIII in the presence or absence of PS lipid. A separate set of animals ($n = 4$) was sacrificed before rechallenge to obtain baseline antibody titers. All animals were sacrificed 2 weeks after the last rechallenge, and the plasma samples were analyzed for the presence of neutralizing antibodies.

Determination of Anti-FVIII Nabs and Total Anti-FVIII Antibodies

The plasma samples were analyzed for anti-FVIII Nab titers by activated partial Thromboplastin time (aPTT) assay following Nijmegen's modified Bethesda assay and expressed in Bethesda units (BU/mL).[9] Total anti-FVIII antibody titers were determined by ELISA as described previously.[10]

Determination of Anti-Ova IgG1 Antibodies by ELISA

Anti-OVA titers were measured using a commercially available kit purchased from Cayman Chemical (Ann Arbor, Michigan). The assay was performed as per manufacturer's instructions. The plate specific factor (PSF) for the assay was calculated as half the difference between the absorbance values for the highest concentration standard and the lowest concentration standard. A linear regression of the plot of the absorbance value versus the log dilution was used to calculate the dilution, which yielded an optical density equal to the PSF. The dilution thus obtained was considered to be the antibody titer value for the sample.