Immunodominant T-cell epitopes in the factor VIII C2 domain are located within an inhibitory antibody binding site

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Summary
Formation of inhibitor antibodies to factor VIII (FVIII) is a major complication of FVIII replacement therapy for hemophilia A patients, and it occurs through a T-cell dependent process. The C2 domain of FVIII contains epitopes that are recognized by antibody inhibitors. We have examined regions of the C2 domain that form epitopes for T cells in mice congenitally deficient in FVIII. We obtained CD4+ T cells from mice immunized by intravenous infusion of therapeutic doses of recombinant human FVIII (rFVIII), or by subcutaneous injections of rFVIII or recombinant human C2 domain in adjuvant. In all cases, the T cells recognized most strongly and consistently two overlapping peptides that spanned residues 2191 to 2220 of the C2 domain.

Keywords
T-cell epitopes, factor VIII, C2 domain

Analysis of the crystal structure of human factor VIII C2 bound to a human monoclonal antibody, BO2C11, showed these residues also constitute part of a human alloimmune B-cell epitope (Spiegel et al., Blood 2001; 98: 13-19). This region includes one of the “hydrophobic spike” protrusions, consisting of M2199 and F2200, as well as the basic residues R2215 and R2220. These residues contribute to membrane binding and to association with von Willebrand factor (vWF). These findings suggest that a major T-cell epitope in the C2 domain recognized by hemophilic mice is located within the same region that binds to inhibitors, vWF, and activated membranes.

Introduction
FVIII is a 2332 amino acid glycoprotein that plays a critical role in hemostasis. Activated FVIII (FVIIIa) is a cofactor for the serine protease factor IXa in the intrinsic coagulation pathway. When the serine protease and its cofactor coalesce at activated phospholipid surfaces the catalytic efficiency towards the substrate, factor X, increases by approximately four orders of magnitude (1). FVIII contains six domains arranged sequentially in the following order: A1, A2, B, A3, C1, and C2. The A domains
are structurally related to the copper-binding protein ceruloplasmin (2, 3), whereas the C domains are members of the discoidin family (4).

The crystal structure of a recombinant factor VIII C2 domain protein (5) revealed a striking feature, two pairs of exposed hydrophobic residues, or “spikes”, which protrude from the tips of β-hairpin turns at one end of the molecule. It was proposed that these side chains insert into the phospholipid membrane as FVIII binds to the membrane surface. Thus, the hydrophobic “spikes” are essential components of both the membrane binding and the von Willebrand factor (vWF) binding motifs (6). Point mutations of the FVIII gene that result in hemophilia A have been mapped within the structure of the C2 domain (7), and also onto a model of the C1 domain (7). Additionally, inversions and deletions in the FVIII gene can lead to loss of functional FVIII (8). The development of antibodies that block the pro-coagulant function of FVIII (inhibitors) is a severe complication of FVIII replacement treatments of hemophilia A. The likelihood of inducing antibody inhibitors remains a critical obstacle to the future development of gene-based therapies for hemophilia A. Significant progress has been made in the identification of genetic mutations in hemophilia A patients who develop inhibitors (9-14). Inhibitors develop in up to a third of hemophilia A patients with major defects in the FVIII gene, such as those caused by large insertions or deletions, intron 22 inversions, or frameshift mutations (10, 12, 13). Most inhibitors bind to sites on the FVIII C2 and A2 domains (11, 15-18). Inhibitors that bind to the C2 domain can block FVIII binding to activated phospholipid surfaces and/or to the FVIII carrier protein, von Willebrand Factor (vWF) (19-22).

Hemophilia A patients carrying missense mutations are generally less likely to develop inhibitors. This might be because the circulating FVIII, although defective in its function, or produced at very low levels, or both, still produces effective immune tolerance. Current treatment options, including tolerance induction regimes and bypass strategies involving administration of procoagulant proteins or complexes, are very expensive, and of limited efficacy. A first step toward improving tolerization protocols is the identification of significant T- and B-cell epitopes on the FVIII protein.

