A Familial Hemorrhagic Diathesis in a Dutch Family: An Inherited Deficiency of $\alpha_2$-Antiplasmin

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This study concerns a case of congenital homozygous deficiency in $\alpha_2$-antiplasmin associated with a severe hemorrhagic diathesis. Heterozygous family members also show a mild bleeding tendency. The propositus is a 17-yr-old male born of white parents and showing a severe hemorrhagic diathesis characterized by spontaneous bleeding in the joints since his early childhood. He was originally suspected of having factor XIII deficiency but was found to have normal functions of the coagulation system and the platelets. Except for $\alpha_2$-antiplasmin, all protease inhibitors showed normal plasma values. With the immediate plasmin inhibition test (synthetic substrate), only 2% of normal functional inhibition was detected, while no reaction with monospecific antisera for $\alpha_2$-antiplasmin was observed. Inhibition of activator-induced fibrinolysis in vitro was reduced. No enhanced spontaneous in vitro fibrinolysis was detected nor were there signs of increased in vivo fibrinolysis during an asymptomatic period. During recovery from a hemorrhagic episode, signs of previous consumption of antithrombin III, $\alpha_2$-macroglobulin, factor XIII, and inter-α-trypsin inhibitor were noted. After the diagnosis was made, treatment with tranexamic acid (4 daily doses of 1 g) was effective for about 2 yr. Among the 37 family members studied, a separate group of 16 individuals (including the father and mother of the propositus) with approximately one-half normal plasma levels of $\alpha_2$-antiplasmin both functionally (59% ± 6%) and immunologically (48% ± 8%) was discovered. The defect appeared to be inherited as an autosomal recessive gene; no ancestral consanguinity could be shown. The group of apparent heterozygotes as a whole showed increased levels of $\alpha_2$-antitrypsin (142% ± 39%; p < 0.01), indicating systemic consequences of the deficiency and reduced binding (±50%) of $\alpha_2$-antiplasmin to fibrin. Six exhibited a mild hemorrhagic diathesis for which no explanation was provided by routine screening of coagulation and platelet functions; also, within the group of heterozygotes, the occurrence of the bleeding tendency did not correlate with differences in residual $\alpha_2$-antiplasmin levels and functions. It is concluded that not only the absence of $\alpha_2$-antiplasmin but also a reduction in its plasma level to ±60% of normal may predispose to a hemorrhagic diathesis.

THE PLASMA PROTEINASE inhibitor $\alpha_2$-antiplasmin is an $\alpha_2$-glycoprotein with an estimated molecular weight of 65,000-70,000 daltons and a plasma concentration established at around 1 μM. Its physiologic function is most likely the strong inhibition of plasmin, the ultimate enzyme of the fibrinolytic system. In recent years, the mechanism of action of the inhibitor has been further elucidated and conclusive evidence for its specificity for fibrinolysis has been provided.

A recently discovered aspect of the mechanism of action of $\alpha_2$-antiplasmin, which is also determinative for its specificity for fibrinolysis, is its covalent binding to fibrin mediated by coagulation factor XIIIa. $\alpha_2$-Antiplasmin bound to the fibrin clot reduces its breakdown.

The physiologic importance of $\alpha_2$-antiplasmin was made apparent by the discovery of congenital deficiencies. Theoretically, the lack of $\alpha_2$-antiplasmin leads to unrestrained fibrinolysis. Its actual consequence was fully realized only with the discovery of a person with a homozygous deficiency in the inhibitor who showed a severe hemorrhagic diathesis.

This report concerns a family showing congenital $\alpha_2$-antiplasmin deficiency and including a homozygous case with a severe hemorrhagic diathesis, emphasizing the relation between the symptoms and the deficiency. In addition, a mild hemorrhagic diathesis is reported in six of the heterozygotes, extending the incidence of clinical manifestations to reduced plasma levels of $\alpha_2$-antiplasmin.

MATERIALS AND METHODS

Materials

Unless otherwise specified, reagents were of analytical grade and were obtained from Merck, Darmstad, West Germany. Microbiologic grade gelatin was purchased from Merck; agarose for electrophoresis, sodium dodecyl sulphate (SDS), ethylene-diamine-tetra-acetic acid disodium salt (EDTA), and sucrose from BDH chemicals, Poole, England. Dextran sulphate, sodium salt (mol wt 500,000) was obtained from Pharmae Ltd., Uppsal, Sweden. Pluvenamic acid (N-a.a.a.-trifluoro-m-tolyl anthranilic acid) was from Aldrich Europe, Beens, Belgium. Carbowax 6000 was from Fluka AG, Buchs, Switzerland; Coomassie brilliant blue R-250 from Serva, Heidelberg, West Germany, adenosine diphosphate (ADP) from Sigma Chemical Co., St. Louis, Mo.; and ristocetin from H.
Lundbeck & Co., Copenhagen, Denmark. Automated APTT (activated partial thromboplastin time) reagent and congenital deficient substrate plasmas for factors VIII, IX, XI, and XII were obtained from General Diagnostics, Mijdrecht, The Netherlands. Bovine thrombin (EC 3.4.21.5) and urokinase (EC 3.4.99.26) were from Leo Pharmaceuticals, Ballerup, Denmark and Cl-inactivator preparation16 from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam. The synthetic tripeptide substrates S-2251 and S-2238, plasmin (EC 3.4.21.7), labeled 15 Casein units/mg protein, and human fibrinogen (grade L) were from AB Kabi, Stockholm, Sweden. The synthetic tripeptide substrates chromozym PK and chromozym PL and the reptilase reagent of Diagnostica Streck, is a product of the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam. The synthetic tripeptide substrate chromozym PK and chromozym PL and the reptilase reagent were from Boehringer Mannheim, Amsterdam, The Netherlands. PT-activated thromboplastin reagent was from DADE, Breuckelen, The Netherlands. Clotrimmun-factor XIII-S, XIII-A, VIII, and plasminogen, rabbit antiserum against alpha-antitrypsin, alpha-antichymotrypsin and M-Partigen plates for alpha-2-macroglobulin, alpha-antitrypsin and plasminogen were from Behringwerke AG, Marburg, West Germany. Rabbit antiserum against 3.8 S-alpha-glycoprotein17 (histidine-rich glycoprotein) was a gift of Dr. N. Heimburger, Behringwerke AG, Marburg, W-Germany. Rabbit antiserum against alpha-antiplasmin were obtained from (I) Nordic, Tilburg, The Netherlands (batch 51179) and (II) as a gift of Dr. D. Collen, Louvain, Belgium (batch DC 2). Adsorbed rabbit antiserum against alpha-antiplasmin initially raised against the plasmin-alpha-antiplasmin complex, were (III) a gift of Dr. D. Collen (batch DC 4) and (IV) a gift of Dr. S. Thorsen, Copenhagen, Denmark. Rabbit antiserum against plasminogen activator inhibitor15 was a gift of Dr. U. Hedner, Malmö, Sweden. Goat antiserum against Cl-inactivator was from Nordic, Tilburg, The Netherlands. Plasminogen was isolated from human Cohn fraction III by lysine-agarose chromatography using a linear gradient from 0 to 0.01 M e-aminocaproic acid for elution. It consisted of a mixture of glu- and lys-plasminogen. Lys-plasminogen further purified from this mixture by gel filtration on Sephadex G150 and chromatography on DEAE-Sephadex was kindly supplied by Dr. D. C. Rijken of our Institute. Plasmin was prepared by activation of human plasminogen using urokinase immobilized on Sepharose.

