Title: Effect of B-cell depletion using anti-CD20 therapy on inhibitory antibody formation to human FVIII in hemophilia A mice

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Running title: B-cell depletion promotes tolerance to FVIII
Abstract

We herein tested the effect of B-cell depletion on tolerance induction to FVIII in a mouse model of hemophilia A. Two subclasses of anti-mouse CD20 monoclonal antibodies with differential depletion effects were used. Thus, IgG1 anti-CD20 selectively depleted follicular B cells and spared marginal zone B cells, while IgG2a anti-CD20 efficiently depleted both. In FVIII primed mice, a single dose of either IgG1 or IgG2a anti-CD20 pretreatment prevented the increase in inhibitor formation in the majority of treated mice by subsequent daily, high dose FVIII i.v. injection as a model for immune tolerance induction (ITI). However, the IgG1 but not the IgG2a anti-CD20 pretreatment led to a significant increase of regulatory T cells in the spleen. Importantly, three months after the partial B-cell depletion with IgG1 anti-CD20, the FVIII-specific hypo-responsive state remained. We suggest a tolerogenic role of the remaining marginal zone B cells as a potential mechanism for anti-CD20 therapy.
Introduction

FVIII replacement therapy is used in hemophilia A patients for treatment of bleeding episodes or for prophylaxis. However, up to 1/3 of the patients develop anti-FVIII inhibitory antibodies (inhibitors), which renders this mode of therapy itself ineffective.1 Hemophilia A patients who recently developed inhibitors (<10 BU) usually undergo immune tolerance induction (ITI) therapy, which requires regular (usually daily) high dose FVIII infusion for months to years. In many patients, inhibitors can eventually be eradicated by ITI therapy with the establishment of long-term tolerance to FVIII. Though ITI has been practiced in the clinic for decades, the mechanism of its action remains largely unknown, nor is there any animal model for this approach. Furthermore, 20-40% of patients still fail the therapy, which inevitably increases their morbidity and mortality.2 Recently, B-cell depletion using rituximab, a mouse/human chimeric anti-CD20 monoclonal antibody 3, has emerged as effective in eliminating inhibitor(s) in some hemophilia A patients who failed ITI.4, 5 However, the evaluation of anti-CD20 therapy often is complicated in the clinical setting by concomitant use of other immune modulating drugs, such as hydrocortisone and IVIG.4 Therefore, it is still not known whether B-cell depletion actually facilitated tolerance induction to FVIII or complemented
immunosuppressive therapies. In this study, we tested whether anti-CD20 therapy \textit{per se} could lead to tolerance after high dose FVIII treatment.
Methods

Animals and reagents

FVIII−/− mice (E16) on C57BL/6 background were maintained from the colony of Dr. Leon Hoyer at the American Red Cross.6, 7 FoxP3-GFP/FVIII−/− mice were generated by crossing FoxP3-GFP knock-in mice8 against E16 mice as described.9 All animals were housed and bred in pathogen-free micro-isolator cages at the animal facilities operated by the University of Maryland School of Medicine, and animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Maryland School of Medicine.

For B cell depletion, mouse IgG1 anti-CD20 mAb,10, 11 IgG2a anti-CD20 mAb,12 and the isotype control mouse IgG1 and mouse IgG2a were as previously described. All these mAbs were kind gifts from Dr. Marilyn Kehry (Biogen Idec, San Diego, CA). Highly purified recombinant human FVIII was kindly provided by Dr. Birgit Reipert (Baxter Bioscience AG).