Several studies have examined the responses of T cells isolated from hemophilic patients to FVIII or to peptides corresponding to the FVIII sequence (23-28). Proliferative responses to FVIII antigens could be detected in many hemophilia A patients, and even in healthy blood donors (29). Studies of the specificity of the immune response in humans, however, have several potential drawbacks. These include limited sample availability, the variable timing of sample collections, and the different treatment history and genetic backgrounds of the patients. In contrast, hemophilic mice, which are deficient in FVIII, provide a controllable model system to examine such specificity (30). In this study, we have examined the proliferative T-cell responses to recombinant human FVIII, to a recombinant human C2 domain construct, and to synthetic peptides corresponding to the human C2 sequence in a mouse model of hemophilia A. Interestingly, the T-cell epitope(s) identified here include residues that contribute to a known B-cell epitope, as they comprise part of the binding site for the inhibitory human monoclonal antibody, BO2C11 (31).

Materials and methods

Mice and antigens

FVIII deficient mice carrying a deletion of exon 16 of the FVIII gene (E-16 mice) have been back-crossed for at least eight generations to the strain C57BL/6 at the Holland Lab, American Red Cross. Recombinant C2 (rC2) was expressed and purified as described previously (32). We used also a library of 16 overlapping peptides, synthesized by manual parallel synthesis (25) and spanning the 162 amino acid sequence of the FVIII C2 domain (residues 2171-2332 of the FVIII precursor). The peptides were 20 residues long (apart from the carboxyl terminal peptide, which was 13 residues), and their sequences overlapped by 10 residues. They are part of a peptide library spanning the entire FVIII precursor sequence, which was used previously to examine the epitope repertoire of FVIII-specific human-derived CD4+ T cells (26, 28).

The peptide length, 20 residues, compares with that of naturally processed class II epitopes, which are 9-14 residues in length (33). Extra residues at either end of an epitope sequence do not affect peptide attachment to the class II molecule binding cleft, which is open at both ends (33). The 10-residue sequence overlap reduces the risk of missing epitopes that might otherwise be “broken” between adjacent peptides. The peptides are identified by residue numbers that refer to the position of the first and last amino acid residue in the corresponding single chain precursor sequence of FVIII. The sequences of the peptides were verified by amino acid composition and their molecular weights were checked by MALDI (Matrix Assisted Laser Desorption Ionization) TOF (Time Of Flight) mass spectroscopy (Bruker Biflex III, Billerica, MA). This analysis confirmed that a compound having the expected molecular weight accounted for most of the material in the sample. Similar peptide libraries have been used previously to map CD4+ epitopes on autoantigens and exogenous antigens (29, 34-38). Human recombinant FVIII (rFVIII) was kindly provided by Baxter, Inc. (Glendale, CA) or purchased from a commercial source (Bayer, Elkhart, IN).

Immunization protocols and T-cell proliferation assays

T-cell reactivity to FVIII and/or its C2 domain epitopes was examined using both therapeutic i.v. immunization, as well as challenge in Complete Freund’s Adjuvant (CFA). The response
of CD4+ T cells to rFVIII was initially measured in mice that received therapeutic doses of rFVIII intravenously without adjuvant, a procedure designed to mimic the intravenous administration of rFVIII to hemophilia A patients. Splenic T cells pooled from 4-5 mice were obtained after three to four rFVIII i.v. infusions, pooled and enriched for CD4+ T cells (depleted of CD8+ cells) using magnetic beads and rat anti-mouse CD8 antibody (Qiagen, Valencia, CA and Pharmingen, San Diego, CA, respectively) as described previously (30). An uncloned FVIII-specific T-cell line was generated from these E-16 mice immunized i.v. as above. We used uncloned T cells to reflect a broad repertoire of specificities (which might be lost by selective subcloning), as well as diverse cytokine profiles. The generation and characterization of this T-cell line has been described elsewhere (30). Briefly, CD4+ T cells were stimulated at 4-10 day intervals in the presence of irradiated splenic feeder cells and 3 µg/ml rFVIII, but in the absence of exogenous IL-2. After 3-4 cycles, the cells were used in proliferation assays. Upon stimulation with rFVIII, this line secreted both IL-4 and IFN-gamma, indicating that it contained both Th1 and Th2 cells. Earlier experiments showed that CD4+ T cells of rFVIII-treated mice recognized peptide pools corresponding to all of the hfVIII domains (30). Thus, hemophilic mice develop an immune response to human FVIII administered intravenously similar to that of hemophilia A patients.

