B lymphocytes producing high titer antibodies to factor VIII (FVIII) are of major concern in the treatment of bleeding episodess in a population of patients with hemophilia A. We hypothesized that an electrostatic, covalent reactive analog of FVIII (FVIII-CRA) can induce antigen-specific tolerance by permanent engagement of B cell FVIII receptors. FVIII-CRA was prepared by derivatizing lysine side chains with phosphonic ester 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− B lymphocytes from FVIII-deficient mice (B6;129F1-Hnsk/J) with FVIII. These freshly isolated B lymphocytes were then restimulated for 6 days with identical doses of FVIII-CRA. The number of antibody secreting cells (ASCs) produced in response to FVIII-CRA was stimulated significantly greater than FVIII alone (Fig. 1A, n = 3). The total number of ASCs 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− B lymphocytes with FVIII-CRA and then added a stimulatory dose of FVIII to the culture. Pretreatment with FVIII-CRA significantly decreased the number of ASCs in response to FVIII. These results suggest that FVIII-CRA may represent a viable method to induce an antigen-specific B cell tolerance in hemophilia.

**MATERIALS AND METHODS**

**FVIII.** Recombinant FVIII (Belgium, Behnken) was dialyzed into a solution of 100mM HEPES, 150 mM NaCl, and 40 mM Tris-PO4. FVIII was stored in small aliquots and stored at -80°C until use.

**FVIII-CRA.** Recombinant FVIII was derivatized at lysine residues using a phosphonic diester precursor with an unsubstituted hapten (1). Unreacted phosphonate groups were removed by gel filtration and phosphonic incorporation was measured based on consumption of free amino (0.80 ± 0.01 phosphonate per moe FVIII; 151 lys residues).

**Mouse immunizations.** FVIII-deficient mice (B6;129F1-Hnsk/J, Jackson Laboratories) were i.c. immunized with 2 µg/mouse of FVIII every 4–5 weeks until serum anti-FVIII titers at 1:50 dilution as determined by ELISA was = 2–40.0.2.0.4.0 above background. Mice were sacrificed 2–6 weeks later.

**Isolation and culture of CD138− plasma cells.** We isolated single cell suspensions of plasma cells from FVIII-immunized mice and removed red blood cells by water lysis. Based on a previously described protocol, (9) cells were mixed with biotinylated anti-CD38 followed by anti-biotin microbeads. The labeled CD38− cells were removed from the cell suspension by passing them over an anti-CD38 column in a MacMacs separation. Depletion of CD38− cells was confirmed by flow cytometric analysis (1% positive cells). The recovered CD38+ cells were then cultured at 1 x 10⁶ cells/ml in supplemented IMDM in the presence of FVIII, FVIII-CRA, or control OVA-CRA for 6 days. After 3 days, the cells were harvested from flasks and viable plasma cells were determined by trypan blue dye exclusion. 40–50% of the cells were mononuclear viable.

**FVIII/CRA Injection.** In a modification of a previous protocol (9), 9–11 intracardiac-peritoneal (IP) injections were given to each mouse (Fisher MAP/SF 200). Complement C5−/− mice were protected. Plates were incubated overnight at 4°C, washed, and azide antibody was detected with a 1:200 dilution of an anti-mouse IgG (antibodies to human anti-human anti-mercapturic acid heavy chains) antibody followed by streptavidin-AP. Spots were visualized by the addition of 3-aminonaphtaldehyde (Acridine). Plates were allowed to dry, and spots were counted using a Leica dissecting microscope.

**REFERENCES**


**RESULTS**

1. **Antigen production of CD138− B lymphocytes**

- Pretreatment of CD138− B lymphocytes with a Covalent Reactive Antigen Analog to FVIII Reduces Antigen Production in Response to FVIII

**ABSTRACT**

A FVIII-CRA, an analog of FVIII (FVIII-CRA) can induce antigen-specific tolerance by permanent engagement of B cell BCRs. To test the effects of FVIII-CRA binding on B lymphocytes, we isolated CD138− B lymphocytes from FVIII-deficient mice with FVIII. These freshly isolated B lymphocytes were then restimulated for 6 days with identical doses of FVIII-CRA. The number of antibody secreting cells (ASCs) produced in response to FVIII-CRA was stimulated significantly greater than FVIII alone (Fig. 1A, n = 3). The total number of ASCs 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− B lymphocytes with FVIII-CRA and then added a stimulatory dose of FVIII to the culture. Pretreatment with FVIII-CRA significantly decreased the number of ASCs in response to FVIII. These results suggest that FVIII-CRA may represent a viable method to induce antigen-specific B cell tolerance in hemophilia.

**CONCLUSIONS**

- Pretreatment of CD138− B lymphocytes with low concentrations of FVIII-CRA reduced their response to stimulation 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 the ability to induce tolerance in mouse models of FVIII and may be an effective method to induce antigen-specific tolerance in hemophilia.

**REFERENCES**