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Phosphatidylserine Converts Immunogenic Recombinant Human Acid Alpha-Glucosidase to a Tolerogenic Form in a Mouse Model of Pompe Disease

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Abstract

Development of unwanted immune responses against therapeutic proteins is a major clinical complication. Recently, we have shown that exposure of Factor VIII (FVIII) in the presence of phosphatidylserine (PS) induces antigen-specific hypo-responsiveness to FVIII re-challenge, suggesting that PS is not immune suppressive, but rather immune regulatory in that PS converts an immunogen to a tolerogen. Since PS is exposed in the outer leaflet during apoptosis, we hypothesize that PS imparts tolerogenic activity to this natural process. Thus, immunization with PS containing liposomes would mimic this natural process. Here, we investigate the immune regulatory effects of PS in inducing tolerance towards recombinant human acid alpha-glucosidase (rhGAA). rhGAA was found to complex with PS liposomes through hydrophobic interactions and incubation PS-rhGAA with dendritic cells resulted in the increased secretion of TGF- β . Immunization with PS-rhGAA or OPLS-rhGAA led to a reduction in anti-rhGAA antibody response which persisted despite re-challenge with free rhGAA. Importantly, the titer levels in a majority of these animals remained unchanged after rechallenge and can be considered non-responders. These data provide evidence that PS liposomes can be utilized to induce tolerance towards therapeutic proteins, in general.

INTRODUCTION

Development of unwanted immune responses against therapeutic proteins compromises the safety and efficacy of many protein based therapeutics. Such responses can not only abrogate biological activity of therapeutic proteins but can also alter their disposition, impacting their circulation half-life and plasma survival¹. Strategies that reduce unwanted immune responses could greatly improve these issues. Recently, we have demonstrated that pre-exposure of Factor VIII (FVIII) in the presence of phosphatidylserine (PS) induces an antigen-specific, hypo-responsiveness towards a re-challenge with FVIII in a mouse model

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of Hemophilia A². Based on these recent observations in our laboratory we concluded that PS could convert an immunogen to a tolerogen. In order to realize the broad utility of this novel property of PS, its ability to reduce unwanted immune responses against other therapeutic proteins was investigated.

Like Hemophilia A, treatment of Pompe Disease is severely hindered by the formation of anti-drug antibodies. Pompe disease is an autosomal recessive disorder caused by mutations in the gene that encode for acid alpha-glucosidase (GAA). These mutations result in an enzyme that is either deficient or completely dysfunctional³. Since GAA is the only enzyme capable of cleaving glycogen stored within the cells throughout the human body into glucose when this occurs, glycogen builds up within the lysosomes. This is what causes the symptoms of Pompe disease. Currently the only treatment for Pompe disease is enzyme replacement therapy (ERT) with rhGAA. While ERT with rhGAA has been shown to be successful for many patients^{4,5}, it has also been shown to be hindered by the very high incidence of the formation of anti-rhGAA antibodies^{6,7}. According to the package inserts for the two products on the market, 89-100% of patients will form anti-rhGAA antibodies when treated^{8,9}. Once a patient develops high sustained antibody titers, therapy with rhGAA is no longer efficacious and only palliative care can be offered to the patient¹⁰.

Since there are no alternative treatment options for patients who have developed an unwanted immune response, many strategies are being investigated to produce either a less immunogenic form of the protein or to develop a tolerance induction regimen to mitigate the immune response towards the current drugs that are used clinically. Currently, a combination regimen of methotrexate, rituximab, and intravenous immunoglobulins (IVIG) is being used as a first line immune tolerance induction regimen in clinical patient with variable degrees of success^{11,12}. However, this strategy and the other current methods utilized involve general immune suppression and have associated toxicities¹². Therefore, there exists a prominent unmet medical need to develop a strategy to mitigate the immunogenicity towards rhGAA. Here, we investigate the ability of PS to convert immunogenic rhGAA to a tolerogenic form of the protein, as well as investigate the key structural components of PS which are needed to elicit this response.

