

# Pretreatment of CD138- B Lymphocytes with a Covalent Reactive Antigen Analog to FVIII Reduces Antibody Production in Response to FVIII

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## ABSTRACT

B lymphocytes producing high titer antibodies to factor VIII (FVIII) are of major concern in the treatment of bleeding episodes in a subpopulation of patients with hemophilia A. We hypothesize that an electrophilic, covalent reactive analog of FVIII (FVIII-CRA) can induce antigen-specific tolerance by permanent engagement of B cell receptors. FVIII-CRA was prepared by derivitizing lysine side chains with phosphonate diester groups. These groups are predicted to bind enzyme-like nucleophilic residues located within the BCR. To test the effects of FVIII-CRA binding on B lymphocytes, we isolated CD138- splenocytes from FVIII-deficient mice (B6:129S4-F8tm1Kaz/J) immunized with FVIII. These putative memory B lymphocytes were then re-stimulated for 6 days with identical doses of FVIII or FVIII-CRA. The number of antibody secreting cells (ASC) produced in response to FVIII-CRA re-stimulation was significantly lower than FVIII re-stimulation (avg of 36 ASC vs 111 ASC,  $p = 0.03$ ). In addition, cell viability following low dose FVIII-CRA treatment was decreased by ~ 22% when compared with identical doses of FVIII. These results suggested that FVIII-CRA may tolerate memory B lymphocytes. To confirm this, we pretreated CD138- splenocytes with FVIII-CRA and then added a stimulatory dose of FVIII to the cultures. Pretreatment with FVIII-CRA resulted in a significant decrease in the number of ASC produced in response to FVIII (by 17-fold compared to control pretreatment). These results suggest that FVIII-CRA may represent a viable means to induce antigen-specific B cell tolerance in hemophilia.

## INTRODUCTION

The main therapy for control of bleeding in hemophilia A patients with FVIII deficiency is infusion of recombinant FVIII. About 20-30% of these patients produce antibodies (Abs) to the infused FVIII, and the FVIII replacement therapy is ineffective in patients with high titer Abs (>10 Bethesda units/ml) to the protein. Tolerance to FVIII can be induced in a majority but not all patients with anti-FVIII Abs. The procedure involves infusion of massive amounts of FVIII over months and even years (Reviewed in 1). Tolerance is evident by complete disappearance of the Abs as measured by the Bethesda assay and normalization of the FVIII half-life. The procedure fails in ~30% of patients potentially because of limitations in the amount of FVIII that can realistically be administered. Immune suppression with medications such as prednisone and cyclophosphamide can be attempted in combination with FVIII therapy but these agents are not very effective in suppressing the Ab levels and may pose the risk of infection and malignancy. The cost of immune tolerance induction is a major problem, as this can run to a million dollars or more per patient.

As exposure to large FVIII concentrations is essential for successful tolerance induction, it may be hypothesized that BCR saturation is a critical tolerogenic signal for B cells. (Figure 1; 2-5). Therefore, we hypothesize that we can induce antigen-specific tolerance in hemophilia A patients through use of a covalent reactive analog of FVIII (FVIII-CRA). The rationale for our hypothesis derives from findings that the combining sites of all Abs examined thus far contain an activated nucleophilic residue that can react covalently with electrophilic phosphonate diesters originally developed as probes for serine proteases (6). Importantly, the covalent reaction of Abs with CRAs is guided by noncovalent recognition of antigenic epitopes, ensuring specificity (6; Fig 2B). We have previously observed that hapten CRAs are bound irreversibly to B cell receptors (BCRs; 7). This suggests that FVIII specific BCRs on memory B cells can be targeted for covalent binding by CRAs. Herein, we report our preliminary findings that FVIII-CRA may have a tolerogenic effect on memory B cells.

## MATERIALS AND METHODS

**FVIII.** Recombinant FVIII (Helixate, Bayer) was dialyzed into a solution of 10mM HEPES, 150 mM NaCl, and 0.025% Tween 20. FVIII was stored in small aliquots and stored at -80 C until use.

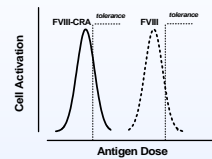
**FVIII-CRA.** Recombinant FVIII was derivitized at Lys residues using a phosphonate diester precursor with an amine-reactive linker (1). Unincorporated precursors were removed by gel filtration and phosphonate incorporation was measured based on consumption of free amines (60-80 mol phosphonate per mole FVIII; 158 Lys residues).

