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-$\alpha$-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% ± 8%) 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 $\mu M$. 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, Darmstadt, 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 Pharmacia Ltd, Uppsala, Sweden. Pluronic acid (N-a.a.a.-trifluoro-m-tolyl anthranilic acid) was from Aldrich Europe, Beerse, Belgium. Carbouw 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.
Casein units/mg protein, and human fibrinogen (grade L) were substrates for factors VIII, IX, XI, and XII substrate plasmas for factors.

VIII, IX, X, and XII substrate plasmas for factors were obtained 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 were from Leo Pharmaceuticals, Ballerup, Denmark and C I -inactivator preparation was kindly performed by R. Zijlstra, Maria Stichting Hospital, Haarlem, The Netherlands. Results were expressed in percentages of the content of pooled normal plasma.

Methods

Immunochemical Techniques

Factor XIIIa and S, α1-antiplasmin (using antiserum I), 3.8 S-α2-γ-glycoprotein, and factor VIII were determined by the Laurell technique and inter-α-trypsin inhibitor, α1-antichymotrypsin, and Cl-inactivator by the Mancini technique using the corresponding monospecific antisera. α2-Macroglobulin, α1-antitrypsin, and plasminogen were determined with M-Partigen plates. 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 α2-macroglobulin, 201 mg·100 ml−1 α1-antitrypsin, and 11.7 mg·100 ml−1 plasminogen.

Clotting times were measured in EDTA buffer with an HCl solution.

Fibrinolysis Techniques

The normal euglobulin fractions of plasma were prepared at pH 5.9 with a plasma dilution of 1:10 as described previously. Precipitates were redissolved in EDTA buffer. Activities were assayed on plasminogen-rich bovine fibrin plates 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. The Cl-inactivator-resistant activator activity, representing specifically the vascular or extrinsic activator activity in blood, 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). 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-dependent 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 resulting in 50% inhibition of the activity, as described previously.

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 clotting and the complete visual disappearance of fibrin. Dilute plasma clot lysis was performed essentially as described by Fearney and Tweed 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 0.075 M NaCl, 0.025 M CaCl2, 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 ml 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 thermostated cuvette holder, and ΔOD(405 nm)·min−1 was recorded.
Plasmin inhibition was expressed as percentage relative to pooled normal plasma, prepared as described elsewhere,13 on 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 2x10^-6 M) involved. To prevent adsorption in our tube/pipette/cuvette system, addition of 2 µg·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 α2-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: α2-macroglobulin (in 16 persons; range in α2-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 (3mg·ml^-1), 0.05 ml thrombin (40 NIH U·ml^-1), and 0.5 ml plasminogen-containing human fibrinogen (2.4 mg·ml^-1) at 0°C, followed by incubation at 37°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 plasmin.24 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.25 Platelet aggregation studies were performed by the turbidimetric method of Born26 using an aggregometer (Payton-Canada). The final concentrations of the aggregating agents used, ADP and ristocetin were 0.8 µg·ml^-1 and 1.1 µg·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 described27 and fibrinogen degradation products (FDP) with the Ihrombo Wellcotest (Wellcome). Antithrombin III was assayed with the chromogenic substrate S-2238.28

**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 CaCl2, 40 mM cysteine, and 0.9 NIH U·ml^-1 thrombin. The formed fibrin was incubated for 2 hr at 37°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-α2-macroglobulin complex, measurable as spontaneous activity of nonactivated plasma samples,29 was assayed on chromozym PL in the presence of soy bean trypsin inhibitor (SBTI) to obtain specificity for α2-macroglobulin-bound plasmin. The activity was followed as the increase in ΔOD·min^-1 at 405 nm for 10 min in the following mixture: 10 µl plasma, 25 µl 1 mM chromozym PL, 190 µl Tris-imidazole-NaCl buffer, µ = 0.15, pH 7.9, with 50 ppm SBTI.