EXPERIMENTAL PROCEDURES

Materials

Recombinant human GAA expressed in human embryonic kidney cells was obtained from Creative Biomart (Shirley, New York). The activity of the protein was confirmed by an enzymatic substrate cleavage assay (described below) and it was found that the product bound to human anti-rhGAA antibodies purchased from Sigma Aldrich by ELISA. Brain phosphatidylserine (PS), dimyristoylphosphatidylcholine (DMPC), and phosphatidylglycerol (PG) were obtained from Avanti Polar Lipids (Alabaster, Alabama). Horseradish peroxidaseconjugated goat anti-mouse IgG antibody, 3,3',5,5'-tertramethylbenzidine substrate (TMB), and 4-methylumbelliferyl- α -D-glucoside (4-MUG) were purchased from Sigma Aldrich (St. Louis, Missouri.)

Preparation of Liposomes

PS liposomes and control PG liposomes were prepared at a 30:70 molar ratio of PS or PG to DMPC as previously described¹³. The protein to lipid molar ratio was prepared at 1:10,000 and the lipid content was confirmed using phosphate assay¹⁴. After extrusion, the size of the liposomes was monitored using a NICOMP Model CW380 particle size analyzer from Particle Sizing Systems (Port Richley, Florida). The protein was loaded into the liposomes by incubating at 37°C for 30 minutes. All formulations were confirmed to be endotoxin negative using a limulus amebocyte assay from Charles River Laboratories, Inc. (Wilmington, Massachusetts) prior to injection.

Preparation of OPLS Solution

OPLS solution was prepared by mixing 5 mg OPLS in 100 l sterile Tris buffer (150 mM sodium chloride and 25 mM Tris.) The pH of the resulting solution was highly acidic and was adjusted with sodium hydroxide pellets to pH=7.4. The osmolarity of the solution was measured using 5500 Vapor Pressure Osmometer from Wescor Incorporated (Logan, Utah). All formulations were confirmed to be endotoxin negative using a limulus amebocyte assay from Charles River Laboratories, Inc. (Wilmington, Massachusetts) prior to injection.

Fluorescence Spectroscopy Studies

Changes in tertiary structure in the presence and absence of PS liposomes were investigated by measuring fluorescence using a Photon Technology International Quantamaster fluorometer (Edison, New Jersey). The concentration of the protein was 5 µg/ml. Samples were excited at 265 nm and emission spectra were collected from 300 nm to 400 nm. The contributions due to scattering of light by liposomes and Raman Band by liposomes were minimized by exciting the samples at 265 nm and also by using a 295 nm long pass filter on the emission path. PS-rhGAA spectra were corrected by subtracting spectra of unloaded PS liposomes. All spectra were normalized to their peak maxima. A collisional quenching study with acrylamide was used to determine the location of rhGAA within the PS liposomes. Quenching of tryptophan residues was monitored following additions of a 5M solution of acrylamide. Samples were excited at 280 nm and an emission scan was collected at 325 nm for one minute then averaged. The scans were repeated after each acrylamide addition.

Substrate Cleavage Assay

The availability of rhGAA to cleave a synthetic substrate in the presence and absence of PS liposomes was quantitated using 4-MUG as a substrate. Briefly samples were incubated in a black 96 well plate for 2 hours at 37°C with a substrate solution that consists of 0.1 M citric acid, 0.2 M disodium hydrogen phosphate, and 1 mM 4-MUG at pH 4.5. After the incubation the reaction was stopped with 0.5 M sodium carbonate solution at pH 10.6. The fluorescence was then measured using SpectraMax Gemini (Sunnyvale, California) at an excitation wavelength of 365 nm and the emission wavelength set at 448 nm.