Tissue plasminogen activator was a partially purified preparation (step 3 material) from human uterus and obtained by extraction with 0.3 M potassium acetate buffer, pH 4.2, ammonium sulphate precipitation, and zinc chelate-agarose chromatography.15 Plasminogen-rich bovine fibrinogen was prepared according to Brakman,16 platelet-poor citrated human plasma and pooled plasma were prepared as previously described,17 and platelet-rich plasma was obtained by centrifugation at 800 g for 10 min. Serum was prepared by incubation of blood at 37°C for 4 hr in plastic tubes before centrifugation. EDTA buffer (µ = 0.15) consisted of 0.05 M sodium diethylbarbiturate, 0.10 M NaCl, 0.25% (w/v) gelatin, and 2.7 mM ethylene-diamine-tetra-acetate (EDTA) adjusted to pH 7.8 with an HCl solution.

Methods

Immunochemical Techniques

Factor XIII and alpha-antiplasmin (using antisera 1), 3.8 S-alpha-glycoprotein, and factor VIII were determined by the Laurell technique and inter-alpha-antitrypsin inhibitor, alpha-antichymotrypsin, and Cl-inactivator by the Mancini technique using the corresponding monospecific antisera. alpha-Macroglubulin, alpha-antitrypsin, and plasminogen were determined with M-Partigen plates. Some of the assays on factor XIII were kindly performed by R. Zijlstra, Maria Stichting Hospital, Haarlem, The Netherlands. Results were expressed in percentages of the content of pooled normal plasma. When compared with a commercial standard plasma or serum, this pool was found to contain 197 mg·100 ml^-1 alpha-macroglubulin, 201 mg·100 ml^-1 alpha-antitrypsin, and 11.7 mg·100 ml^-1 plasminogen.

Ouchterlony double diffusion analysis, electroimmunodiffusion, and the Laurell and Mancini techniques were performed in 1% (w/v) agarose gels (1 mm) buffered with 0.03 M sodium diethylbarbiturate, pH 8.6. Electrophoresis was carried out overnight at 100 V and 10°C; for the electroimmunodiffusion, electrophoresis was two-fold prolonged (90 min) as compared to standard procedures to obtain a more complete separation of lys- and glu-plasminogen. Diffusion was allowed to proceed for 48 hr at 4°C. The agarose plates were stained with Coomassie brilliant blue R-250.

Fibrinolysis Techniques

The normal euglobulin fractions of plasma were prepared at pH 5.9 with a plasma dilution of 1:10 as described previously.15 Precipitates were redissolved in EDTA buffer. Activities were assayed on plasminogen-rich bovine fibrin plates16 and results expressed in diameters of lysed zones in the plates after 18-hr incubation at 37°C. Recorded were the spontaneous activity of 30 µl of the fraction and the activity obtained after elimination of inhibitors by addition of 5 µl 14 mM sodium flufenamate to the 30-µl drop.15 The Cl-inactivator-resistant activator activity, representing specifically the vascular or extrinsic activator activity in blood,15,16 was assayed by addition of 5 µl purified Cl-inactivator to the 30 µl drop to obtain a final concentration of approximately 125% Cl-inactivator (relative to pooled plasma).17 Total plasminogen activator plus proactivator level in plasma was assayed with the blood activator inventory test. The activity of the dextran sulphate euglobulin fraction was expressed in arbitrary blood activator units (BAU·ml^-1) as described previously. One-hundred BAU·ml^-1 has been shown to correspond to about 0.7 International Units of urokinase activity. The contributions of the factor-XII-dependent and XII-independent proactivator systems can be revealed separately by inhibition of the factor-XII-independent system in plasma containing baseline levels of activity. For this purpose, an extract of Schistosoma mansoni is added to a final concentration of 8 mg dry weight/ml, usually revealing ±50% inhibition of the activity, as described previously.21 Euglobulin clot lysis time methods were performed by clotting 0.2 ml normal euglobulin fraction with 0.1 ml thrombin (10 NIH units·ml^-1) in 0.075 M NaCl, CaCl₂ (0.025 M), or alternatively, with 0.1 ml thrombin (20 NIH units·ml^-1) in 0.15 M NaCl. The lysis time is the time elapsing between the observation of complete visual disappearance of fibrin. Dilute plasma clot lysis was performed essentially as described by Fearney and Tweed22 with serial plasma dilutions in EDTA buffer from 1/2 to 1/128, pH 7.4. Each dilution (0.2 ml) was clotted with either 0.1 ml thrombin (10 NIH units·ml^-1) in 0.15 M NaCl, or 0.1 ml thrombin (20 NIH units·ml^-1) in 0.15 M NaCl. The number of clots lysed after 24 hr is expressed by a score of plus (+) signs. For whole blood clot lysis, spontaneously clotted blood held at 37°C was observed.

