The development of inhibitory antibodies to factor VIII in patients affected by a mild form of hemophilia A (factor VIII > 0.05 IU/mL) is considered a rare event. In this study, we evaluated the relationship between genotype and anti-factor VIII antibody formation in a patient with mild hemophilia A. Mutation analysis showed that a missense mutation in the factor VIII gene leading to replacement of Arg 593 by Cys in the A2 domain of factor VIII was associated with hemophilia A in this patient. The anti-factor VIII antibodies present in the patient’s plasma were characterized using metabolically labeled factor VIII fragments expressed in insect cells. The anti-factor VIII antibodies, composed of subclasses IgG2 and IgG4, reacted with both the fragment corresponding to the factor VIII heavy chain and the A2 domain. The Arg<sup>593</sup> → Cys substitution was introduced into the cDNA encoding the A2 domain of factor VIII and the resulting construct was expressed in insect cells. Strikingly, the metabolically labeled A2 domain carrying the Arg<sup>593</sup> → Cys mutation was not recognized by the anti-factor VIII antibodies present in the plasma of the patient. These data indicate that the anti-factor VIII antibodies are exclusively directed against exogenous factor VIII. This strongly suggests that the Arg<sup>593</sup> → Cys substitution results in recognition of wild-type factor VIII as nonself and is thereby related to the formation of anti-factor VIII antibodies after factor VIII replacement therapy in this particular patient.

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Lab Ltd, Crawley, UK). Oligonucleotide primers, protein G Sepharose-4FF, CNBr-activated Sepharose-4B, and Gelatin Sepharose-4B were obtained from Pharmacia-LKB (Woerden, The Netherlands). The Taq track sequencing system was obtained from Promega (Madison, WI). Pfu-polymerase was purchased from Stratagene (Cambridge, UK). Radioactive chemicals were obtained from Amersham (‘s Hertogenbosch, The Netherlands). The plasmid pAcGP67B and the Baculogold Baculovirus Autographa californiae DNA were purchased from Pharmingen (San Diego, CA). Spodoptera frugiperda (SF-9) insect cells and Trichoplusia ni High Five insect cells were obtained from Invitrogen (Leek, The Netherlands). Characterization of monoclonal antibodies (MoAbs) CLB-CAg 9 and CLB-CAg 117 has been described previously.

Mouse MoAbs directed against human IgG1, IgG2, IgG3, IgG4, and IgM were obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service and coupled to CNBr-activated Sepharose-4B.

**Coagulation assays.** Blood was collected for coagulation assays by venapuncture in tubes containing 9 vol blood and 1 vol 3.2% (wt/vol) trisodium citrate. The blood sample was centrifuged for 10 minutes at 12,000 g. Plasma was collected and immediately stored at −70°C in 0.5 to 1.0-mL aliquots. Factor VIII procoagulant activity was assayed by a one-stage clotting assay.

Inhibitor titers were measured in plasma using the Bethesda assay essentially as described previously.

**Molecular genetic studies.** Blood was collected by venapuncture in EDTA for isolation of peripheral blood lymphocytes. Chromosomal DNA was extracted from the lymphocytes by Qiagen columns (Qiagen GMBH, Hilden, Germany) according to the manufacturer’s instructions. The 26 exons of the factor VIII gene with the bordering intron regions were amplified by polymerase chain reaction (PCR) using oligonucleotide primers that will be described elsewhere (manuscript in preparation). PCR products were analyzed by single-stranded conformation polymorphism (SSCP) and PCR products displaying an aberrant SSCP pattern were analyzed by direct sequencing.