Animals

6^{neo}/6^{neo} GAA knockout (KO) mice were first produced by Raben et al.¹⁵. Embryonic stem cells from 129/Sv Rw4 mice with the targeted disruption of the GAA gene were injected

into C57BL/6J blastocysts and the resulting male chimeric mice were then bred with C57BL/6J mice. Breeding pairs of these mice were purchased from The Jackson Laboratories (Bar Harbor, Maine) and a homozygous colony of GAA KO mice were bred in house. The presence of the homozygous mutation was confirmed in offspring using a standard polymerase chain reaction as described by The Jackson Laboratories¹⁶. All animal experiments were conducted under approval and following the guidelines of the Institutional Animal Care and Use Committee of the University at Buffalo.

PS Immunogenicity and Tolerance Induction Studies

The relative immunogenicity and ability to induce tolerance of PS-rhGAA was carried out in GAA KO mice. PG-rhGAA liposomes were used as a negative, charge matched control for PS-rhGAA liposomes. Mice (n=6-8) received 4 weekly subcutaneous (sc) injections of 1 µg of either free rhGAA, PS-rhGAA, or PG-rhGAA near the base of the tail. This pre-treatment was followed by a two week washout period. A plasma sample was collected from the saphenous vein to determine the relative immunogenicity of rhGAA and lipidic rhGAA and then all mice were rechallenged with 4 weekly sc doses of 1 µg free rhGAA to determine whether PS induces tolerance towards rhGAA. The dosing regimen can be seen in Figure 2A. Two weeks after the final injection, plasma samples were collected via cardiac puncture into a 10% acid citratedextrose (ACD) solution. Plasma samples were stored at -80°C until analysis. Anti-rhGAA antibodies were measured by ELISA.

OPLS Immunogenicity and Tolerance Induction Studies

The relative immunogenicity and ability to induce tolerance of OPLS-rhGAA was carried out in GAA KO mice. Mice (n=6-8) received 4 weekly subcutaneous (sc) injections near the base of the tail of 1 µg of either free rhGAA, OPLS-rhGAA together delivered mixed in the same injection, or OPLS and rhGAA delivered into the same injection site 5 minutes apart by two separate, sequential injections. This pre-treatment was followed by a two week washout period. A plasma sample was collected from the saphenous vein to determine the relative immunogenicity of the formulations and then all mice were rechallenged with 4 weekly sc doses of 1 µg free rhGAA to determine whether OPLS induces tolerance towards rhGAA. Two weeks after the final injection, plasma samples were collected via cardiac puncture into a 10% ACD solution. Plasma samples were stored at -80°C until analysis. Anti-rhGAA antibodies were measured by ELISA.

Anti-rhGAA Antibody ELISA

Total anti-rhGAA IgG antibody titers were measured using an ELISA assay modified from procedure that was previously described¹⁷. 96-well plates were coated with 5 µg/ml of rhGAA in 0.2 M sodium carbonate-bicarbonate buffer overnight at 4°C. The following day the plates were washed and then blocked with 0.1% bovine serum albumin in phosphate buffered saline for 2 hours at 37°C. The plates were washed again and a two-fold serial dilution of sample plasma was added to the plate and incubated for 1 hour at 37°C. The plates were washed again followed by the addition of horseradish peroxidase-conjugated goat anti-mouse IgG secondary detection antibody and incubated for 1 hour at 37°C. The plates were washed again and TMB substrate was added. The plate was developed for 15 minutes in the dark at room temperature. The reaction was then stopped with 1 N

hydrochloric acid and the absorbance was read at 450 nm. Titers were determined using a statistically significant cutoff determined from sham treated animals as described previously¹⁸.

In Vitro Cytokine Expression

Bone marrow derived dendritic cells (BMDCs) were harvested from the femur and tibiae of a naïve GAA KO mice as described by Lutz et al.¹⁹. Cells were then cultured following methods previously described by Gaitonde et al.²⁰. On day nine of culture, BMDCs were counted, replated, and then exposed to treatments and controls for 72 hours. A combination of dexamethasone and vitamin D3 were used a control for tolerogenic dendritic cells²¹. The supernatant was then collected and analyzed for TGF- β secretion by ELISA. The TGF- β ELISA was done using a DuoSet ELISA kit from R&D Systems (Minneapolis, Minnesota) following manufacturer's instructions.