E-16 mice also were immunized subcutaneously with 0.5 µg rC2 (six mice) or with 5 µg rFVIII (three mice), each diluted 1:1 in CFA (Sigma, St. Louis, MO). Two weeks later, two of the rFVIII mice and two of the rC2 mice were sacrificed. Their lymph nodes were removed, and the cells were enriched for CD4+ T cells and cultured with FVIII, C2 or its peptides as above. The remaining E-16 mice immunized with rC2/CFA were then boosted seven weeks later with C2/CFA subcutaneously at the base of the tail. One week later, they were sacrificed and their spleens were removed. The spleen cells were pooled and enriched for CD4+ cells using the same protocol as above. Proliferation assays were carried out as before.

To examine the specificity of the response to FVIII, CD4+ T cells from mice immunized as above were seeded in quadruplicate or sextuplicate in 96-well U-bottom plates (200 µl/well). Serial dilutions of rFVIII and rC2 or a series of 20-mer overlapping C2 peptides (sterilized by irradiation) at 10 µg/ml (~5 µM) were added to the replicate wells in Enhanced Eagles Media (EHAA, Biofluids, Rockville MD) supplemented with 2.5 mM sodium pyruvate, 4 mM L-glutamine, 50 µM 2-mercaptoethanol, 10 mM HEPES, 100 µM penicillin, 100 µg/ml streptomycin, 2% FBS, and 0.5% serum from naïve E-16 hemophilic mice. One µCi of [3H]-thymidine per well (ICN Pharmaceuticals) was added after 24 hours culture at 37°C and cultures were harvested and assayed 16 hours later using a Packard Matrix 9600 Harvester/Counter. The counts per minute (cpm) were measured according to the manufacturer’s protocol.

Finally, a second set of rFVIII immunization with adjuvant experiments was carried out in order to confirm and further evaluate CD4+ T-cell responses to the 20-mer peptides. E-16 mice were injected subcutaneously, 3-4 times every 3-5 weeks with 4 µg human FVIII (Bayer, Elkhart, IN) in CFA. Two days after the last boost, spleen cells from two identically treated mice were collected, pooled, and depleted of CD8+ cells, as described above. The resulting CD4+ enriched cells were suspended in standard medium (2 × 10^6 cells/ml). The cells were seeded with 10 µg/ml (~5 µM) of each individual synthetic peptide added to quadruplicate wells; a peptide pool containing 1 µg/ml (~500 nM) of each peptide: 0.25 µg/ml (~1.5 nM) and 0.5 µg/ml (~3 nM) FVIII and 10 µg/ml phytohemagglutinin (Sigma, St. Louis, MO) were used as positive controls. Additional wells cultured without any stimulus provided the basal proliferation rates of the cells. After four days of culture the cells were labeled for 16 hours with [3H]-thymidine and harvested using a Skatron cell harvester (Titertek, Sterling, VA), and the [3H]-thymidine incorporation measured as above.

When an antigen induced a statistically significant (p < 0.05) increase in the proliferation of the cells as compared with the basal rate of proliferation (assessed using a two-tailed Student’s t test), the stimulation index (the ratio of the average cpm of cultures in the presence of the antigen to the average basal proliferation of the same cells) was considered significant. The use of SI’s normalizes the results, and allows comparison of experiments carried out at different times or using cells from different mice.

**Results**

Figure 1A shows responses of an uncloned T-cell line from hemophilic mice that received repeated i.v. infusions of rFVIII. These cells had characteristics of both Th1 and Th2 cells (30). Only the peptides spanning the region 2191-2220 elicited a response significantly above the baseline rates, although the T-cell line initially responded to both the heavy and light chains of rFVIII, as would be expected for uncloned T cells reflecting the repertoire of these mice (data not shown). This cell line reacted weakly if at all to the other C2 peptides. This FVIII specific T-cell line responded to the rC2 protein but less strongly than to peptides 2191-2210 and 2201-2220. This may have been due to the different molar concentrations of the stimulating antigens, which were much higher for the synthetic peptides (~5 µM) than for rC2 (10 nM). Peptides corresponding to C2 sequences in the region 2221-2280 also elicited a proliferative response in some experiments that tested the response of CD4+ spleen cells, but these responses were not seen consistently. The peptide-induced responses were dose-dependent and could be detected at peptide concentrations as low as 1 µg/ml, or 0.3 µM (data not shown).