Immunologic assays

FACS analysis for B cell phenotype and the induction of Tregs were performed using a LSR-II (BD Biosciences), and data analyzed using FlowJo software (Tree Star, Inc.). ELISA and Bethesda assays for measuring
anti-FVIII IgG titer and for the FVIII inhibitor titer, respectively, were performed as previously described.\textsuperscript{13, 14}

Statistics

Student’s \textit{t} test or non-parametric Mann-Whitney U test were used where it is appropriate to evaluate the significance of results. A \textit{p} value less than 0.05 was considered as significant.
Results and Discussion

The extent of B-cell depletion by anti-CD20 varies according to the target antigen (human versus mouse CD20), the tissues examined and among different mouse genetic backgrounds.\textsuperscript{15} To test the efficacy of B-cell depletion in E16 mice (C57BL/6 background), we examined the number and phenotype of splenic B cells two weeks after \textit{i.v.} injection of either IgG1 or IgG2a anti-mouse CD20 monoclonal antibodies. As shown in figure 1, IgG2a anti-CD20 efficiently depleted 98\% of the splenic B cells, including both marginal zone (MZ, CD19\textsuperscript{+}CD23\textsuperscript{int}CD21\textsuperscript{hi}) and follicular (FO, CD19\textsuperscript{+}CD23\textsuperscript{hi}CD21\textsuperscript{low}) B cells, as compared with the mice that received control IgG. However, B-cell depletion using IgG1 anti-CD20 was less complete. While 95\% of FO B cells were depleted, MZ B cells were largely spared and comprised about 39\% of the residual splenic B cells (Fig. 1B and C). The reason MZ B cells were spared by IgG1 anti-CD20 is presumably due to the inability of this mouse IgG subclass to activate complement, since complement C3 has been shown to be absolutely required for depletion of MZ B cells using anti-CD20 antibodies.\textsuperscript{15} It is of note that the Fc region in the chimeric Rituximab originated from human IgG1, which can fix complement.\textsuperscript{3} However, effect of Rituximab on splenic MZ B cell subpopulation in hemophilia A patients has not been reported.
We next tested the effect of B-cell depletion using IgG1 anti-CD20 on inhibitor formation and its potential for tolerance induction to FVIII in FVIII primed E16 mice. As outlined in figure 2A, we first primed the E16 mice (n=51) i.v. with four weekly therapeutic doses (0.2µg) of FVIII to more closely mimic the situation in hemophilia A patients. As shown in Supplemental Figure 1A, over 80% of the mice developed inhibitor titers up to 150 BU (mean = 30.7± 4.8 BU). Groups of mice were then treated with a single dose of either IgG1 anti-CD20 or isotype control IgG1, intravenously. Compared with the control IgG, B-cell depletion using IgG1 anti-CD20 alone did not significantly decrease the inhibitor titer in the mice (supplemental Fig. 1B). This is not surprising since CD20 is specifically expressed on the cell surface of pre-B to mature B cells, including memory B cells, but not on plasma cells.

To test whether B-cell depletion could facilitate tolerance induction to FVIII, we initiated daily, “high” dose FVIII i.v. injections in an attempt to mimic the clinical ITI procedure in hemophilia A patients. The E16 mice in this experiment were given daily i.v. injections with a moderately high dose of FVIII (10 IU) for 19 consecutive days (Fig. 2A). This modified ITI protocol is equivalent to 500 IU/kg body weight. Despite this high dose, the majority of control mice given control IgG responded with increased titers of
inhibitors (Fig. 2B, left panel) and total IgG anti-FVIII (data not shown). This does not necessarily define failure of ITI, since the mice were only exposed to high dose FVIII for 19 days and this may not be long enough for achieving tolerance to FVIII. In addition, a temporary marked increase of inhibitor titer after initiation of ITI is also often seen in hemophilia A patients.17

In contrast, the dramatic increase in inhibitor titer seen in the control group was largely prevented by the pretreatment of a single dose of IgG1 anti-CD20, which depleted most of FO B cells and spared MZ B cells (Fig. 2B, right panel). A similar result was also seen when the experiment was independently repeated with the more complete B-cell depletion using IgG2a anti-CD20 (Fig. 2D). It is known that inhibitor formation is T cell dependent in both humans and mice,6,18-20 and it has been reported that B cells are required for optimal CD4 T cell function.21 Thus, the elimination of the majority of B cells may be responsible for the lack of boosting.