Statistical Analysis

One-way ANOVA followed by Tukey's post-hoc analysis was performed using GraphPad Prism Version 6 for Windows, GraphPad Software (La Jolla, California). Results will be considered significant at a p-value less than 0.05.

RESULTS

Biophysical Characterization of PS-rhGAA Interaction

Prior to investigating the immunomodulatory effects of PS liposomes, the interaction between PS liposomes and rhGAA was examined. The protein was loaded into PS liposomes by incubating rhGAA with liposomes at 37°C for 30 minutes and tertiary structural changes of the protein was investigated by fluorescence spectroscopy (Figure 1A). The peak maxima for free rhGAA was observed at 332 nm compared to a peak maxima for PS-rhGAA observed at 328 nm. This substantial spectral blue shift of 4 nm for PS-rhGAA is consistent with a more hydrophobic location for rhGAA and suggests that some portion of protein has inserted in the hydrophobic bilayer of the PS liposomes²². This location is further confirmed by two separate approaches: collisional quenching with acrylamide and the accessibility of rhGAA to cleave a fluorescent substrate. If the protein is associated with liposomes, it will be less available for acrylamide quenching or to cleave a substrate. Using the Stern-Volmer relationship²³, the accessibility of the tryptophan residues of rhGAA to the quencher in the presence and absence of PS was assessed. The quenching constant, K_{SV} , or slope, of the Stern-Volmer plots (Figure 1B) was decreased for liposome-associated protein. For free rhGAA, K_{SV} was found to be 6.1 M^{-1} but that substantially decreased for PS-GAA to $K_{SV}=0.93 \text{ M}^{-1}$. This suggests that a majority of the tryptophan residues of rhGAA that were previously available to interact with the acrylamide when rhGAA was free were now inaccessible to the quencher due to their interaction with the PS liposome. The interaction between rhGAA and PS liposomes was further supported by the reduced ability of PS-rhGAA to cleave a substrate. Multiple trials ($n=4$) of an *in vitro* assay showed an average reduction in enzymatic cleavage of $64.5\% \pm 12\%$ when rhGAA is associated with PS liposomes compared to free rhGAA at equivalent amounts. Together, these experiments

suggest that rhGAA is associated with liposomes with a configuration where rhGAA is intercalated within the lipid bilayer of PS liposomes.

Immunogenicity Studies

The immune regulatory properties of PS liposomes with rhGAA were investigated *in vivo* using a mouse model of Pompe Disease. GAA KO mice are a good model of both infantile (early onset) Pompe disease with respect to the complete lack of function of GAA and a model for the late onset Pompe disease with the rate of accumulation of glycogen within the muscles²⁴. Further, this pre-clinical model has been shown to display a robust, reproducible immune response to exogenously administered rhGAA²⁴. The sequence homology between mouse and human GAA is about 80% and is highly conserved in T-cell epitope regions²⁵.