Figure 1B shows representative stimulation responses of CD4+ spleen cells isolated and pooled from mice immunized
The CD4+ T cells responded vigorously to an equimolar pool of the overlapping C2 peptides and to a few individual peptides. Two sets of stimulation assays were carried out using these cells, under identical conditions, with similar results. Interestingly, the response was primarily against the same two peptides that elicited proliferation by T cells from i.v. immunized mice.

Figure 1C shows the responses of CD4+ spleen cells from mice immunized subcutaneously with rC2 in Freund’s adjuvant proliferate in response to C2 domain peptides. In Fig. 1B, the panels show two representative proliferation experiments (reported as SIs) using pooled splenic CD4+ T cells challenged with two different concentrations of rFVIII, or with an equimolar (400 nM each) pool of the C2 peptides; phytohemagglutinin (PHA) was used as a positive control. The two sets of cells were treated identically, and the variation in response shows that the mice had a fairly heterogeneous response to the peptides. The basal proliferation rates for experiments in 1A and 1B were less than 1800 cpm. See text for experimental details. In Fig. 1C, splenic CD4+ T cells from rC2-immunized mice were challenged in vitro with 10 µg/ml (~5 µM) peptides. Results shown are the responses from pooled splenic cells, and assays were carried out in sextuplicate. Basal proliferation was 2109 ± 163 cpm, and ConA was used as a positive control, as indicated. These cells did not respond to peptides with sequences derived from the FVIII C1 domain (not shown).
shown). The results indicated that in these measurements the limiting concentration of rFVIII required to induce a proliferative response over baseline levels was about 0.2 nM, whereas proliferation in response to the C2 protein was elicited only at higher levels, around 20-60 nM. This is presumably because FVIII epitopes outside the C2 domain contributed significantly to T-cell proliferation.

Discussion

As a first step to analyzing the epitope repertoire of CD4+ T cells specific for FVIII in the mouse model of hemophilia A, we focused our efforts on the C2 domain, because it contains the binding sites for both von Willebrand factor (vWF) and phospholipids, and epitopes that are commonly recognized by antibody inhibitors. We used three different immunization approaches to elicit responsiveness to identify T-cell epitopes. The first immunization protocol mimicked the therapeutic intravenous doses of FVIII given to hemophilia patients, and which has been shown to lead to both T-cell and B-cell responses to FVIII that resemble those seen in hemophilia patients. Another utilized CD4+ T cells from spleens and lymph nodes of mice that were immunized subcutaneously with either rFVIII or rC2 in CFA. In all cases, CD4+ T cells were challenged in vitro with individual 20-mer overlapping peptides spanning the C2 domain sequence. Despite different immunization protocols, the response to one region of the C2 domain predominated in these hemophilia A mice. The finding that these CD4+ T cells primarily recognized the same sequence region, 2191-2220, suggests that the route of immunization and the use of adjuvant did not alter the selection and presentation of immunodominant epitopes of the FVIII C2 domain. In this communication, we use the term “immunodominant” to refer to the relative responses to C2-derived peptides, not to the proliferation seen in response to the C2 domain versus other domains of FVIII.

Figure 3 shows the location in the human C2 domain of the two peptide sequences that most consistently formed epitopes for the mouse CD4+ T cells. We show also some of the amino acid side chains that form the binding site for the inhibitory antibody BO2C11, to allow comparison of T- and B-cell epitopes, with the caveat that a mouse T-cell epitope is being compared to a human B-cell epitope. The patient-derived antibody BO2C11 recognizes residues on several adjacent loops of the C2 domain (31). A crystal structure of the C2 domain complexed with BO2C11 identified this B-cell epitope (31). This antibody blocks FVIII binding to both membranes and vWF (22). The antibody epitope visualized in the crystal structure includes both of the hydrophobic spike regions, M2199-F2200 and L2251-L2252, as well as residues R2215, R2220, Q2222, V2223, H2315, and Q2316. These residues provide salt links, hydrogen bonds and hydrophobic interactions that stabilize the bimolecular complex between FVIII and the antibody. The present findings indicate a much stronger T-cell response to the first hydrophobic spike, contained in peptides 2191-2220, than to peptides corresponding to other regions of the C2 domain sequence. It is not clear which residues within this 30 residue
region contribute to its immunodominance, only that T cells in E-16 mice preferentially respond to >10-mer processed peptides bound within MHC class II grooves in this area.