To test if the remaining MZ B cells after the IgG1 anti-CD20 pretreatment are able to promote tolerance to FVIII, we re-challenged treated mice with FVIII three months after the initiation of B cell depletion using IgG1 anti-CD20, when the number of peripheral B cells had recovered 60 % or more. After FVIII challenge, the inhibitor titers remained significantly lower in the
IgG1 anti-CD20 group (Fig. 2C). Importantly, after the mice were subcutaneously challenged with an unrelated antigen, OVA, there was no significant difference in anti-OVA antibody response between the two groups (data not shown).

It has been shown that MZ B cells are necessary for the systemic tolerance phenotype induced through an immune privileged site such as the eye.\textsuperscript{22} We speculated that MZ B cells might be also a previously unappreciated candidate as tolerogenic antigen-presenting cells for FVIII during ITI therapy. The location of MZ B cells would make them easily accessible to immune complexes derived from the blood. In addition, MZ B cells also express higher levels of B7.1 and B7.2 than FO B cells do, thus facilitating effective antigen presentation to CTLA-4\textsuperscript{+} regulatory T cells.\textsuperscript{23, 24} Through FACS analysis, we found that the B7 co-stimulatory molecules were further upregulated upon IgG1 anti-CD20 treatment + high dose FVIII exposure. In contrast, control IgG + FVIII treatment had no significant effect on this phenotype of MZ B cells, compared to non-treated naïve mice (Fig. 3).

To directly compare IgG1 versus IgG2a anti-CD20 in their effects on inhibitor formation and Tregs induction, we performed an additional experiment using the FoxP3-GFP/FVIII\textsuperscript{−/−} mice, in which the CD4\textsuperscript{+}/FoxP3\textsuperscript{+} Tregs can be easily tracked by GFP expression. Considering the dramatic
boosting and lack of tolerance to FVIII in the control IgG treated mice in the previous experiments, we further modified the ITI protocol by using a more intensive albeit less lengthy procedure. Thus, groups of FVIII primed mice were pretreated with either IgG1 or IgG2a anti-CD20, followed by *i.v.* injection with 2µg FVIII twice daily (instead of daily) for 5 consecutive days. As shown in Table 1, the total number of Tregs significantly increased in the spleen from the mice with IgG1 anti-CD20 pretreatment compared to naïve mice, but the number of Tregs with IgG2a anti-CD20 treatment was not significantly changed. Again, both IgG1 and IgG2a anti-CD20 pretreatment largely prevented the increase of inhibitor formation in majority of the mice (16/20 with BU < 5) following the intensive FVIII exposure (Fig. 4). Under this shortened protocol, however, one can not conclude whether tolerance has been induced. Nonetheless, caution needs to be exercised using reagents for complete B-cell depletion in treating hemophilia A patients with inhibitors.

ITI therapy in hemophilia A patients is not only extremely expensive, but also practically very challenging. It requires regular (usually daily) administration of high dose FVIII for a minimum 9 months and up to 33 months.² Our results herein support the notion that an IgG1 subclass anti-mouse CD20 monoclonal antibody, that selectively depleted FO B cells
while sparing MZ B cells, can facilitate tolerance to FVIII during our mouse model of ITI. Thus, protocols to achieve selective partial B-cell depletion by anti-CD20 in this animal model may provide insight into future tolerogenic therapies for hemophilia A patients with inhibitors.
Acknowledgments

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Authorship

Contribution: A.H.Z. designed the research, analyzed data, and wrote the paper; J.S. designed the research and analyzed data; and D.W.S. designed the research, analyzed data and wrote the paper.
References


23. Oliver AM, Martin F, Kearney JF. IgMhighCD21high lymphocytes enriched in the splenic marginal zone generate effector cells more rapidly than the bulk of follicular B cells. *J Immunol.* 1999;162(12):7198-7207.