Inhibitor Assays

Immediate plasmin inhibition assay: This assay is a modification of the Coatest antiplasmin procedure of Kabi Vitrum (a gift of C. Kortmann) and the procedure described by Gallimore and coworkers. To 120 µl buffer (0.05 M Tris, 0.11 M NaCl, pH 7.4, with 1.4 mg·ml^-1 Carbowax) equilibrated in a polystyrene tube (5 x 1 cm) at 37°C, 0.2 µl plasma (or dilutions) and 40 µl 3.5 mM S-2251 were added. The reaction was started with 40 µl (approx. 0.15 CU·ml^-1) plasmin in 50% glycerol added with a Hamilton syringe. The mixture was rapidly transferred to a cuvette (type 178, Hellma Benelux, The Hague, The Netherlands), placed in a thermostatically controlled cuvette holder, and ΔOD(405 nm)·min^-1 was recorded.

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Plasmin inhibition was expressed as percentage relative to pooled normal plasma, prepared as described elsewhere. In a linear dose-response curve of residual plasmin activity versus amount of plasma between 0%–50% inhibition. To prevent deterioration of plasmin activity, Carbowax was found to be necessary as a supplement to the cuvette buffer. The assay of the control plasmin activity (without addition of plasma) presents a problem, possibly because of the very low plasmin concentrations (about \(2 \times 10^{-6} M\)) involved. To prevent adsorption in our tube/pipette/cuvette system, addition of 2 \(\mu g\cdot ml^{-1}\) poly-D-lysine to the buffer, the presence of plasma proteins, and treatment of the cuvettes with 5 \(N\) NaOH were found to be of value. The specificity of the method used for \(\alpha_2\)-antiplasmin, characterized by the recording of immediate (plasmin) inhibition and the addition of components in reverse order, was confirmed by (A) the observation that the propositus had only 2% residual inhibition; (B) the absence of correlation with levels of the other two major inhibitors of plasmin: \(\alpha_2\)-macroglobulin (in 16 persons; range in \(\alpha_2\)-macroglobulin: 46%–222%), and antithrombin III (in 11 persons; range in antithrombin III: 98%–135%). Inhibition of the fibrinolytic activities of plasmin, urokinase, and human tissue plasminogen activator was done by a fibrin clot lysis method. The fibrin clot was formed by mixing 0.1 ml enzyme, 0.1 ml plasma dilution, 0.05 ml human plasminogen (3 mg.ml\(^{-1}\)) at 0\(^\circ\)C, followed by incubation at 37\(^\circ\)C. The time between clotting and lysis was determined and used for calculation of the residual activity of the enzyme. Results of individual plasmas were compared with those of the normal plasma pool and, using a standard curve obtained with a series of normal plasma dilutions, expressed as percent inhibition.

Plasmin inhibition by plasma was also evaluated on fibrin plates containing the plasma. The plates were prepared by mixing the fibrinogen solution with plasma, usually 0.5% (v/v) final concentration, before clotting with thrombin. The inhibition of plasmin on fibrin plates with patients’ plasma was compared with that on plates containing normal pooled plasma and expressed as a percentage of the inhibition by normal plasma.

**Assay of Platelet Function**

The bleeding time was determined by the method of Ivy. Platelet aggregation studies were performed by the turbidimetric method of Born using an aggregometer (Payton-Canada). The final concentrations of the aggregating agents used, ADP and ristocetin were 0.8 \(\mu g\cdot ml^{-1}\) and 1.1 \(\mu g\cdot ml^{-1}\), respectively.

**Assay of Coagulation Factors**

The activated partial thromboplastin time (automated APTT reagent), prothrombin time, and reptilase time were measured on a Coagulizer (Sherwood Medical Industries, Bridgeton, Miss.). Factors VIII, IX, XI, and XII were assayed in a one-stage assay on the Coagulizer using congenitally deficient plasma as substrate. Fibrinogen was measured as previously described and fibrinogen degradation products (FDP) with the Ihrombo Wellcotest (Wellcome). Antithrombin III was assayed with the chromogenic substrate S-2238.

**Other Methods**

The capacity of plasma to induce cross-linking of fibrin was evaluated qualitatively by clotting citrated plasma with an equal volume of 0.1 \(M\) Tris-HCl, pH 7.4, containing 40 \(mM\) CaCl\(_2\), 40 \(mM\) cysteine, and 0.9 \(NIH\ U\cdot ml^{-1}\) thrombin. The formed fibrin was incubated for 2 hr at 37\(^\circ\)C. Cross-linking was assessed by polyacrylamide gel electrophoresis in sodium dodecyl sulphate after dissolution of the clot in phosphate buffer, pH 7.0, containing SDS and 2-mercaptoethanol.

Plasmin-\(\alpha_2\)-macroglobulin complex, measurable as spontaneous activity of nonactivated plasma samples, was assayed on chromozym PL in the presence of soy bean trypsin inhibitor (SBTI) to obtain specificity for \(\alpha_2\)-macroglobulin-bound plasmin. The activity was followed as the increase in \(\Delta OD\cdot min^{-1}\) at 405 nm for 10 min in the following mixture: 10 \(\mu l\) plasma, 25 \(\mu l\) 1 \(mM\) chromozym PL, 190 \(\mu l\) Tris-imidazole-NaCl buffer, \(\mu = 0.15\), pH 7.9, with 50 ppm SBTI.