**Plasmid constructions.** The previously described plasmid pCLB-BPVdB695 has been used as template in the construction of plasmids encoding fragments of factor VIII. Reaction conditions for PCR were 5 minutes at 95°C; 2 minutes at 50°C; 3 minutes at 72°C; 17 cycles of 45 seconds at 95°C, 90 seconds at 50°C, and 3 minutes at 72°C; and 5 minutes at 65°C in the presence of 1 mmol/L dNTPs, Pfu-polymerase reaction buffer, 50 pmol of each oligonucleotide primer, and 2.5 U Pfu-polymerase. The PCR product was purified and digested with KpnI and NdeI fragments were cloned together into the pAcGP67B plasmid, yielding pCLB-GP67-80K. The plasmid pCLB-BPVdB695 plasmid was digested with NdeI (nucleotide position 5521 of factor VIII) and NotI (nucleotide position 5779 of factor VIII) and the resulting construct, designated pCLB-GP67-A2, cDNA encoding the leader peptide of the acidic glycoprotein GP67. The plasmid pAcGP67B-A2 was digested with NdeI and NotI and cloned into plasmid pAcGP67B. In the resulting construct, designated pCLB-GP67-A2, cDNA encoding amino acid sequence Ser72→Arg40 of factor VIII is fused to that encoding the leader peptide of the acidic glycoprotein GP67. The pCLB-GP67-90K plasmid encoding amino acids Ala1→Arg40 of the heavy chain of factor VIII was constructed by amplifying a 548-bp fragment using oligonucleotide primers 90K-1 (5′TCTCCATTGG-GTGCACCCAGAAGATAC3′; nucleotide position 58-75 of factor VIII, sense) and 90K-2 (5′ACATACTAGTGGGCTC3′; nucleotide position 577-594 of factor VIII, antisense). The 548-bp PCR product was purified and digested with NcoI and NdeI fragment and the resulting fragment was cloned into the pAcGP67B plasmid together

with the Nco I-Nde I fragment and the Nde I-Kpn I fragment. The resulting plasmid was termed pCLB-GP67-90K. The pCLB-GP67-80K plasmid, encoding amino acids Glu714→Ty722 of the light chain fragment of factor VIII, was constructed by amplifying a fragment using oligonucleotide primers 80K-1 (5′GCCCATGGGGGAAA-

TAACTGTACTACTCTC3′; nucleotide position 5002-5020 of factor VIII, sense) and 80K-2 (5′CTGACTGTCTGTGGCTC3′; nucleotide position 5659-5679 of factor VIII; antisense). The PCR product was purified and digested with NcoI and NdeI. The pCLB-BPVdB695 plasmid was digested with Nde I (nucleotide position 5521 of factor VIII) and Not I. The resulting Nco I-Nde I and Nde I-Not I fragments were cloned together into the pAcGP67B plasmid, yielding pCLB-GP67-80K. The plasmid pCLB-GP67B-A2-R593C encoding the A2 domain of factor VIII in which the Arg593 is replaced by Cys was constructed by site-directed mutagenesis using overlap extension. Oligonucleotide primer R593C-1 (5′GAGAGGAGA-

TTCTGTTCTCTCC3′; nucleotide position 1822-1844 of factor VIII; sense) was used together with oligonucleotide primer A2-2 to amplify a part of the A2 domain. Oligonucleotide primer R593C-2 (5′GGGAGAAAGAGTGATAT3′; nucleotide position 1822-1844 of factor VIII; antisense) and oligonucleotide primer A2-1 were used to amplify a fragment corresponding to the amino terminus of the A2 domain. Both amplified fragments were purified and used as template in a PCR using primer A2-1 and A2-2 to amplify a fragment that was purified and digested with NcoI and NotI and cloned into the pAcGP67B plasmid, yielding pCLB-GP67B-A2-R593C. The nucleotide sequence of all constructs was verified by oligonucleotide sequencing.

**Expression and metabolic labeling of selective domains of factor VIII in insect cells.** Recombinant baculoviruses expressing the various recombinant factor VIII fragments were obtained after transfection of SF-9 cells according to the instructions of the manufacturer. High Five cells were infected with recombinant baculoviruses at a multiplicity of infection of 7. The cells were maintained in culture medium containing 25% (vol/vol) Grace’s insect medium and 75% (vol/vol) of EX-CELL 401 medium supplemented with 2.5% fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. At 24 hours postinfection, the cells were pulse-labeled with [35S]-methionine (50 μCi/mL; specific activity, >800 Ci/mmol) for 24 hours in a similar culture medium lacking methionine. Medium of metabolically labeled cells was collected in an equal volume of 2 times concentrated immunoprecipitation buffer (IPB). IPB consists of 50 mmol/L Tris (pH 7.6), 1 mmol/L NaCl, 1.2% (vol/vol) Triton X-100, 0.1% (vol/vol) TWEEN-20, 1.0% (wt/vol) bovine serum albumin, 35 mmol/L EDTA, 10 μg/mL soybean trypsin inhibitor, 10 mmol/L benzamidine, and 5 mmol/L N-ethylmaleimide.