Table 1. The effect of IgG1 versus IgG2a anti-CD20 pretreatment on the induction of Tregs in FoxP3-GFP/FVIII<sup>-/-</sup> mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total splenocytes (&lt;x10^6&gt;)</th>
<th>Ratio of CD4&lt;sup&gt;+&lt;/sup&gt; T cells (%)</th>
<th>Ratio of Tregs (% of CD4&lt;sup&gt;+&lt;/sup&gt; T cells)</th>
<th>Total Tregs in the spleen (&lt;x10^5&gt;)</th>
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<tbody>
<tr>
<td>Non-treated naïve mice</td>
<td>38.9 ± 6.2</td>
<td>19.4 ± 2.0</td>
<td>12.4 ± 0.4</td>
<td>8.4 ± 0.7</td>
</tr>
<tr>
<td>IgG1 anti-CD20 + FVIII</td>
<td>27.7 ± 2.8&lt;sup&gt;*&lt;/sup&gt;</td>
<td>31.4 ± 1.3&lt;sup&gt;*&lt;/sup&gt;</td>
<td>13.0 ± 0.3&lt;sup&gt;n.s.&lt;/sup&gt;</td>
<td>11.1 ± 1.0&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>IgG2a anti-CD20 + FVIII</td>
<td>22.7 ± 2.2&lt;sup&gt;*&lt;/sup&gt;</td>
<td>37.2 ± 2.7&lt;sup&gt;*&lt;/sup&gt;</td>
<td>12.5 ± 0.8&lt;sup&gt;n.s.&lt;/sup&gt;</td>
<td>10.1 ± 0.8&lt;sup&gt;n.s.&lt;/sup&gt;</td>
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<sup>*</sup>, p< 0.05 and <sup>n.s.</sup>, not significant, compared to non-treated naïve mice (student’s t test).
Figure legends

Figure 1. The differential effect of B-cell depletion by two subclasses mouse anti-mouse CD20 monoclonal antibodies. E16 mice (n=3 or 4) were i.v. injected with 250 µg of either IgG1 or IgG2a anti-CD20, or the same dose of control mouse IgG1 + IgG2a. Two weeks after the antibody treatment, the extent of B-cell depletion was evaluated using the splenic cells by cell counting and FACS analysis of B-cell surface markers. (A) The representative bar graphs for the percentage of B cells (CD19⁺), (B) the representative dot plots for the percentage of MZ B cells (CD23intCD21hi) and FO B cells (CD23hiCD21low) after gating on CD19⁺cells, and (C) the absolute numbers of total B cells (left panel), marginal zone B cells (middle panel) and follicular B cells (right panel) are shown. Data are expressed as Mean ± SEM. Representative data from more than 3 independent experiments with similar results are shown here.
Figure 2. Partial B-cell depletion using IgG1 anti-CD20 promotes tolerance to FVIII during an ITI protocol. (A) Outline of the experimental protocol. B-cell depletion using a single dose of anti-CD20 antibody treatment in the FVIII primed E16 mice was followed by a daily high dose FVIII \textit{i.v.} injection to model clinical ITI therapy. The mice were bled at various time points for evaluation of anti-FVIII antibody levels and inhibitor formation. (B) Inhibitor formation during the course of an ITI protocol in IgG1 anti-CD20 mAb or control IgG1 treated mice. The FVIII primed mice (n = 7 to 11) received IgG1 anti-CD20 mAb (right panel) or control IgG1 (left panel) were further treated with daily \textit{i.v.} injections of high dose FVIII (2 µg per mouse) to mimic the clinical ITI therapy. The mice were bled before (day 56), during (day 69) and one week after the end of an ITI protocol (day 83). Inhibitor titers were evaluated by Bethesda assay. A single dose of IgG1 anti-CD20 pretreatment prevented the increase in inhibitor formation in the majority of treated mice. (C) Inhibitor formation upon delayed challenge after the IgG1 anti-CD20 treatment. Three months after the IgG1 anti-CD20 or control IgG1 treatment followed by an ITI protocol, the surviving mice were boosted again by intraperitoneal injection of 2µg of FVIII. The inhibitor titer remained significantly lower in the IgG1 anti-CD20 group than in the control group. (D) The effect of B-cell depletion with IgG2a anti-
CD20 on inhibitor formation during the course of an ITI protocol. In an independent experiment, FVIII primed mice (n = 15) received either IgG2a anti-CD20 mAb (right panel) or control IgG2a (left panel) and were further treated with daily i.v. injection of high dose FVIII (2 µg per mouse) to mimic the clinical ITI therapy. The mice were bled before (day 56), during (day 69) and one week after the end of an ITI protocol (day 83). Inhibitor titer was evaluated by Bethesda assay. n.s. not significant and *P < 0.05, compared to control IgG treatment in terms of increase for inhibitor titers at day 69 and day 83, respectively (B and D); *, P < 0.05 compared with control (C) (Mann-Whitney U test, one-tailed).
Figure 3. IgG1 anti-CD20 treatment plus high dose FVIII exposure upregulated B7.2 and B7.1 co-stimulatory molecules on the MZ B cells. Groups of FVIII primed E16 mice (n = 9 to 11) were treated with either IgG1 anti-CD20 or control IgG (250µg per mouse), followed by twice daily i.v. injection of 2µg FVIII for 5 days. One week after the final i.v. FVIII injection, spleen B cells were analyzed for B7 molecules expression. A group of naïve mice (n=8) was included as an additional control. (A) Representative dot plot shows the gates for FO (CD23\textsuperscript{hi}CD21\textsuperscript{low}) and MZ (CD23\textsuperscript{int}CD21\textsuperscript{hi}) B cells. Cells were gated on live CD19\textsuperscript{+} B cells. (B) Representative overlay histographs show the B7.2 and B7.1 expression on MZ B cells from naïve mice (solid gray), mice with control IgG pretreatment (line), and mice with IgG1 anti-CD20 pretreatment (dashed line). (C) Bar graphs show the quantitative results of mean fluorescence intensity (MFI) for B7.2 and B7.1, respectively. *, $P < 0.05$; n.s., not significant (Student’s $t$ test, two-tailed).
Figure 4. A direct comparison of IgG1 versus IgG2a anti-CD20 pretreatment in their effect on inhibitor formation. Groups of FVIII primed FoxP3-GFP/FVIII<sup>−/−</sup> mice (n = 9 to 11) received 250µg of IgG1 anti-CD20 (A) or IgG2a anti-CD20 (B). Two weeks later, the mice were given an intensive FVIII treatment, which was twice daily i.v. injection of 2µg FVIII for 5 days. The mice were bled one week after priming with 3 weekly i.v. injection of 0.2µg FVIII (day 21) and 5 days after the final high dose FVIII exposure (day 46). Inhibitor titers were evaluated by Bethesda assay. n.s., not significant between IgG1 and IgG2a anti-CD20 pretreatment (Mann-Whitney U test, one-tailed).
Fig. 1