Binding of \(\alpha_2\)-antiplasmin to fibrin was studied by clotting 180 \(\mu l\) citrated plasma with a 120 \(\mu l\) calcium chloride (37.5 \(mM\)), thrombin (4 \(NIH\ U\cdot ml^{-1}\)), NaCl (37.5 \(mM\)) mixture for 1 hr at 37\(^\circ\)C. In the serum supernatant and in a plasma sample incubated with 120 \(\mu l\) 0.15 \(M\) NaCl, \(\alpha_2\)-antiplasmin activity was assayed by the above described immediate plasmin inhibition test. The difference represented the amount of \(\alpha_2\)-antiplasmin bound to fibrin.

Prekallikrein concentrations in plasma were assayed after optimal conversion to kallikrein with 25 \(\mu g\cdot ml^{-1}\) dextran sulphate at 0\(^\circ\)C as described previously. Activity on chromozym PK was expressed in nkat\(-ml^{-1}\).

**RESULTS**

**Case History**

The patient is a 17-yr-old male born of white parents and who has had a hemorrhagic diathesis since his early childhood. He showed no abnormal umbilical bleeding at birth. At the age of 1 yr, the first hemorrhagic complication appeared. While playing, a fish-hook lodged in his throat. After the hook was removed, bleeding persisted for 14 days. No special treatment was given. The following years were characterized by rapidly developing hematomas after a minor trauma. After the age of 6, spontaneous joint bleedings occurred, especially in the knees and ankles. In addition, subarachnoid and muscle bleeding in the upper left arm were noted. At the age of 10 yr, a nevus pigmentosus was removed; this was complicated by bleeding for several days. Treatment with plasma and cryoprecipitate was unsuccessful. This complication also occurred after surgical incision of a paronychium. Severe bleeding in the thigh and episodes of hematuria were also observed.

At the age of 16 yr, he developed paraplegia of the legs, without a demonstrable trauma. Before surgical intervention, a routine coagulation study was performed (plasma A1). This showed a low level of factor XIII according to the 5 \(M\) urea solubility method and a high FDP titer. During the operation, a large epidural hematoma localized at the first lumbar vertebra was found. The surgical intervention was complicated by bleeding, which started some hours after the operation (plasma A2). He was first treated with plasma and cryoprecipitate without success. After later administration of tranexamic acid, the bleeding soon stopped. Since the case was diagnosed, the patient has been treated successfully with tranexamic acid at 1 g four
times per day. During a 2-yr period on this treatment, minor bleedings have occurred only twice.

The family history revealed that his father was already known for a hemorrhagic diathesis with a bleeding episode after an appendectomy and hematomas after small trauma. Five other members of his family also showed symptoms such as rapidly developing hematomas and bleeding after venipuncture, and three of them have had bleedings for some hours after tooth extraction, for which they had already consulted a general practitioner. One such episode lasted for 1 wk. In these persons, routine screening for coagulation and platelet function provided no explanation for the symptoms.

**Factor XIII**

Factor XIII deficiency was initially suspected to be involved in the bleedings of the propositus (O.J.). Table 1 shows an immunochemical and functional evaluation of this factor in the plasma of the propositus and his parents. The factor XIII (subunits A and S) levels and the cross-linking capacity returned to normal after the propositus had recovered from the bleeding episode (sample B, 2 wk after A2, normal sedimentation and acute-phase globulin levels; Table 2). The data obtained with the plasma of his parents were normal, which further excluded an inherited defect involving factor XIII.

**Absence of α₂-Antiplasmin**

Immediate plasmin inhibition assayed in the plasma of the propositus (Fig. 1) by a synthetic substrate method was only 2% of that of pooled normal plasma. Addition of a constant amount of plasma of the propositus to the assay system did not significantly change the degree of inhibition by a range of pooled plasma dilutions (Fig. 1). Preincubation of plasmas for 15 min did not change this pattern. This confirmed the very low level of α₂-antiplasmin in the propositus plasma and demonstrated the failure of restoration of inhibition by addition of propositus plasma to normal plasma in various ratios. It also provided evidence against the presence of factors such as antibodies directed towards α₂-antiplasmin and interfering with the assay in the patients’ plasma, although the possibility of “low titer” antibody, the effect of which is time dependent, is not eliminated by this experiment.

The rocket immunoelectrophoresis (Laurell technique) with monospecific antiserum (antiserum I) against α₂-antiplasmin showed no detectable precipitation with the propositus plasma. The detection limit was assessed at 0.2% of the normal plasma level. Ouchterlony triangles with antisera I and II (Fig.

**Table 1. Studies on Factor XIII**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Factor XIII Subunit A (%)</th>
<th>Factor XIII Subunit S (%)</th>
<th>Cross-linking</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal plasma</td>
<td>100*</td>
<td>100*</td>
<td>+</td>
</tr>
<tr>
<td>F-XIII-deficient plasma</td>
<td>0</td>
<td>30</td>
<td>-</td>
</tr>
<tr>
<td>Propositus (A1)</td>
<td>45</td>
<td>70</td>
<td>±</td>
</tr>
<tr>
<td>Propositus (B)</td>
<td>80</td>
<td>90</td>
<td>+</td>
</tr>
<tr>
<td>Mother</td>
<td>80</td>
<td>85</td>
<td>+</td>
</tr>
<tr>
<td>Father</td>
<td>140</td>
<td>150</td>
<td>+</td>
</tr>
</tbody>
</table>

(+) Almost complete cross-linking; (±) incomplete α-chain cross-linking; (−) no cross-linking.

*Set at 100%.