**Immunoprecipitation.** Immunoprecipitation was performed essentially as described previously.

Conditioned media were preclariied by incubation for 2 hours at room temperature with Gelatin Sepharose 4B and two successive incubations with protein G Sepharose 4FF. Specific adsorption was performed overnight at 4°C by adding preformed complexes of protein G Sepharose 4FF and 30 μL of patient plasma. MoAbs CLB-CAg 9 (1 μg/mL) directed against the A2 domain of factor VIII and CLB-CAg 117 (1 μg/mL) directed against the light chain of factor VIII were used to monitor the expression of the factor VIII fragments. Plasma of several healthy donors was used to demonstrate the specificity of the adsorption.

For antibody subclass typing, specific adsorption was performed using αG1, αG2, αG3, or αG4 covalently linked to CNBr-activated Sepharose-4B. Immunoprecipitates were extensively washed with IPB and finally with 20 mmol/L Tris-HCl buffer, pH 7.6. Bound protein was eluted by boiling for 5 minutes in sodium dodecyl sulfate (SDS) sample buffer and analyzed under reducing conditions on a 10% (wt/vol) SDS-polyacrylamide gel. After electro-phoresis, gels were fixed in 30% methanol and 10% acetic acid and
Table 1. Patient Characteristics Over Time

<table>
<thead>
<tr>
<th>Time</th>
<th>Factor VIII Activity (IU/mL)</th>
<th>Inhibitor Concentration (BU/mL)</th>
<th>Half-Life (h)</th>
<th>Bleeding Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before 1980</td>
<td>0.20</td>
<td>0.0</td>
<td></td>
<td>Mild</td>
</tr>
<tr>
<td>1980</td>
<td>0.01</td>
<td>22</td>
<td>2-3</td>
<td>Severe muscle hematomas</td>
</tr>
<tr>
<td>1981</td>
<td>0.08</td>
<td>1.2</td>
<td></td>
<td>Mild</td>
</tr>
<tr>
<td>1992</td>
<td>0.14</td>
<td>0.7</td>
<td></td>
<td>Mild</td>
</tr>
<tr>
<td>1994</td>
<td>0.14</td>
<td>0.7</td>
<td>5.5</td>
<td>Mild</td>
</tr>
<tr>
<td>1995</td>
<td>0.14</td>
<td>0.4</td>
<td></td>
<td>Mild</td>
</tr>
</tbody>
</table>

Plasma levels of factor VIII and inhibitor were determined as described in the Materials and Methods. The factor VIII antigen levels (measured by an enzyme-linked immunosorbent assay using two different MoAbs directed towards the light chain of factor VIII) were 0.08 IU/mL in the plasma samples of 1992, 1994, and 1995. No cross-reactivity of the inhibitor against porcine factor VIII was observed in 1992.

Patient. Patient A, born in 1961, was affected by mild hemophilia A with a factor VIII level of about 0.20 IU/mL. His HLA-identical twin brother, patient B, also suffered from mild hemophilia A, with a similar factor VIII level. In 1980, patient A developed an inhibitor after a period of extensive perioperative replacement therapy. He had frequent hematomas and spontaneous muscle bleeding episodes, irrespective to factor VIII administration, that could be controlled by Feiba (Immuno AG, Vienna, Austria). Concomitant with an increase in the titer of the inhibitor, the level of circulating factor VIII decreased to less than 0.01 IU/mL (Table 1). Apart from the inhibitor, no further abnormalities could be detected when extensive laboratory evaluations and immunopathologic tests were performed. After 15 months, the level of the inhibitor decreased to less than 1 BU/mL and remained at a low level. During the next 12 years there were only five bleeding episodes, all successfully treated with factor VIII concentrate. In 1992, the patient received extensive perioperative factor VIII replacement therapy again. In 1994, the in vivo recovery of immuno-affinity purified factor VIII concentrate was decreased (0.52 IU/mL after the administration of 50 IU/kg) and the factor VIII half-life was rather short (5.5 hours). This suggested that antibodies directed against factor VIII were present 14 years after the first clinical signs of the inhibitor. Strikingly, the patient had a normal response after 1-deamino-8-D-arginine vasopressin (DDAVP) administration (0.3 µg/kg) when this was tested 1 week later. His factor VIII increased from a baseline level of 0.15 IU/mL to 0.55 IU/mL. Plasma samples from 1992 onwards have been used in the present study to characterize the anti-factor VIII antibodies by immunoprecipitation.