**Mouse immunizations.** FVIII-deficient mice (B6:129S4-F8tm1Kaz/J; Jackson Laboratories) were i.v. immunized with 2 µg/mouse of FVIII every 7 days for 4-5 weeks until serum anti-FVIII titer at 1:500 dilution as determined by ELISA was > than 0.2 O.D. 490 units above background. Mice were sacrificed 2-6 weeks later.

**Isolation and culture of CD138- splenocytes.** We isolated single cell suspensions of splenocytes from FVIII-immunized mice and removed red blood cells by water lysis. Based on a previously published protocol (8), cells were treated with biotinylated anti-CD138 followed by anti-biotin microbeads. The labeled CD138- cells were removed from the cell suspension by placing them over an LD column in a MidiMACS separator. Depletion of CD138+ cells was confirmed by flow cytometry staining (< 1% positive cells). The recovered CD138- splenocytes were then cultured at 1 x 10<sup>6</sup> splenocytes/ml in supplemented DMEM in the presence of FVIII, FVIII-CRA, or control OVA-CRA for 5 days. After 5 days, cells were harvested from flasks and viable splenocytes were determined by trypan blue dye exclusion. 40-70% of the cells recovered were viable.

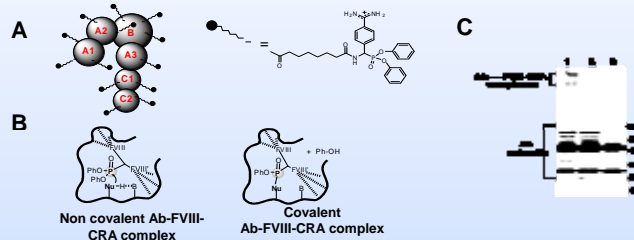
**FVIII-specific ELISPOT.** In a modification of a previous protocol (9), 96 well nitrocellulose-bottomed plates (Fisher MAIP N45 50) were coated with 20 µg/ml FVIII in PBS. Plates were incubated overnight at 4 C, blocked with 10% FCS in RPMI, then CD138- splenocytes (0.5-2.0 x 10<sup>6</sup>) were added to the wells. Plates were incubated overnight at 37 C, washed, and secreted antibody was detected by addition of 2 µg/ml biotinylated goat anti-mouse heavy chain Ab followed by streptavidin-HRP. Spots were visualized by the addition of 3-amino-9-ethylcarbazole (AEC). Plates were allowed to dry, and spots were counted using a Leica dissecting microscope.

## RESULTS

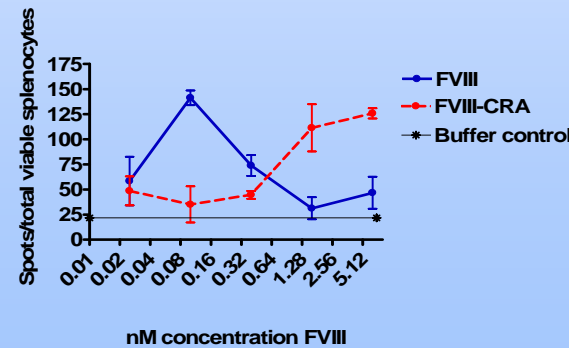


**Fig. 1.** Potential for induction of B cell tolerance by treatment with FVIII-CRA

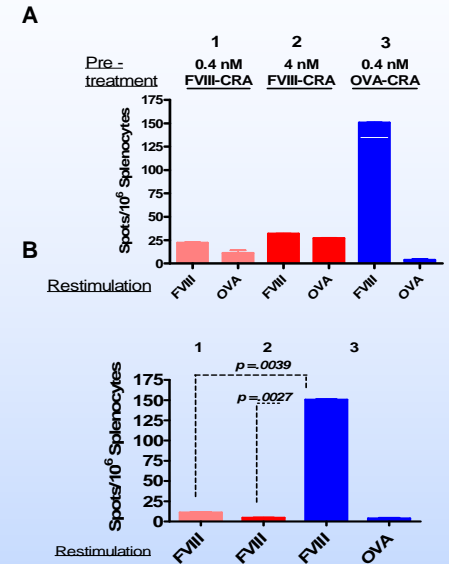
Noncovalent BCR saturation with excess conventional antigens is well known to induce B cell clonal silencing (5-8). However, conventional antigens dissociate from BCRs because the noncovalent binding is a reversible process. In contrast, the strong covalent bonding activity of CRAs precludes their dissociation, resulting in more facile saturation of BCRs, and eventually driving the B cells into the anergic and apoptotic pathways. Therefore, the use of FVIII-CRA may represent a powerful method for inducing B cell tolerance in patients with anti-FVIII inhibitor antibodies.



