Properties of human antibodies to factor VIII defined by phage display
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CHAPTER 6

Longitudinal analysis of factor VIII inhibitors in a previously untreated mild hemophilia A patient with an Arg$^{593}$→Cys substitution

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ABSTRACT

Recent studies suggest that certain missense mutations associated with mild to moderate hemophilia A predispose to inhibitor development. In this study, we present a longitudinal analysis of the epitope specificity of an inhibitor that developed in a mild hemophiliac with an Arg→Cys mutation. Immunoprecipitation studies revealed the presence of antibodies directed toward the light chain and A2 domain of factor VIII. Limited reactivity was observed with metabolically labeled C2 domain. Almost complete inhibitor neutralization was achieved upon addition of A2 domain. Binding of the inhibitor was not affected by the presence of the Arg→Cys substitution in the recombinant A2 fragment. Evaluation of the epitope specificity of anti-factor VIII antibodies in plasma samples obtained at different time-points of inhibitor development revealed initial development of a low titer inhibitor directed toward the A2 domain and factor VIII light chain. A second period of factor VIII replacement therapy resulted in a dramatic rise in factor VIII inhibitor titer, which maintained their original epitope specificity. Based on the results of this and previous studies (Fijnvandraat et al., 1997; Thompson et al., 1997) it is argued that inhibitor development in patients with the Arg→Cys mutation may proceed via a similar mechanism.

INTRODUCTION

A rare complication in hemophilia care is the development of factor VIII inhibiting antibodies in nonseverely affected hemophilia A patients. Exposure to circulating endogenous factor VIII in mild and moderate hemophiliacs may induce tolerance for administered factor VIII, which could account for the lower incidence of inhibitor development in this group. Interestingly, certain missense mutations appear to be more common in mild and moderate severity hemophilia A patients with an inhibitor. In 16 inhibitor patients, 9 different missense mutations were identified which were mainly located in the C1 and C2 domains. Besides mutations in the border region of the C1/C2 domain, mutations in the A2 domain have been observed in mild hemophilia A patients with inhibitors. The above findings may suggest that certain genetic defects are related to inhibitor development. This is illustrated by 2 reports, which describe inhibitor characteristics in patients with an Arg→His substitution. Strikingly, inhibitory antibodies observed in these patients were less reactive with endogenous (His) compared to exogenous factor VIII (Arg) providing evidence for a possible relation between this missense mutation and inhibitor development. Recently, we studied the characteristics of factor VIII inhibitors present in the plasma of a patient with an Arg→Cys substitution. Remarkably, anti-factor VIII antibodies obtained at a late stage of inhibitor development were able to discriminate between wild-type (Arg) and variant (Cys) factor VIII. Thus, inhibitors in patients with the Arg→Cys or Arg→His substitution discriminate between exogenous and endogenous factor VIII. These observations suggest that factor VIII genotype may relate to
inhibitor development. However, in another patient with the Arg\(^{593}\)→Cys substitution factor VIII inhibitory antibodies with a different epitope specificity were observed.\(^6\) Reactivity of this patient's antibodies with factor VIII was not affected by the presence of the Arg\(^{593}\)→Cys substitution. Detailed epitope mapping revealed that anti-factor VIII antibodies were directed against the region Arg\(^{484}\)→Ile\(^{508}\), which constitutes a major inhibitor epitope in the A2 domain.\(^8\)

To get further insight in origin and development of factor VIII inhibitors in patients with mild hemophilia we studied inhibitor characteristics in another patient with the Arg\(^{593}\)→Cys substitution. We report on the relation between epitope specificity and factor VIII genotype. Additionally, we provide a longitudinal analysis of factor VIII inhibitor epitope specificity from the onset of factor VIII replacement therapy.

**MATERIALS AND METHODS**

**Assays**

Citrated plasma samples used in this study were collected and stored in 0.5-1.0 mL aliquots at -70°C. Factor VIII procoagulant activity (factor VIII:C) was measured by a one-stage clotting assay and expressed as IU/mL.\(^9\) Inhibitor titers were measured according to the Bethesda assay essentially as described previously.\(^10\)

**Molecular genetic studies**

Genomic DNA was obtained from peripheral blood lymphocytes using the QIAamp Blood Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The 26 exons of the factor VIII gene with their bordering intron sequences were amplified by polymerase chain reaction (PCR). Amplification was performed using one specific and one oligonucleotide primer elongated with the M13-derived sequence 5'-GTAAAACGACGGCCAGT-3'. PCR fragments were sequenced by dye-labeled-21M13 primers using the Thermo Sequenase fluorescent labeled primer cycle sequencing kit (Amersham, 's Hertogenbosch, The Netherlands). Sequence analysis was performed using an Applied Biosystems 373a automated DNA sequencer.