Patient B developed a transient inhibitor around the same time as patient A. His inhibitor was lower (peak, 9.0 BU/mL) and the clinical picture was less severe. He had received factor VIII replacement therapy on about 5 occasions before he developed an inhibitor.

Characterization of the anti-factor VIII antibodies. To characterize the antibodies against factor VIII present in the plasma of patient A, immunoprecipitation analysis was performed with metabolically labeled factor VIII fragments expressed in insect cells. These experiments showed the presence of antibodies in the patient’s plasma directed against the heavy chain of factor VIII (Fig 1). Further analysis indicated that these antibodies bound to the carboxyterminal part of the heavy chain, the A2 domain. In contrast, no detectable binding of the antibodies to the metabolically labeled light chain of factor VIII was observed, whereas MoAb CLB-CAg 117 reacted readily with this fragment. A plasma sample of patient B from 1993 was analyzed in a similar manner but no antibody binding to metabolically labeled domains of factor VIII could be detected upon immunoprecipitation analysis (data not shown).

Subclass determination was performed by immunoprecipitation using subclass specific antibodies. The anti-factor VIII...
A2R593C was present in the conditioned medium in a similar concentration as the wild-type A2 domain (Fig 4, lanes 1 and 2). Human antibodies, present in the plasma of two other hemophilia A inhibitor patients and one spontaneous inhibitor patient, also bound to both wild-type and modified A2 domain (data not shown). However, the antibodies present in the plasma of patient A did not react with the modified A2 domain. This observation provides evidence that the anti-factor VIII antibodies react solely with wild-type factor VIII and are not directed towards the mutant factor VIII synthesized by the patient. Thus, in this particular patient, our data suggest a link between the missense mutation causing factor VIII deficiency and antibody development.

DISCUSSION

We report a patient affected by mild hemophilia who developed anti-factor VIII antibodies that appeared to be exclusively directed to wild-type A2 domain when analyzed by immunoprecipitation. The antibodies did not bind to the A2 domain containing the Arg
\[ ^{593} \text{Cys} \]
substitution associated with hemophilia A in this patient.

Inhibitor development in patients with mild hemophilia is relatively rare. Thirty-two cases concerning inhibitor development in moderate or mild hemophilia have been published.11,12,23-38 In some of these patients inhibitor formation is reported to be related to a period of intensive factor VIII replacement therapy. In patient A, antibody formation was

Relation of the genetic defect associated with hemophilia A and antibody formation. To study a relation between the genotype of hemophilia A and antibody formation in this patient, we screened the factor VIII gene for a genetic defect by SSCP. An aberrant migration pattern was observed for the fragment corresponding to exon 12 of the factor VIII gene. Direct sequencing showed a point mutation (C \[ \rightarrow \text{T} \]) that predicted replacement of Arg
\[ ^{593} \text{Cys} \] in this patient, in agreement with a previous report.22

We hypothesized that this mutation could be related to the epitope of the antibody. To address this issue, we introduced the Arg
\[ ^{593} \text{Cys} \] in the construct that encodes for the A2 domain. The resulting construct, termed pCLB-GP67-A2-R593C, was expressed in insect cells. Immunoprecipitation of the metabolically labeled modified A2 domain, termed A2R593C, with MoAb CLB-CAg 9 showed that antibodies present in the plasma of patient A consisted of subclasses IgG2 and IgG4 (Fig 2). We next examined the reactivity of the anti-factor VIII antibodies in plasma samples of patient A from 1992 to 1995. The amount of metabolically labeled A2-domain reactive with the anti-factor VIII antibodies was found to decrease, indicating a decline in the antibody concentration in plasma over time (Fig 3).