Binding of α2-antiplasmin to fibrin was studied by clotting 180 µl citrated plasma with a 120 µl calcium chloride (37.5 mM), thrombin (4 NIH U·ml^-1), NaCl (37.5 mM) mixture for 1 hr at 37°C. In the serum supernatant and in a plasma sample incubated with 120 µl 0.15 M NaCl, α2-antiplasmin activity was assayed by the above described immediate plasmin inhibition test. The difference represented the amount of α2-antiplasmin bound to fibrin.

Prekallikrein concentrations in plasma were assayed after optimal conversion to kallikrein with 25 µg·ml^-1 dextran sulphate at 0°C as described previously.30 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 fishhook 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 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. 1) showed no precipitations in the patients' plasma. The detection limit was assessed at 0.2% of the normal plasma level.

### Table 1. Studies on Factor XIII

<table>
<thead>
<tr>
<th>Plasma 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>85</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>α1-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>α1-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."
ANTIPLASMIN DEFICIENCY AND BLEEDING

Fig. 2. Ouchterlony triangle of pooled normal plasma (PP), the propositus plasma (O.J., sample B), and monospecific antiserum (Ab) against a2-antiplasmin (Ab).

2), both raised against purified α2-antiplasmin, showed no reaction with the plasma of the propositus. Similar experiments with antisera III and IV raised against the α2-antiplasmin-plasmin complex confirmed these results. Thus, the plasma of the propositus studied in an asymptomatic period showed no functional α2-antiplasmin and no immunochemical precipitation reaction with four independently and differently obtained antisera against α2-antiplasmin.

Other Inhibitors

The levels of inhibitors other than α2-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 α1-antitrypsin and α1-antichymotrypsin remained slightly elevated. In the samples taken shortly after the bleeding episodes, the levels of the acute-phase reactive inhibitors α1-antitrypsin and α1-antichymotrypsin were markedly increased (Table 2). The levels of α2-macroglobulin, antithrombin III, and inter-α-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
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.

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.

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-antiplasin. 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 (ΔOD·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-Antiplasin 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-antiplasin. Also, by immunocchemical assay, the same group showed about half-normal plasma concentrations of α₂-antiplasin (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.

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**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.
A hemorrhagic diathesis is a tendency to bleed excessively or to have prolonged bleeding. It can be a symptom of a variety of medical conditions. In this context, the study examines the inheritance pattern and the clinical manifestations of a hemorrhagic diathesis in heterozygotes.

**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 a2-antitrypsin concentrations between the two groups of heterozygotes. Noteworthy may be lower values for a2-macroglobulin (97% ± 20% versus 125% ± 44%), histidine-rich 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 a2-antitrypsin levels in the heterozygote group as a whole (142% ± 39%) were significantly lower than the normal level.
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 \( \alpha_2 \)-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 \( \alpha_2 \)-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 \( \alpha_2 \)-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 \( \alpha_2 \)-antiplasmin and \( \alpha_2 \)-macroglobulin in activator-induced fibrinolysis. Correlations with \( \alpha_1 \)-antitrypsin levels were poor \( (p < 0.6) \). Detailed studies on the tests mentioned will be reported elsewhere (Wijngaards et al. in preparation). These functional fibrinolytic inhibition tests may more realistically reflect residual fibrinolytic inhibition in the deficient cases.