**Expression, metabolic labeling, and immunoprecipitation of selective domains of factor VIII**

Expression and metabolic labeling of recombinant factor VIII fragments were performed essentially as described previously.\(^5,11\) Recombinant baculoviruses expressing the various recombinant factor VIII fragments were obtained after transfection of SF-9 cells. High-Five\(^{\text{TM}}\) cells were infected with recombinant baculoviruses and maintained in culture medium consisting of serum-free Insect-XPRESS medium (BioWhittaker, Alkmaar, The Netherlands) supplemented with 100 U/mL penicillin and 100 \(\mu\)g/mL streptomycin (GIBCO, Breda, The Netherlands). Medium of metabolically labeled cells was collected in an equal volume of 2 times concentrated immunoprecipitation buffer. Immunoprecipitation was performed as described previously.\(^5\) Expression of radiolabeled factor VIII fragments was monitored by
monoclonal antibodies specific for the C2 domain (CLB-CAg 117) and the A2 domain (CLB-CAg 9). Normal plasma served as a negative control.

**Inhibitor neutralization assay**

Recombinant factor VIII fragments corresponding to the A2 domain, the A2 domain with the Arg$^{593}$→Cys mutation (designated A2-R593C), and the factor VIII light chain were derived from High-Five™ cells infected with recombinant baculoviruses encoding these fragments. Recombinant fragments present in culture medium were dialyzed against 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl. The concentration of factor VIII light chain and C2 domain in the conditioned medium was determined by ELISA.$^{11}$ Quantification of recombinant wild-type A2 domain and A2-R593C was performed by a method outlined previously.$^{12}$ Pooled normal human plasma served as a standard. Inhibitor neutralization was performed essentially as described previously.$^{11,13}$

**RESULTS**

**Patient**

Patient AMC-67, a 63-year-old previously untreated mild hemophiliac with factor VIII procoagulant levels of 0.29 IU/mL received extensive perioperative factor VIII replacement therapy (monoclonally purified plasma-derived factor VIII) for surgical correction of a hernia inguinalis and symptomatic varices. No bleeding complications occurred. At a routine control, 6 weeks later, an inhibitor of 1.2 BU/mL and factor VIII procoagulant levels of 0.04 IU/mL were measured. No clinical symptoms of bleeding were present at that time. Three months

![Figure 1. Inhibitor development in patient AMC-67. Bars in the graph indicate periods of factor VIII replacement therapy and treatment of bleeding episodes with factor VIIa. On the left y-axis the titer of the inhibitor is given in BU/mL (●, closed circles). On the right y-axis factor VIII procoagulant activity is depicted in IU/mL (O, open circles). On the x-axis the time is given in days. The sample corresponding to day zero has been taken a day prior to surgery.](image-url)
later, the patient presented with a spontaneous hemorrhage in the left psoas muscle. Upon administration of high doses of factor VIII concentrate (continuous infusion 300-750 IU/h for 5 days) the bleeding was arrested. Remarkably, factor VIII recovery was strongly reduced and an anamnestic rise in inhibitor titer to a peak value of 250 BU/mL was observed (Figure 1). Subsequently, the inhibitor titer gradually decreased and was undetectable 5 months later. During this period 2 bleeding episodes were successfully controlled with recombinant factor VIIa. Meanwhile, factor VIII procoagulant levels increased to a value of 0.11 IU/mL. The clinical course of this patient has been described in more detail elsewhere.14

Characterization of factor VIII inhibiting antibodies present in plasma of patient AMC-67

A plasma sample corresponding to a titer of 250 BU/mL was evaluated for epitope specificity by immunoprecipitation analysis with radiolabeled factor VIII fragments. The antibodies present in the patient's plasma reacted with A2 domain and the light chain of factor VIII (Figure 2A). Only limited reactivity was observed with metabolically labeled C2 domain of factor VIII. In order to address the functional epitope of the inhibiting antibodies, factor VIII neutralization experiments were performed using recombinant factor VIII fragments.