The experimental design (Figure 2A) involves pre-treatment with a sub-therapeutic, subcutaneous dose of PS-rhGAA or control treatment for four weeks. As seen in Figure 2B, after pre-treatment with PS-rhGAA, the mean titers for animals in the PS-rhGAA treatment group (374 ± 122 mean titers $\pm$ SEM) are significantly lower ($p < 0.05$) than observed for animals given free rhGAA (3431 ± 788 mean titers $\pm$ SEM) and control, charge matched PG-rhGAA (2155 ± 609 mean titers $\pm$ SEM), suggesting that PS-rhGAA is a less immunogenic form of the protein. Treatment with PS liposomes was stopped after week four and the immunized animals from all treatment groups were then re-challenged with free rhGAA to determine whether the reduction in immune response is due to immune tolerance induction. If it is caused by immune suppression, the animal will respond upon rechallenge. If it is due to tolerance then titers levels will remain unchanged. Terminal blood samples were taken and again antibody development was measured (Figure 2C.) The mean titers for animals pre-treated with PS-rhGAA (535 ± 218 mean titers $\pm$ SEM) are significantly lower ($p < 0.05$) than the animals pre-treated with free rhGAA (5456 ± 1589 mean titers $\pm$ SEM) and PG-rhGAA control (4562 ± 511 mean titers $\pm$ SEM). The saphenous vein sampling made it feasible to follow changes in the titer level in the same immunized animal before and after the rechallenge to evaluate the induction of tolerance (Figure 2D). Importantly, in the PS-rhGAA treated animals, titer levels in four out of eight animals remained relatively unchanged after rechallenge and that these animals can be considered as nonresponders. Whereas four out of the six animals in the free rhGAA control group and seven out of eight animals in the PG-rhGAA control group saw substantial multi-fold changes in titer level. Overall, these studies indicate that PS has converted rhGAA into a tolerogenic form of the protein that produced hypo-responsiveness towards a rechallenge.

In order to explore the structural and spatial requirements of PS that elicits a hypo-responsiveness, immunization studies were carried out in the presence of OPLS, the head group of the PS phospholipid, given either in the same injection with rhGAA (OPLS-rhGAA together) or sequentially as two separate injections, OPLS and then rhGAA, into the same injection site (OPLS-rhGAA sequential). The immunization schedule is the same as described for the PS immunogenicity study. During the immunization of free rhGAA and OPLS-rhGAA, the mean titers $\pm$ SEM for free rhGAA was found to be 3135 ± 910 but were significantly ($p < 0.05$) reduced to 787 ± 155 for OPLS-rhGAA given together in the same injection or to 1399 ± 501 for OPLS and rhGAA given sequentially in separate injections

(Figure 3A). These observations are similar to those observed for PS containing liposomes and also indicate that OPLS, given with rhGAA or just prior to rhGAA at the same injection site, is a less immunogenic form of the protein. Upon re-challenge, the mean titer level for OPLS-rhGAA given together (895 ± 335 mean titers $\pm$ SEM) or sequentially (1884 ± 671 mean titers $\pm$ SEM) remained significantly lower ($p < 0.05$) than animals that were pre-treated with free rhGAA (8775 ± 236 mean titers $\pm$ SEM) (Figure 3B). Figure 3C compares the titers in individual animals before and after the re-challenge with free rhGAA. Again, as was seen for PS-rhGAA treated animals, titers remained unchanged in half of animals, three out of six, that had been pre-treated with either OPLS-rhGAA administered together or sequentially. The titers for the animals that were pre-exposed to OPLS-rhGAA given together were low during pre-treatment stage and remained low after re-challenge, suggesting that OPLS induced a hypo-responsiveness towards rhGAA. This observation also demonstrates that the head group, phospho-serine, is one of the key structural entities needed for the induction of tolerance. It is important to note that while animals treated OPLS and rhGAA given sequentially also had significantly lower titers than free rhGAA, compared to OPLS-rhGAA given together, the mean titers levels for these animals were higher. As rhGAA was injected into the same area in the subcutaneous space occupied by OPLS, it is likely that while many DCs were engulfing rhGAA simultaneously with OPLS, there may have been a substantial population of DCs encountering rhGAA alone, thereby responding in an immunogenic manner and resulting in the observed higher formation of anti-rhGAA antibodies. This study suggests that giving OPLS and rhGAA in two separate injections is less efficient at modulating the immune response.