**Table 2. Inhibitor Levels in Plasma of the Propositus**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Sample A1 (%)</th>
<th>Sample A2 (%)</th>
<th>Sample B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α₂-Macroglobulin</td>
<td>72</td>
<td>88</td>
<td>99</td>
</tr>
<tr>
<td>α₁-Antitrypsin</td>
<td>390</td>
<td>341</td>
<td>153</td>
</tr>
<tr>
<td>C1-Inactivator</td>
<td>157</td>
<td>133</td>
<td>137</td>
</tr>
<tr>
<td>Inter-α-trypsin inhibitor</td>
<td>63</td>
<td>68</td>
<td>103</td>
</tr>
<tr>
<td>α₂-Antichymotrypsin</td>
<td>531</td>
<td>431</td>
<td>149</td>
</tr>
<tr>
<td>Antithrombin III</td>
<td>70</td>
<td>73</td>
<td>100</td>
</tr>
<tr>
<td>Histidine-rich glycoprotein</td>
<td>NT</td>
<td>NT</td>
<td>132</td>
</tr>
<tr>
<td>Activator inhibitor*</td>
<td>+</td>
<td>+</td>
<td>NT</td>
</tr>
</tbody>
</table>

Data are in percent relative to pooled normal plasma; NT, not tested; (+) present according to Ouchterlony analysis. Samples A1 and A2 were obtained shortly after the bleeding episodes, B after recovery.

*Described by Hedner.¹¹

Fig. 1. Titration of plasmin with plasma in the immediate plasmin inhibition test. (O) Plasma of the propositus O.J., sample B; (♀) plasma pool; (Δ) plasma pool with 1 μl plasma of the propositus added in all assays. The residual plasmin activity on the ordinate is recorded as ΔOD · min⁻¹ in the assay (see Methods).
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Fig. 2. Ouchterlony triangle of pooled normal plasma (PP), the propositus plasma (O.J., sample B), and monospecific antiserum (II) against a2-antiplasmin (Ab).

2), both raised against purified a2-antiplasmin, showed no reaction with the plasma of the propositus. Similar experiments with antisera III and IV raised against the a2-antiplasmin-plasmin complex confirmed these results. Thus, the plasma of the propositus studied in an asymptomatic period showed no functional a2-antiplasmin and no immunochemical precipitation reaction with four independently and differently obtained antisera against a2-antiplasmin.

Other Inhibitors

The levels of inhibitors other than a2-antiplasmin were studied in three plasma samples of the propositus: two obtained shortly after the bleeding episodes and one 2 wk later after recovery (Table 2). After recovery, most plasma inhibitor levels studied showed values within normal ranges; only a1-antitrypsin and a1-antichymotrypsin remained slightly elevated. In the samples taken shortly after the bleeding episodes, the levels of the acute-phase reactive inhibitors a1-antitrypsin and a1-antichymotrypsin were markedly increased (Table 2). The levels of a1-macroglobulin, antithrombin III, and inter-a-trypsin inhibitor were decreased in that period, suggesting consumption. The plasminogen activator inhibitor described by Hedner12 was studied by the Ouchterlony technique and found to be normally present. The concentration of the recently described histidine-rich glycoprotein11,31 was normal after recovery.

Functional inhibition of plasmin, urokinase, and human tissue plasminogen activator by plasma was tested by a clot lysis time method. The propositus's plasma showed greatly reduced values of 42%, 28%, and 19% for sample A2 and 49%, 21%, and 18% for sample B, respectively, as compared with inhibition exerted by pooled normal plasma (see Fig. 6).

Coagulation

Results of coagulation studies of the propositus in an asymptomatic period were all normal as evidenced by routine coagulation tests (prothrombin time, activated partial thromboplastin time, reptilase time) and by factor assays (fibrinogen, factor VIII AHF, factor VIII AG, factors IX, XI, XII, and prekallikrein). There were no abnormalities in platelet count or function (ADP aggregation) or von Willebrand factor activity (ristocetin aggregation). In retrospect, the bleeding time has been found to be within normal limits (1–4 min) since early childhood (3 yr).

Fibrinolysis Studies

Fibrinolytic parameters of the propositus’s plasma obtained shortly after the bleeding episodes and after recovery are listed in Table 3. Screening for the

| Table 3. Fibrinolysis Studies of the Propositus |
|-------------------------------|-------|------|-------|-------|--------|
|                               | A1    | A2   | B     | PP    | Normal Range |
| Fibrin plate method           |       |      |       |       |         |
| Plasma                        | 0     | 0    | 0     | 0     | 0 mm    |
| Regular euglobulin fraction   | 6.8   | 5.5  | 9.2   | 12.0  | 9-15 mm |
| Idem + flufenamate            | 14.0  | 11.3 | 13.0  | 15.6  | 12-18 mm|
| C1-inactivator resistant activity | SL   | SL   | SL    | 6.7   | 6-8 mm  |
| Blood activator inventory test | 73   | 66   | 80    | 100   | 85-115 BAU · ml⁻¹ |
| Clot lysis method             |       |      |       |       |         |
| Whole blood                   | NT    | NT   | >36   | N.T.  | >36 hr  |
| Dilute plasma (EDTA)          | NT    | NT   | +     | +     |         |
| Dilute plasma (Ca⁺⁺)          | NT    | NT   | 0     | 0     |         |
| Euglobulin fraction (EDTA)    | NT    | NT   | 384   | 434 min |
| Euglobulin fraction (Ca⁺⁺)    | NT    | NT   | 369   | 281 min |
| Plasminogen                   | 85    | 87   | 89    | 100   | 75%-125% |
| FDP                           | 30-40 | NT   | <10   | <10   | 10 µg · ml⁻¹ |

NT, not tested, SL, starting of lysis at 18 hr; PP, pooled normal plasma. Samples A1 and A2 after the bleeding, B after recovery.