Figure 2. Characterization of anti-factor VIII antibodies present in plasma of patient AMC-67 (A) Binding of antibodies to metabolically labeled factor VIII fragments corresponding to the A2 domain (A2), the factor VIII light chain (LCh), and C2 domain (C2) as assessed by immunoprecipitation. Plasma of patient AMC-67 (67), positive control (+), negative control (-) are indicated for every fragment tested. At the right of the figure molecular weight markers (in kDa) are depicted. (B) Neutralization of inhibitor activity by recombinant factor VIII fragments: C2 domain (□, open squares), factor VIII light chain (●, closed squares), A2 domain (○, closed circles), and A2-R593C harboring the arginine to cysteine substitution at position 593 (○, open circles). Residual factor VIII activity is indicated on the y-axis as a percentage of the control sample. On the x-axis the concentration of factor VIII fragment added is given.
Approximately 70 percent neutralization was achieved when increasing concentrations of recombinant A2 domain were added (Figure 2B). Although the factor VIII light chain was recognized by the patient's antibodies in immunoprecipitation studies, only limited neutralization was observed upon addition of this fragment.

Analysis of the factor VIII gene of patient AMC-67 revealed a C → T substitution in exon 12, which predicts replacement of an Arg$^{593}$ in the A2 domain by Cys. Previously, we have described a patient with an identical molecular defect in which anti-factor VIII antibodies selectively recognized a recombinant A2 domain with an Arg at position 593. Therefore, we evaluated whether binding of the antibodies in patient AMC-67 was also related to the Arg$^{593}$ to Cys substitution. The ability of the recombinant fragment A2-R593C to neutralize the activity of the inhibitory antibodies was investigated. Addition of A2-R593C resulted in a dose-dependent increase in factor VIII activity, which overlapped the pattern of inhibitor neutralization observed for the nonmodified A2 domain (Figure 2B). These results indicate that binding of the inhibitor to the A2 domain is not affected by the Arg$^{593}$ → Cys substitution.

To obtain information on the epitope specificity of anti-factor VIII antibodies over time, plasma samples collected during inhibitor development were analyzed by immunoprecipitation (Figure 3). Samples up to 5 weeks after initial factor VIII treatment remained negative in immunoprecipitation (samples 1 - 4; data not shown). Subsequently, weak but significant reactivity with factor VIII light chain and both recombinant wild-type and variant A2-R593C domain was observed in agreement with the low titers determined in the Bethesda assay (samples 5 - 8 containing 0.3 - 1.1 BU/mL) (Figure 3). A marked increase in signal was seen for factor VIII light chain, A2 domain, and A2-R593C in samples 10 and 11. This corresponds with a sharp increase in titer of the inhibitor (samples 9 - 14) which is followed by a more gradual decrease in inhibitor levels. The transient rise in inhibitor titer is reflected by the amount of radiolabeled protein bound by the antibodies in the patient's plasma. The latest sample, which contains detectable reactivity, is sample 18, which corresponds with a titer of 2 BU/mL. The above analysis was complemented by neutralization assays for samples 9 and 17. For both samples, a similar pattern of nearly complete inhibitor neutralization was observed following addition of recombinant A2 domain or A2-R593C (Table 1). Our results indicate that initial development of a low titer inhibitor is characterized

Table 1. Inhibitor neutralization by recombinant factor VIII fragments.

<table>
<thead>
<tr>
<th>Sample number (days)</th>
<th>Titer BU/mL</th>
<th>Light chain (A3-C1-C2)</th>
<th>C2</th>
<th>A2</th>
<th>A2-593</th>
</tr>
</thead>
<tbody>
<tr>
<td>9 (141)</td>
<td>250</td>
<td>10 ± 4</td>
<td>8 ± 1</td>
<td>67 ± 8</td>
<td>65 ± 3</td>
</tr>
<tr>
<td>14 (147)</td>
<td>8 ± 1</td>
<td>11 ± 1</td>
<td>71 ± 6</td>
<td>74 ± 2</td>
<td></td>
</tr>
<tr>
<td>17 (183)</td>
<td>58</td>
<td>13 ± 2</td>
<td>12 ± 2</td>
<td>84 ± 5</td>
<td>72 ± 9</td>
</tr>
</tbody>
</table>

Inhibitor neutralization is given in percentages. Values represent the means ± S.D. of 3 independent determinations.
by the presence of antibodies directed against the A2 domain and the factor VIII light chain. A second period of factor VIII replacement therapy resulted in an anamnestic rise in level of factor VIII inhibitors, which maintain their original epitope specificity.