As the immune regulatory properties of PS are mediated by TGF- β , the role of this cytokine was evaluated *in vitro*. BMDCs isolated from naive GAA KO mice were incubated with PS-rhGAA or control formulations and the supernatant was then analyzed for TGF- β secretion. As a control for a positive phenotype of a tolerogenic DC, a group of cells were treated with dexamethasone and vitamin D3 (Dex-D3) as previously demonstrated²¹. As seen in Table 1, there was increased TGF- β secretion by cells treated with PS-GAA (1283 ± 130 ng/ml) which was comparable to the secretion of TGF- β by the positive control, Dex-D3 (1302 ± 258 ng/ml). This is compared to BMDCs cultured with only free rhGAA (724 ± 310 ng/ml) and liposome control PG-30-rhGAA (915 ± 113 ng/ml) which secreted lower levels of TGF- β . While statistical significance could not be achieved, the trends of the data suggest that PS is able to convert DCs to a tolerogenic phenotype and suggest the involvement of TGF- β as a key player in this response.

DISCUSSION

The robust antibody response in Pompe disease patients has been shown to correlate with a decrease in therapeutic efficacy⁴. Mitigating the immune response is a priority since patients have no alternative treatment options once ERT fails to be efficacious. Our goal is to utilize the tolerogenic properties of PS that we described previously for FVIII² to reduce an unwanted immune response and induce immunological tolerance towards rhGAA.

The interaction of rhGAA with PS containing liposomes was first investigated. Based on previous experience, the context of co-displaying the protein of interest in the presence of

the PS is of high importance. The signals derived from the interaction of PS with PS receptors, such as TIM-4, tip the balance from immunity towards tolerance²⁶. If a protein is processed by antigen presenting cells without the signals of PS then an immune response will continue as observed for free protein. This is supported by previous experiments, where we have shown that the immune response is maintained to an unrelated antigen administered at site distal to the administration of PS-FVIII²⁷. If PS and rhGAA are delivered as a complex, there is a higher likelihood that the desired tolerogenic immune response will occur. Therefore, it is important to study the interaction of the PS liposome with rhGAA. As part of its mechanism of action in the blood coagulation cascade FVIII has been demonstrated to bind to PS present on membrane surface of activated platelets via interactions in the C2 domain of the protein²⁸. There are no similar mechanism related reasons to believe that rhGAA would specifically bind to PS. In order for PS to be utilized as a more broadly applicable therapy for therapeutic proteins other than FVIII, interactions between PS and the protein of interest, in this case rhGAA, would need to occur in a manner that does not rely upon receptor-ligand interactions.

The results from the biophysical studies in Figure 1 demonstrate an interaction between PS and rhGAA. The data from these studies, as well as the tendency for two hydrophobic moieties to interact in an aqueous milieu, suggests that it is likely that the catalytic binding pocket of rhGAA is the portion of the protein that is intercalating into the hydrophobic bilayer of the PS liposomes. Further, one of the predicted immunodominant T-cell epitopes, residues 341-352, lies at the border of the catalytic β/α barrel domain which spans residues 347-732 of GAA²⁹. If the proposed interaction is occurring, this immunodominant epitope could now be either shielded or sterically hindered by the PS liposomes from being accessed by immune cells. This sequestration of the epitope could be contributing to the reduction of anti-rhGAA antibody titers, possibly contributing to the reduction in immune response due to immunological ignorance.

The immunogenicity studies showed that a statistically significant difference is observed when comparing the relative immunogenicity of PS-rhGAA to the control treatments after the animals had been treated for four weeks. However, as the experiment was extended to include a rechallenge it was shown that the lower anti-rhGAA antibody response for animals pre-exposed to PS-rhGAA remained statistically lower, but this was not observed for the other treatments (Figure 2C). This occurs despite that fact that treatment with PS had been halted at week 4 and mice were administered a series of re-challenges with free protein. In addition, since a similar reduction was observed when mice were administered OPLS-rhGAA which lacks vesicular structure, these data together suggest that the PS-mediated reduction of immunogenicity goes beyond a mere shielding of the epitopes involved. The observations revealed that administering rhGAA in the context of the PS liposomes or OPLS solution is not only less immunogenic, but is able to induce a hypo-responsiveness, as the re-challenge of rhGAA to PS-rhGAA or OPLS-rhGAA (together) pre-treated groups did not elicit significant immune response. Further, a majority of animals treated with PS-rhGAA (Figure 2D) or OPLS-rhGAA (together) (Figure 3C) did not show any increase in titer levels when rechallenged with free rhGAA and thus, can be considered non-responders. These results mirror the results seen when hemophilia A mice are given FVIII with PS liposomes or OPLS^{2,30}.