**Fig 2.** Structure and hypothetical reaction of FVIII-CRA BCR, and demonstration of covalent binding to soluble Ig. **A.** FVIII-CRA structure. The electrophilic phosphonate diesters are located on Lys side chains of the protein. Multiple phosphonates can be placed within a single protein molecule allowing presentation of the electrophile in conjunction with several antigenic epitopes. **B.** Hypothetical representation of irreversible Ab reactions with FVIII-CRA. The Ab forms the initial noncovalent complex by conventional epitope-paratope interactions. Activated by a general base (-B), the nucleophile (Nu-H) attacks the phosphonate group located within the antigenic epitope, forming a stable covalent phosphonyl-Ab adduct. B, general base; B-H, general acid; NuH, nucleophile. **C.** Irreversible FVIII-CRA binding to anti-FVIII mAb. Immunoblots of denaturing SDS-gels showing anti-Ig stained high molecular weight complex of FVIII-CRA with a monoclonal anti-FVIII Ab (clone ESH8; lane 1). Lane 2 shows the control FVIII devoid of phosphonates incubated with the anti-FVIII Ab, and lane 3, the FVIII-CRA incubated with an irrelevant, isotype-matched Ab (IgG clone c23.5). Reaction conditions, IgG 0.7 µM; FVIII-CRA or FVIII 0.2 µM; 2 h, 37°C. Reaction mixtures boiled (5 min) in 2% SDS prior to electrophoresis. Anti-Ig stained bands at 50kD and 25kD are the heavy and light chains, respectively. Bands at 70 and 80 kD represent anomalously migrating Ab subunits.



**Fig 3.** Lower doses of FVIII-CRA do not stimulate anti-FVIII producing CD138- cells. CD138- splenocytes were isolated as described and cultured for 5 days in the presence of the indicated concentrations of FVIII or FVIII-CRA. Splenocytes isolated from 6 day culture in media alone were used as a control for baseline production of FVIII-specific antibody secreting cells (ASC). The number of ASC produced is calculated per total number of viable splenocytes recovered from the restimulation culture in order to account for death of splenocytes under different culture conditions. Results are representative of four separate experiments.



**Fig 4.** Pre-treatment of CD138- splenocytes with FVIII-CRA prevents restimulation of FVIII-specific ASC. **A.** CD138- splenocytes were pre-treated for 6 hours with 0.4 nM FVIII-CRA (Group 1), 4 nM FVIII (Group 2) or control 4 nM OVA-CRA (Group 3). After 6 hours, a restimulation dose of 4 nM FVIII or OVA was added to the cultures as indicated. Cells were cultured for a total of 5 days, then harvested and used in a FVIII-specific ELISA. Results are expressed as number of spots produced/10<sup>6</sup> splenocytes. **B.** Results are represented with the baseline response to the pre-treatment FVIII-CRA (with 4 nM OVA restimulation) subtracted. These values are compared to the ASC produced in response to FVIII restimulation alone (blue bar). Statistical differences were determined by use of an unpaired t test with Welch's correction. Results are representative of 3 experiments.

## CONCLUSIONS

- Derivatization of FVIII to include the CRA moiety may reduce its antigenicity. Thus, loss of non-covalent binding to the BCR may impair the overall efficiency of the covalent reaction, with the result that stimulation of antibody synthesis by the B cells requires FVIII-CRA concentrations greater than control FVIII.

- Pre-treatment of CD138- splenocytes with low concentrations of FVIII-CRA reduced their response to restimulation with FVIII. These results suggest that covalent binding by FVIII-CRA may prove to be a viable, antigen-specific method for suppressing Ab synthesis by memory B cells. Interestingly, FVIII-CRA possesses its tolerance-inducing property despite loss of antigenic potency, suggesting that distinct epitopes are responsible for tolerogenicity and antigenicity.

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