(+) 1 dilution lysed; (++) 2 dilutions lysed at 24 hr (see Methods).
fibrinolytic activity of plasma samples A1 and A2 showed a low fibrinolytic activity in the regular euglobulin assay on fibrin plates. In sample B, the activity had increased to within the normal range. The low values for samples A1 and A2 stem from increased inhibition, since flufenamate restores normal activity. The immunologically determined level of C1-inactivator, mainly responsible for the residual fibrinolysis inhibition in euglobulin fractions, was found to be elevated to, respectively, 62% and 81% of the pooled plasma concentration as compared to ±30% usually present in these fractions. Since the levels of C1-inactivator (Table 2) are only modestly elevated in patient plasma, this indicates a disproportionate deposition of C1-inactivator in the euglobulin fraction as also observed before in certain diseases.32

The C1-inactivator-resistant activity, specifically representing the plasma level of vascular-extrinsic activator, was low in all samples. The total activity of plasminogen activators plus proactivators was close to normal in sample B (80 BAU·ml⁻¹) and slightly decreased in samples A1 and A2, suggesting consumption of proactivators at that time. The ratio factor-XII-independent/factor-XII-dependent proactivator potential in sample B as assessed by inhibition with extracts of Schistosoma mansoni was close to 1 as usual.21

Euglobulin fibrinolytic activity of the propositus assessed in clot lysis assays, with clotting induced either by EDTA/thrombin or by Ca⁺⁺/thrombin, showed values close to those of pooled normal plasma. Also, plasma clot lysis was not significantly different from that of pooled normal plasma at 24 hr reading (shown in Table 3) or 48 hr reading. Plasma of the propositus (and that of several heterozygous family members) did not show fibrinolytic activity on fibrin plates. FDP levels were elevated only shortly after the bleeding episode. Plasminogen levels were normal and apparently unchanged by the bleeding episodes. Thus, extensive evaluation of blood fibrinolysis did not reveal signs of enhancement of fibrinolysis in vitro. Only shortly after the bleeding episode did the FDP levels indicate in vivo fibrinogenolysis or fibrinolysis.

α₂-Macroglobulin-Bound Plasmin Activity

The propositus’s plasma sample (A1) containing FDP as a sign of recent fibrinolysis was checked for the presence of plasmin-α₂-macroglobulin complexes that may have emerged in the absence of α₂-antiplasmin. The activity of the plasma sample of the propositus on chromozym PL in the presence of soy bean trypsin inhibitor as a measure for plasmin-α₂-macroglobulin complexes was very low (AOD·min⁻¹ = 0.0046) and identical with the activity of pooled normal plasma and plasmas of normal individuals (range 0.004–0.005). Apparently, the plasma level of plasmin-α₂-macroglobulin complexes was not significantly elevated.

Degraded Plasminogen

A sign of uncontrolled plasmin action in plasma can be the presence of degraded plasminogen (lys-plasminogen). Lys-plasminogen when added to the propositus plasma or pooled plasma, could be demonstrated to a level of 25% (Fig. 3) and maximally down to 10% of the plasma plasminogen concentration. As shown in Fig. 3, the propositus’s plasma showed no signs of the presence of the degraded form of plasminogen by the technique used.

Family Study

α₂-Antiplasmin Plasma Levels

Thirty-seven members of the family of the propositus were examined for their immediate plasmin inhibition in plasma. Sixteen were clearly distinguished from the others by a reduced plasma level of 59% ± 6% (SD) as compared to 107% ± 9% (SD) inhibition (Fig. 4). The group with approximately half-normal inhibition levels is considered to represent the heterozygotes for the deficiency in α₂-antiplasmin. Also, by immunochromatographic assay, the same group showed about half-normal plasma concentrations of α₂-antiplasmin (48% ± 8%).

There was no significant difference between heterozygotes and other family members in plasma concentrations of the second important plasmin inhibitor α₂-macroglobulin; the values were, respectively, 114% ± 34% and 97% ± 67% of normal plasma.

Fig. 3. Electroimmunodiffusion of plasma to separate Glu and Lys-plasminogen in plasma milieu. From top to bottom: (1) 3 μl plasma of the propositus (sample A1); (2) idem + 0.12 mg.ml⁻¹ Lys-plasminogen added; (3) idem + 0.06 mg.ml⁻¹ Lys-plasminogen added; (4) idem + 0.03 mg.ml⁻¹ Lys-plasminogen added; (5) 3 μl pooled normal plasma. The wells contain 80 μl of monospecific antiserum to plasminogen.
Hemorrhagic Diathesis in Heterozygotes

In retrospect, 6 of the 16 heterozygotes showed a mild hemorrhagic diathesis characterized by postoperative bleeding, excessive bleeding after tooth extraction and easy bruising after a slight trauma. Three of these had manifest bleedings only after tooth extraction. Other heterozygotes showed no manifestations, several neither after surgery nor after tooth extraction (see also "Case history").

The individuals afflicted with a hemorrhagic diathesis are labeled in Fig. 5. Routine screening for coagulation and platelet function showed no abnormalities in the six heterozygotes with a bleeding tendency. In comparison with the other heterozygote family members, there was no difference in a2-antiplasmin concentrations functionally or immunochemically assayed nor in factor XIII/Ca f-mediated a2-antiplasmin binding to fibrin.

Functional inhibition tests for plasmin and tissue activator also showed no differences. Apparently, the expression of bleeding in heterozygotes did not correlate with fibrinolysis inhibition or a2-antiplasmin residual activity.

The search for other factors possibly responsible for expression of bleeding in heterozygotes showed no significant difference in age, sex, or family origin or in factor XIII subunits A and 5, antithrombin III, or α1-antitrypsin concentrations between the two groups of heterozygotes. Noteworthy may be lower values for α2-macroglobulin (97% ± 20% versus 125% ± 44%), histidine-nich glycoprotein (100% ± 31% versus 117% ± 26%) and urokinase inhibition (52% ± 24% versus 66% ± 9%) and higher levels of plasminogen (116% ± 27% versus 98% ± 23%) for the 6 symptomatic heterozygotes versus 10 asymptomatic ones. All of the above are differences favoring a hemorrhagic diathesis. It was also striking that of 5 of 37 subjects with an elevated plasminogen level (above mean + 2 SD), 4 were heterozygotes, 3 of whom were symptomatic.