PS is a naturally occurring phospholipid that is present in the inner leaflet of healthy cells. However, during apoptosis PS is flipped to outer leaflet. It has been established by several investigators that during efferocytosis (phagocytic uptake of apoptotic cells) externalization of PS in apoptotic cells provides “find me”, “eat me” and “ignore me” signals to maintain central tolerance³¹. However, our efforts to reduce immunogenicity of replacement therapies (such as Factor VIII for Hemophilia A), where there is lack of central tolerance, has taught us that apoptosis is not a “silent process” that down regulates immune response but rather an active process in which “teaching and learning” about the antigen occur. Hemophilia A mice pre exposed to PS-FVIII showed hypo-responsiveness upon rechallenge with free FVIII which was not observed for FVIII-dexamethasone or other lipid treatment groups². It is interesting to note that the animals “remember” the antigen even after the PS therapy was stopped, suggesting that during exposure of the antigen in the presence of PS, then immune system learns to tolerate the antigen. This observation led to our conclusion that the teaching is due to a novel molecular property of PS that it converts an immunogen to a tolerogen. To our knowledge, this molecular property of PS has not been identified previously. We hypothesize that only this property of PS could explain previous observations such as the tolerogenic potential of apoptotic cells (which expose PS in their outer leaflet) whereas the “ignore me” signal (to maintain central tolerance) cannot. If the later mechanism is operative, it would have led to immunological ignorance where the animals would have responded normally to re-challenge as there is no “active learning” occurred during antigen exposure in the presence of PS. For example, that administration of a foreign antigen loaded in apoptotic cells induced tolerance to ovalbumin³² and defective apoptotic pathways led to autoimmune conditions³³. This is further supported by our observation that administration of an antigen in the presence of PS increased the production of regulatory T-cells that are likely to be antigen specific². This novel property could shed light on how pathogens and parasites evade immune surveillance of host for their survival and infection³¹⁻³³. Our approach is to harness this molecular property for clinical applications such as to reduce the immunogenicity of therapeutic proteins.

It has been postulated in literature that PS interactions with its receptors are able to mediate immune suppressive functions³⁴. There are several direct and indirect receptors that have been discussed in literature which recognize and interact with PS including stabilin-2 and members of the T-cell/transmembrane, immunoglobulin and mucin (TIM) family of receptors. The TIM family of receptors, especially TIM-4, specifically recognize PS and have been indicated to be involved in maintaining tolerance²⁶. The structural analysis of the TIM4/PS complex showed a binding pocket for phospho-serine. The studies carried out with OPLS, the head group of PS, demonstrate that phospho-serine is one of the key structural requirements for the PS mediated effects. Further, the OPLS immunogenicity study began to evaluate the necessity of rhGAA to be complexed with the PS moiety and the spatial and temporal requirements of dendritic cells encountering the two components. This study begins to illustrate that when the two components, rhGAA and OPLS, are not given as a complex mixed in the same solution and delivered at the same moment in time, that there is a decreased likelihood that DCs encounter both components at the same moment and an increased likelihood that the DCs will encounter rhGAA alone, responding as if the drug was free. This is shown by a higher mean anti-rhGAA antibody titer level for animals treated

with OPLS-rhGAA sequentially. Separating the injections further, both spatially and temporally, would help to define this scenario more completely.