Figure 3. Longitudinal analysis of factor VIII inhibitors in patient AMC-67. Plasma samples obtained at different time points during inhibitor development were characterized by immunoprecipitation. Upper panel: inhibitor titer (BU/mL) at different time points of inhibitor development. On the y-axis the inhibitor titer is indicated in BU/mL. Below each bar the number of days is depicted according to data presented in Figure 1. Lower panel: immunoprecipitation with plasma samples, which correspond to the numbers indicated on the x-axis of Figure 3. Radiolabeled fragments correspond with the factor VIII light chain (LCh), the A2 domain (A2) and A2 domain with the Arg$^{593}$→Cys mutation (A2-R593C). Anti-factor VIII antibodies of subclass IgM could only be detected in samples 8 and 9. These antibodies were directed toward the light chain of factor VIII. We were unable to detect IgM antibodies directed toward the A2 domain of factor VIII.
DISCUSSION

Factor VIII inhibitors are infrequently observed in patients with previously untreated mild hemophilia A and tend to associate with certain genetic defects. Here, we describe a previously untreated mild hemophiliac with an Arg$^{593}$→Cys substitution who developed a factor VIII inhibitor after intensive factor VIII replacement therapy.

The epitope specificity of the inhibitory antibodies in patient AMC-67 is remarkably similar to that of patient F described in a previous report. In plasma of both patients, who both received intensive factor VIII replacement therapy after surgery, inhibiting antibodies directed against the A2 domain have been identified. Part of the anti-factor VIII antibodies possesses light chain specificity. This observation suggests that inhibitor formation may proceed via a similar mechanism in both patients. In the patient described by Thompson and coworkers the anti-A2 antibodies were directed against a common binding site (Arg$^{484}$-Ile$^{508}$) for factor VIII inhibitors. Although it has not been addressed in full detail in this study, it is likely that the anti-A2 antibodies observed in patient AMC-67 are directed toward the same region. It has been proposed that exposure of cryptic epitopes in combination with a high antigen load may explain development of inhibitors in patients with the Arg$^{593}$→Cys mutation. We hypothesize that following treatment of patients with the Arg$^{593}$→Cys mutation and subsequent processing of factor VIII antigen, peptides containing amino acid Arg$^{593}$ may evoke T-helper cell activation. This may result in loss of tolerance, which coincides with formation of B-cell clones expressing antibodies directed toward the major inhibitor epitopes on factor VIII. Following initial treatment of patient AMC-67 a low titer inhibitor developed. Alternative treatment of the psoas muscle bleed with DDAVP or recombinant factor VIIa may have prevented anamnesis. Furthermore, our analysis indicates that frequent monitoring may be useful for early detection and management of patients at risk of inhibitor development.

In this study we were not able to demonstrate any change in inhibitor specificity over time. Previously we have described an inhibitor patient with the same factor VIII mutation. In that patient baseline factor VIII was reduced at the time the inhibitor first appeared. At a late stage of inhibitor development the baseline factor VIII of the patient increased and the inhibitor appeared to be exclusively directed toward wild-type A2 domain, not recognizing the patient's endogenous, variant (Cys$^{595}$) factor VIII. The difference in the natural history of inhibitor development between the presently and previously studied patient most likely originates from factors beyond the factor VIII genotype.

Antibodies exclusively recognizing wild-type factor VIII may be induced by antigen driven stimulation of a specific B-cell clone. In the case of a patient with the Arg$^{593}$→Cys mutation this would require continuous presentation of the Arg$^{593}$ containing peptide by an appropriate MHC class II molecule capable of supporting proliferation of B-cell clones specific for the Arg$^{593}$→Cys mutation. In the absence of the appropriate MHC class II allele inhibitor development may depend on “antigen load” as proposed by Thompson and coworkers. The hypothetical model outlined above may explain the different inhibitor epitope specificity in patients with the Arg$^{593}$→Cys mutation and predicts a prominent role for MHC class II
molecules in inhibitor development in patients with mild hemophilia A. Presently, we are evaluating whether inhibitor development in patients with the Arg^{593}→Cys mutation can be linked to a particular MHC class II allele. The result from this analysis may provide a rationale for inhibitor development in a subset of patients with mild and moderate hemophilia A.

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REFERENCES