While these T-cell epitopes correspond to linear stretches of amino acids, most antibodies bind to “discontinuous” epitopes, formed by residues that are in close proximity in the folded protein but that may be remote in the linear amino acid sequence. Indeed, the fact that B and T cell epitopes do not usually overlap is due to the conformational nature of antibody recognition, although T cell epitopes may occur in linear sequences within these regions. Antibodies from many hemophilia A inhibitor patients bind to the FVIII C2 domain, blocking binding to phosphatidylserine and/or to von Willebrand Factor (20, 21). Studies of the binding to inhibitors of recombinant FVIII proteins harboring substitutions of specific amino acids on the C2 surface have greatly improved our understanding of the B-cell epitopes on the C2 domain surface (39-41). The mouse anti-FVIII monoclonal antibody NMC-VIII/5 recognizes an epitope on the human FVIII C2 domain (21). Even though BO2C11 and NMC-VIII/5 both block FVIII binding to membranes, Bethesda assays of their binding to mutant FVIII constructs indicated that their B-cell epitopes differed (41). In this case a comparison was made between a mouse monoclonal antibody and a patient-derived monoclonal antibody, but our results show that there is also heterogeneity in the T-cell immune response, even among mice with a genetically similar background. This indicates that the heterogeneity of antibodies against FVIII, which is also seen in humans (41), will continue to be a challenge in attempting to design improved versions of FVIII.

The present findings indicate that, in the mouse model of hemophilia A, the segment 2191-2220 of the C2 domain sequence, which was immunodominant in these experiments for the sensitization of CD4+ T cells, regardless of the route of immunization and the form of the antigen-containing construct, also forms binding sites for inhibitory antibodies in humans. The sequence region 2191-2220 contains a segment (2198-2201) that forms a hairpin turn in the C2 domain. This region contributes residues essential to both the membrane and vWF binding motifs of FVIII (6). Two recent studies have evaluated the stimulation of human blood-derived CD4+ cells with this same panel of overlapping C2 peptides (26, 28). The sequence region 2291-2330 was recognized most consistently by four groups of subjects: healthy individuals (1), hemophilia A patients without (2) and with (3) inhibitors, and nonhemophilic patients with autoantibodies to FVIII (4). Interestingly, the region 2191-2220 was recognized by T cells from all groups except the hemophilia A patients with inhibitors (28). Moreover, the results with this set of (hemophilia) patients appeared to be independent of HLA phenotype, perhaps due to the promiscuity of binding of certain peptides to human HLA-D (see below).

Considering that the E16 hemophilic mice we used are highly inbred (on an H-2d background), whereas the hemophilia patients are of diverse HLA phenotypes, it is somewhat surprising that these epitopes are relatively immunodominant. However, it is also possible that additional epitopes may be found in the murine model of hemophilia A when additional T-cell lines are developed and tested from hemophilia mice on different H-2 backgrounds (42, 43). Nonetheless, the fact that the dominant T-cell epitopes in knockout mice and some hemophilia patients are similar and appear in the same region to which inhibitors bind could reflect the accessibility of this region and its importance in the function of FVIII, rather than the inbred nature of the mice we employed.

The relative lack of recognition of this region by patients with high titers of inhibitors (28) could be due to ‘epitope spreading’ in the patients, who have been exposed to FVIII for longer time periods, and to the selective pressure of the high affinity antibody response. Thus, these patients may have a more avid or mature immune response with high affinity inhibitors compared to the hemophilic mice. Further studies in hemophilic mice on different H-2 backgrounds, after long-term immunization to achieve similar high inhibitor titers, may resolve this issue.

These findings might be applicable to the development of inhibitor treatments or tolerization regimes that will target T-cell epitopes and/or larger regions of FVIII. It will be important in future studies to locate additional T-cell epitopes in other domains of FVIII, and to characterize the relationships between their recognition by CD4+ T cells and their binding to inhibitory antibodies. A better understanding of the specific sequences recognized by T cells will likely lead to the development of improved strategies to induce immune tolerance. In addition, the identification of a limited number of T-cell epitopes suggests that it may be possible to introduce point mutations at these regions to create a less immunogenic version of the FVIII protein. It will be interesting to see if mutations introduced at known antigenic sites affect the immunogenicity of the protein as well. Advances in treatment options to prevent and/or manage inhibitory antibody responses will improve the efficacy of treatment and quality of life for patients with anti-FVIII allo- and auto-antibodies.

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