While the data herein support induction of hypo-responsiveness by PS for rhGAA, in order to demonstrate that PS liposomes impart tolerogenicity, studies to confirm antigen specificity are required and will be carried out in the future. It was shown here that when BMDCs are exposed to PS-GAA or a tolerogenic DC control, Dex-D3, TGF- β secretion is increased (Table I). The secretion of TGF- β by DCs has been shown to be a key initiator of tolerogenic responses³⁵. This data corroborates previous mechanistic studies carried out with PS and FVIII, suggesting that PS possesses the immune regulatory mechanism for the induction of tolerance. The mechanism involves PS mediated conversion of DCs from immunogenic to tolerogenic phenotype with TGF-beta secretion. In addition to the study carried out here, more mechanistic studies are needed to confirm that PS-mediated tolerance induction is functioning in an antigen specific manner, which is independent of the antigen that is co-administered, to induce tolerance. To accomplish this, future studies will be carried out for rhGAA as they were for FVII to further demonstrate the roles of TGF- β , regulatory T cells, and memory B cells in vivo, and to compare the results to what was previously observed for FVIII³⁶.

The immune regulatory properties of PS can be exploited to induce tolerance with a low, sub-therapeutic dose of rhGAA via the subcutaneous route. Noticeably, this approach deviates from the clinical dosage regimen given to Pompe disease patients who receive 20 mg/kg biweekly by intravenous infusion. The subcutaneous route was chosen for this tolerizing regimen as it specifically targets the resident and migratory dendritic cells within the subcutaneous space³⁷ which are crucial in tipping the balance from immunity towards tolerance. This certain cell type would not be encountered if the PS-rhGAA formulation was given via intravenous injection. Therefore, an ideal therapy here would consist giving the tolerizing PS-rhGAA pretreatment regimen as described here either just prior to, or in conjunction with conventional IV dosing of rhGAA a dose proven to be clinically efficacious. Future studies will be aimed at determining the feasibility of these alternate dosing schemes, with the joint goal of mitigating immune responses as well as being an efficacious therapy.

CONCLUSIONS

Collectively, the results of these experiments illustrate that PS converts an immunogen to a tolerogen which could be clinically exploited as a promising treatment option to reduce immunogenicity and induce tolerance towards therapeutic proteins. The efforts here highlight the use of PS to convert an immunogen into a tolerogen by mimicking a natural process to induce peripheral tolerance. Further investigation is certainly necessary to realize the full potential of this approach and to translate it into the clinic.

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FIGURE 1.

A) Fluorescent spectra to examine the tertiary structure of rhGAA in the presence and absence of PS liposomes. The solid black line represents rhGAA and the dashed gray line represents PS-rhGAA. B) Acrylamide quenching to examine location of rhGAA within the PS liposome bilayer. The solid black line represents rhGAA and the dashed gray line represents PS-rhGAA

FIGURE 2.

Immunogenicity and tolerance induction study with PS-rhGAA. (A) Immunization schedule where an arrow indicates an injection and an asterisk (*) indicates blood sample. (B) Anti-rhGAA antibody (mean $\pm$ SEM) titer response in animals that received 4 weekly injections of pre-treatment with formulation: free rhGAA (circles), PS-rhGAA (squares), or PG-rhGAA (triangles). (C) Anti-rhGAA antibody titer response after re-challenge with free rhGAA. (D) Comparison of anti-rhGAA antibody titer in individual animals before and after re-challenge with free rhGAA.

FIGURE 3.

Immunogenicity and tolerance induction study with OPLS-rhGAA given together and sequentially. (A) Anti-rhGAA antibody titer response (mean $\pm$ SEM) in animals that received 4 weekly injections of pre-treatment with formulation: free rhGAA (circles), OPLS-rhGAA together (triangles), or OPLS-rhGAA sequential (diamonds). (B) Anti-rhGAA antibody titer response after re-challenges with free rhGAA. (C) Comparison of anti-rhGAA antibody titer in individual animals before and after re-challenge with free rhGAA.

Table 1TGF- β levels for *in vitro* treated BMDC

Treatment Group	Mean TGF- β Level (pg/mL) $\pm$ SEM
rhGAA	724 $\pm$ 310
PS-rhGAA	1283 $\pm$ 130
PG30-rhGAA	915 $\pm$ 113
DEX-D3	1302 $\pm$ 258