Binding of \( \alpha_2 \)-Antiplasmin to Fibrin

Clotting of plasma samples with \( \text{CaCl}_2 / \text{thrombin} \) causes a considerable loss of functional and immunochemical \( \alpha_2 \)-antiplasmin in the resulting serum (Fig. 7). After coagulation of normal plasma with thrombin/EDTA or of factor-XIII-deficient plasma with \( \text{CaCl}_2 / \text{thrombin} \), the serum still contained almost as much \( \alpha_2 \)-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 \( \alpha_2 \)-antiplasmin to fibrin. The binding of \( \alpha_2 \)-antiplasmin onto fibrin during coagulation of plasma was assessed in normals and heterozygotes (Table 4). The amount of \( \alpha_2 \)-antiplasmin bound to fibrin was significantly less \( (\mp 50\%) \) in heterozygotes than in normals. This reduced binding of \( \alpha_2 \)-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
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. A nonimmunochemically recognizable residue. Theoretically, there may be a remainder of nonfunctional, i.e., immunochemically nonrecognizable residue. Evidence against the presence of circulating quenching suggests that not only the complete absence of the inhibitor, but also reduction to half-normal plasma levels, may result in clinical symptoms. Apart from this, the occurrence of two molecular forms of a2-antiplasmin as having a severe hemorrhagic diathesis. The patient with homozygous a2-antiplasmin deficiency and hemorrhagic diathesis. Apart from this, the clinical symptoms of bleeding in both cases are very similar, supporting a causal relationship between this deficiency and hemorrhagic diathesis. In experiments in vitro and after thrombolytic therapy, the study of three plasma samples of the propositus provided insight into the reaction patterns of some components of the fibrinolytic system. For a2-macroglobulin, the decrease in the plasma level has been reported. Its physiologic role is still obscure. For plasminogen, the changes are puzzling, since no a-trypsin inhibitor and a2-macroglobulin. For interdiction in the level of plasminogen proactivators. The antithrombin III and factor XIII suggested intravascular coagulation, and high levels of FDP indicated fibrinolysis. The rather pronounced reduction in factor XIII had led to the initial suspicion that the propositus had a defect in this coagulation factor. Consumption by active fibrinolysis possibly explains the slight reduction in the levels of fibrinogen and factor XIII. The rather pronounced reduction in fibrinogen level, however, was rather stable. Other acute phase reactants a1-antitrypsin and a1-proteinase inhibitor. Reduction in the levels of fibrinogen and factor XIII were found fibrinolytic components such as plasminogen and the anti-fibrinolytic drug tranexamic acid. Therefore, the isolated abnormality, the absence of a2-antiplasmin, appears to be responsible for the severe hemorrhagic diathesis. In this case, the disease was associated with an increased activity of the propositus. The rational treatment of the patient with the antifibrinolytic drug tranexamic acid has improved significantly. During the period following the CNS bleeding provided evidence from congenital or other deficiencies. The understanding of the importance of the fibrinolytic system.

Table 1. Binding of a2-Antiplasmin to Fibrin

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Number</th>
<th>Original Plasma Level</th>
<th>Supernatant Bound to a-Antiplasmin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>normals</td>
<td>12</td>
<td>9 ± 57</td>
<td>6 ± 36</td>
</tr>
<tr>
<td>heterozygotes</td>
<td>16</td>
<td>9 ± 57</td>
<td>6 ± 36</td>
</tr>
</tbody>
</table>

Table 2. Binding of a2-Antiplasmin to Fibronectin

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Number</th>
<th>Original Plasma Level</th>
<th>Supernatant Bound to a-Antiplasmin (%)</th>
</tr>
</thead>
<tbody>
<tr>
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<tr>
<td>heterozygotes</td>
<td>16</td>
<td>9 ± 57</td>
<td>6 ± 36</td>
</tr>
</tbody>
</table>
The most striking observation is that a mild hemorrhagic diathesis occurs in 6 out of 16 heterozygotes. It should be concluded, therefore, that a similar a2-antiplasmin deficiency in relatives and possibly in other members of the family, none of the heterozygotes were reported to suffer from bleedings. This finding was that a2-antitrypsin was at a statistically significant higher level in symptomatic heterozygotes than in normal family members. Routine coagulation and platelet function tests did not provide an explanation for the symptoms. The most obvious absence of a clear-cut expression of the functional defect is inherited as a non-sex-linked autosomal recessive trait and could also be detected in the classification of heterozygotes.