However, none of the differences recorded were statistically significant (p < 0.05). It was noted that α1-antitrypsin levels in the heterozygote group as a whole (142% ± 39%) were significantly lower than in the normal population.
Fibrinolytic inhibition tests may more realistically reflect residual fibrinolytic inhibition in the deficient cases.

**Binding of α₂-Antiplasmin to Fibrin**

Clotting of plasma samples with CaCl₂/thrombin causes a considerable loss of functional and immunochemical α₂-antiplasmin in the resulting serum (Fig. 7). After coagulation of normal plasma with thrombin/EDTA or of factor-XIII-deficient plasma with CaCl₂/thrombin, the serum still contained almost as much α₂-antiplasmin as did the plasma. These results are in agreement with those of Sakata and Aoki, demonstrating calcium and factor-XIII-dependent covalent linking of α₂-antiplasmin to fibrin. The binding of α₂-antiplasmin onto fibrin during coagulation of plasma was assessed in normals and heterozygotes (Table 4). The amount of α₂-antiplasmin bound to fibrin was significantly less (±50%) in heterozygotes than in normals. This reduced binding of α₂-antiplasmin to fibrin may contribute to increased fibrinolysis in heterozygotes. There is no difference, however, in binding between symptomatic and asymptomatic heterozygotes.

**DISCUSSION**

Fibrin serves a temporary function in hemostasis, and after exerting its activity, is removed by fibrinolysis: a process of proteolytic liquefaction of the fibrin protein matrix. In theory, too rapid removal of the fibrin by increased fibrinolysis can result in renewed bleeding. An increased fibrinolysis may be due to a decreased inhibition of the process. Until recently, such theoretical considerations of the relevance of fibrinolytic inhibition tests may more realistically reflect residual fibrinolytic inhibition in the deficient cases.

**Fibrinolysis Inhibition by Plasma**

Inhibition of plasmin, urokinase, and human tissue plasminogen activator by plasmas of normal and heterozygote family members and the propositus as determined by a clot lysis method is shown in Fig. 6. Decreased inhibition of the fibrinolytic activities was observed in the heterozygous and the homozygous α₂-antiplasmin deficiencies. The decrease in inhibition was least pronounced for plasmin and most pronounced for tissue plasminogen activator. All tests showed good correlations with the α₂-antiplasmin levels (immediate plasmin inhibition assay). Tissue activator inhibition showed the best correlation ($r = 0.98$), followed by urokinase ($r = 0.96$) and plasmin ($r = 0.90$).

Further, the inhibition in all tests correlated rather well with α₂-macroglobulin levels in the group of heterozygotes. The correlation coefficients were, respectively, 0.91, 0.81, and 0.80 for plasmin, urokinase, and tissue activator inhibition. This indicates a function of both α₂-antiplasmin and α₂-macroglobulin in activator-induced fibrinolysis. Correlations with α₁-antitrypsin levels were poor ($p < 0.6$). Detailed studies on the tests mentioned will be reported elsewhere (Wijngaards et al. in preparation). These functional binding of α₂-Antiplasmin to Fibrin

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antiplasmin has been recently demonstrated, and the possibility of a partial deficiency of only one of these plasma protease inhibitors, notably a2-macroglobulin. Antibodies was provided by experiments showing the complete absence of the inhibitor in the circulation. The 2% residual inhibition in the immediate plasmin inhibition test is considered to be due to other plasma had been mixed with normal plasma (c.f., Fig. I 6 of whom also show a bleeding tendency. This congenital deficiency reported here represents an analogous case. The theoretical, immunochemically non-recognizable residue. Theoretically, there may be a remainder of non-functional, i.e., immunochemically non-recognizable residue. The propositus appears to be homozygously deficient in the fibrinolysis inhibitor a2-antiplasmin. Therefore, are major contributions to this hemorrhagic diathesis. Apart from this clinical symptoms of bleeding in both cases are very similar, supporting a causal relationship between this clinical disease in a2-antiplasmin deficiency and hemorrhagic diathesis. The rational treatment of the propositus with the antifibrinolytic drug tranexamic acid was successful for about 24 mo, during which the acute phase reactants a1-antitrypsin and antithrombin III and factor XIII suggested intravascular coagulation, and high levels of FDP indicated consumption. This suggests that, in the period following the CNS bleeding provided insight into the reaction patterns of some components of the coagulation and fibrinolytic systems during the acute phase. The rather pronounced reduction in factor XIII activity was successful for about 24 mo, during which minor bleeding occurred only twice and morbidity had decreased significantly. Therefore, the isolated abnormality, the absence of a2-antiplasmin, appears to be responsible for the severe hemorrhagic diathesis. The rational treatment of the propositus improved significantly. The study of three plasma samples of the propositus revealed a2-macroglobulin as having a severe hemorrhagic diathesis. In agreement with the participation of a2-macroglobulin in the fibrinolytic system, such a role of a2-macroglobulin has been demonstrated in experiments in vitro and after thrombolytic therapy. In agreement with the participation of a2-macroglobulin in the fibrinolytic system, such a role of a2-macroglobulin has been demonstrated in experiments in vitro and after thrombolytic therapy. Such a role of a2-macroglobulin has been demonstrated in experiments in vitro and after thrombolytic therapy.