We have examined the binding of the antibodies over time (Fig 3). Immunoprecipitation analysis of antibodies binding to the A2 domain of factor VIII, with plasma samples of patient A from various time points. Lanes 1 through 4, plasma of patient A from 1992, 1993, 1994, and 1995; and lanes 5 and 6, plasma of patient A from 1992 and 1995. Some nonspecific binding of a protein of approximately 100 kD was observed in this particular experiment. A molecular weight marker is given on the right of the figure.
indeed preceded by a period of intensive factor VIII replacement therapy. The low frequency of inhibitor formation in patients with mild and moderate hemophilia may result from tolerance to administrated factor VIII, induced by endogenously synthesized factor VIII. Our data indicate that a missense mutation that alters the immunogenic determinants of the endogenously synthesized factor VIII may predispose to antibody development upon initiation of factor VIII replacement therapy. This suggests that wild-type factor VIII is recognized as nonself by the immune system of this patient.

The Arg to Cys mutation at amino acid position 593 in the factor VIII gene has been identified in other patients, but it has not been associated with inhibitor development. This and the milder clinical picture of the twin brother illustrates the importance of additional factors besides the genotype.

It should be noted that immunoprecipitation was performed with plasma samples obtained 12 to 15 years after the inhibitor was first observed. At this point, the inhibitory activity had decreased considerably according to the Bethesda assay. Because the factor VIII specificity of inhibitors may change over time, it cannot be excluded that the antibodies present in the tested samples differ in epitope specificity from the antibodies present at the time of the initial inhibitor detection. An antibody with a different epitope could explain the decrease in circulating factor VIII levels to less than 0.01 IU/mL in 1980, which is unlikely to result from the anti-factor VIII antibodies described in this report. We assume that the antibodies binding to the A2 domain of factor VIII are the cause of the slight inhibition of factor VIII activity in the Bethesda assay, present in the plasma samples from 1992 to 1995. However, the inhibitory nature of the antibodies could not be ascertained, because neutralization experiments using wild-type A2 domain and modified A2 domain were unsuccessful, most likely due to the very low inhibitor titer. It appears that this patient has had the following sequence of immunologic events. After the initial loss of immune tolerance involving shared epitopes, the patient subsequently developed immune tolerance to all but the foreign epitope. This observation is reminiscent of the sequence of events in posttransfusion purpura (PTP).

In PTP, an immune response is elicited by alloantigens present on platelets in transfused blood products. The transient thrombocytopenia observed in these patients is thought to result from reactivity of the antibodies with autologous platelets.30

The Arg to Cys mutation at amino acid position 593 in factor VIII may change the conformation of the A2 domain of factor VIII.41 Furthermore, we cannot exclude that the baculovirus-expressed A2R593C domain has an altered structure due to an S-S bond peptide. This would then provide an alternative explanation for lack of binding of the patient’s antibody to the mutated A2 domain. However, gross alterations in the mutant A2 domain seem unlikely, because the mutant A2 domain is bound by antibodies present in the plasma of two other hemophilia A inhibitor patients and one spontaneous inhibitor patient.

Two other missense mutations leading to a new cysteine residue at amino acid position 2105 and 2229 of factor VIII have been associated with inhibitor development in mild and moderately severe hemophilia A.42 Furthermore, as in our patient, a discrepant response to factor VIII concentrate and DDAVP has been reported in two brothers with mild hemophilia caused by an Arg2150→His mutation.33 Together with the findings in the present report, these observations indicate that certain missense mutations in the factor VIII gene may predispose to inhibitor formation. Our findings may provide a molecular basis for the increased risk of inhibitor formation after factor VIII replacement therapy in certain families affected by mild hemophilia A.43

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Aveolar soft part sarcoma. A 25-year-old woman presented with an 18-month history of an enlarging thigh mass. At the time of removal of this mass, multiple bilateral pulmonary metastases were present; other sites of spread were absent. The diagnosis of alveolar soft part sarcoma was confirmed by the electron microscopic identification of unique membrane-bound, intracellular rhomboid crystals seen in longitudinal (A) and cross-sectional (B) views. (Original magnification [A] × 171,000 and [B] × 251,000.) (Courtesy of Ann M. Dvorak, MD, Department of Pathology, Beth Israel Hospital, Harvard Medical School, 330 Brookline Ave, Boston, MA 02215.)
The Missense Mutation $\text{Arg}^{593} \rightarrow \text{Cys}$ Is Related to Antibody Formation in a Patient With Mild Hemophilia A

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