A

Control IgG

IgG1 anti-CD20

IgG2a anti-CD20

# Cells

CD19

33.3 66.7

B

Control IgG

IgG1 anti-CD20

IgG2a anti-CD20

CD23

CD21

24.7 51.2 17.6

22.6

75.2 7.9

C

Total B cells

MZ B cells

FO B cells

Cell numbers x 10^6

Control IgG

IgG1 anti-CD20

IgG2a anti-CD20
Fig. 2

A

Days 1 28 35 42 57 69 76 83 132 139

Blood samples obtained

FVIII priming
αCD20 or control IgG
ITI
FVIII boost

B

Control IgG1 + ITI

IgG1 anti-CD20 + ITI

Bethesda Units per ml

Day 56 69 83

C

Control IgG1 + ITI

IgG1 anti-CD20 + ITI

Bethesda Units per ml

D

Control IgG2a + ITI

IgG2a anti-CD20 + ITI

Bethesda Units per ml

Day 56 69 83

n.s.

*
Fig. 3

A

CD23

CD21

38.6

B

CD21

B7.2

MFI of B7.2

0

200

400

600

800

1000

1200

1400

C

MFI of B7.1

0

200

400

600

800

1000

1200

Baseline

Control IgG

IgG1 anti-CD20

n.s.

*
Fig. 4

A

B

Bethesda Units per ml

IgG1 anti-CD20 + ITI

IgG2a anti-CD20 + ITI

Day 21 46

Day 21 46

n.s.
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