Table 4. Binding of a2-Antiplasmin to Fibrin

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<tr>
<th>Plasma Level</th>
<th>Original</th>
<th>Clot</th>
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<tbody>
<tr>
<td></td>
<td>9 57±6 36</td>
<td>8 44±23</td>
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Number of individuals; a2-antiplasmin is assayed as immediate plasmin inhibition tests for plasmin, urokinase, and tissue activators. The function of a2-antiplasmin in neutralizing plasmin is illustrated in experiments in vitro and after thrombolytic therapy. Such a role of a2-macroglobulin has been demonstrated in experiments in vitro and after thrombolytic therapy. In agreement with the participation of a2-macroglobulin in the fibrinolytic system, such a role of a2-macroglobulin has been demonstrated in experiments in vitro and after thrombolytic therapy. Such a role of a2-macroglobulin has been demonstrated in experiments in vitro and after thrombolytic therapy. In agreement with the participation of a2-macroglobulin in the fibrinolytic system, such a role of a2-macroglobulin has been demonstrated in experiments in vitro and after thrombolytic therapy.
Family could be excluded. The inheritance pattern supported the presence of these conditions coagulation, which also result in reduced plasma levels of a2-antiplasmin. Erroneous classification could occur in the case of liver cirrhosis and disseminated intravascular coagulation, since not all individuals have been similarly exposed to, factors that could also have been important were searched for. Groups of symptomatic and asymptomatic heterozygotes showed bleeding problems. Also, in the Japanese series of patients, the incidence of hemorrhagic diathesis occurs in 6 out of 16 heterozygotes. It should be concluded, therefore, that the reduced plasma levels of a2-antiplasmin in heterozygotes is higher than expected (50%) as a consequence of an acute phase reaction. The clot lysis time assays with whole blood, plasma, and cryoprecipitate had no effect. The ineffectiveness of fresh frozen plasma is in contrast to the experience of Japanese patients. This may be related to the presence of an antithrombin which is the need for detection of heterozygotes. It can be speculated that the mean value of a2-antiplasmin is at a statistically significant, higher level in heterozygotes (59%) than in normal family members.

Laboratory tests used for fibrinolytic activity showed normal values. These tests included fibrin plate assays of fibrinolytic activity as a contributory factor to the hemorrhagic diathesis. The most striking observation is that a mild hemorrhagic diathesis associated with the heterozygous state for a2-antiplasmin deficiency in relatives and possibly depends on various other factors of the hemostatic balance that could not be definitely established. A comparative study between the Japanese and other series of patients with a2-antiplasmin deficiency, the homozygote offspring. In the management and prevention of complications. Furthermore, partners of known heterozygotes are being screened to predict the risk of bleeding due to a2-antiplasmin deficiency, the major consequence of the finding of a hemorrhagic diathesis. This is emphasized by the fact that the results are in part different from what was observed in our case.
those of Aoki et al. for the Japanese case of α₂-antiplasmin deficiency. They found increased in vitro fibrinolysis by testing for whole blood clot lysis and euglobulin clot lysis. The low resting level of extrinsic or vascular activator in our case (Table 3) may have reduced the expression of the deficiency in α₂-antiplasmin in whole blood clot lysis. Methods to increase circulating vascular activator, such as venous occlusion tests or exercise, could have provided an answer, but were not considered ethical. The difference in results obtained by euglobulin methods may be due to arbitrary differences in methodology. We previously demonstrated that α₂-antiplasmin is not normally precipitated in the euglobulin fraction. Its occurrence in euglobulin fractions is only possible as a contaminant on the walls with tissue. We found this procedure to be more satisfactory than washing the precipitate once with 0.015 M sodium chloride, pH 5.9 (unpublished). Aoki et al. reported that α₂-antiplasmin added to the plasma had repercussions in the fibrinolytic activity of euglobulin fractions, demonstrating that, in their modification of the technique, α₂-antiplasmin occurs to a significant extent in euglobulin fractions.

Expression of α₂-antiplasmin is also not unambiguous in functional assays of fibrinolysis inhibition, as could be elevated by using the deficient plasmas. Plasmin inhibition studied by the fibrin plate assay appeared to be close to normal (80%) in the propositus, while the clot lysis time variant showed 42% residual plasmin inhibition. Plasmin, urokinase, and tissue activator inhibition as determined by clot lysis time methods showed reasonable correlations with α₂-antiplasmin levels of, respectively, \( r = 0.90 \), \( r = 0.96 \), and \( r = 0.98 \), but α₂-macroglobulin contributed significantly to the inhibition.

With respect to the molecular mechanisms that may explain how the lack of, or reduction in, α₂-antiplasmin results in bleedings, various hypotheses have been advanced. Aoki stressed the importance of plasminogen adsorption to fibrin, which is increased at decreased α₂-antiplasmin concentrations. Low levels or the absence of α₂-antiplasmin thus enhance lysis. This aspect of the mechanism, however, has to be reevaluated because of the recent finding by Lijnen et al. of the histidine-rich glycoprotein, which more strongly exerts this aspect of α₂-antiplasmin action. Based on data of Lijnen et al., it was calculated that changes in free plasminogen level (not complexed with histidine-rich glycoprotein and α₂-antiplasmin) are due to fluctuations in the plasma level of histidine-rich glycoprotein in heterozygotes rather than to the reduction in the α₂-antiplasmin level from 100% to 50%. Collen placed emphasis on the direct fibrinolytic activity of plasmin, which is unrestrained in the absence of α₂-antiplasmin. In addition to this, other actions of plasmin, such as formation of lys-plasminogen from native plasminogen and generation of activators from intrinsic proactivators, may also be enhanced and may contribute to the lysis. No evidence for systemic lys-plasminogen formation was obtained in the homozygous case, in agreement with Aoki et al. However, a more rapid than normal increase in its formation remains a possibility during fibrinolysis in α₂-antiplasmin deficiency.

A new aspect of the mechanism of action of α₂-antiplasmin concerns its immobilization on fibrin by action of factor XIII, rendering the resulting clot less susceptible to lysis. Heterozygotes actually bind only about half as much α₂-antiplasmin as normals. This may significantly contribute to the bleeding risk in general. An argument in favor of the importance of this aspect may be the clinical symptoms seen in factor XIII deficiency, where normal α₂-antiplasmin levels exist but no binding of this inhibitor to fibrin occurs. Such patients show a severe bleeding tendency.

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