When compared to the Japanese patient,4 this may be related to the acute phase reaction. Antitrypsin is an acute phase protein and it can be expressed clinically. This variability in hemostasis with the Japanese heterozygotes may be coincidental; this incidence may be even higher, since not all individuals have been similarly exposed to, for instance, surgical intervention or other conditions that could also have been important factors that could also have been important. Thus far, groups of symptomatic and asymptomatic heterozygotes were compared, but no factors that could also have been important were searched for. Groups of symptomatic and asymptomatic heterozygotes were compared, but no factors that could also have been important were searched for. Groups of symptomatic and asymptomatic heterozygotes were compared, but no factors that could also have been important were searched for. Groups of symptomatic and asymptomatic heterozygotes were compared, but no factors that could also have been important were searched for. Groups of symptomatic and asymptomatic heterozygotes were compared, but no factors that could also have been important were searched for. Groups of symptomatic and asymptomatic heterozygotes were compared, but no factors that could also have been important were searched for.
those of Aoki et al.\textsuperscript{9} for the Japanese case of $\alpha_2$-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 $\alpha_2$-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 $\alpha_2$-antiplasmin is not normally precipitated in the euglobulin fraction.\textsuperscript{15} Its occurrence in euglobulin fractions is only possible as a contaminant of supernatant fluid. Contamination is reduced to a minimum in our technique by wiping the test tube 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.\textsuperscript{9} reported that $\alpha_2$-antiplasmin added to the plasma had repercussions in the fibrinolytic activity of euglobulin fractions, demonstrating that, in their modification of the technique, $\alpha_2$-antiplasmin occurs to a significant extent in euglobulin fractions.

Expression of $\alpha_2$-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 $\alpha_2$-antiplasmin levels of, respectively, $r = 0.90$, $r = 0.96$, and $r = 0.98$, but $\alpha_2$-macroglobulin contributed significantly to the inhibition.

With respect to the molecular mechanisms that may explain how the lack of, or reduction in, $\alpha_2$-antiplasmin results in bleedings, various hypotheses have been advanced. Aoki\textsuperscript{9,41} stressed the importance of plasminogen adsorption to fibrin, which is increased at decreased $\alpha_2$-antiplasmin concentrations. Low levels or the absence of $\alpha_2$-antiplasmin thus enhance lysis. This aspect of the mechanism, however, has to be reevaluated because of the recent finding by Lijnen et al.\textsuperscript{31} of the histidine-rich glycoprotein, which more strongly exerts this aspect of $\alpha_2$-antiplasmin action. Based on data of Lijnen et al.,\textsuperscript{31} it was calculated that changes in free plasminogen level (not complexed with histidine-rich glycoprotein and $\alpha_2$-antiplasmin) are due to fluctuations in the plasma level of histidine-rich glycoprotein in heterozygotes rather than to the reduction in the $\alpha_2$-antiplasmin level from 100\% to 50\%. Collen\textsuperscript{42} placed emphasis on the direct fibrinolytic activity of plasmin, which is unrestrained in the absence of $\alpha_2$-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,\textsuperscript{20} 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.\textsuperscript{4} However, a more rapid than normal increase in its formation remains a possibility during fibrinolysis in $\alpha_2$-antiplasmin deficiency.

A new aspect of the mechanism of action of $\alpha_2$-antiplasmin concerns its immobilization on fibrin by action of factor XIII, rendering the resulting clot less susceptible to lysis.\textsuperscript{7} Heterozygotes actually bind only about half as much $\alpha_2$-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,\textsuperscript{33} where normal $\alpha_2$-antiplasmin levels exist but no binding of this inhibitor to fibrin occurs. Such patients show a severe bleeding tendency.

\textbf{ACKNOWLEDGMENT}

We would like to acknowledge the contributions of A. F. H. Jie and E. Groeneveld and the early provision of a preprint of reference 31 by Dr. H. R. Lijnen, Leuven, Belgium.

\textbf{REFERENCES}

6. Wiman B, Lijnen HR, Collen D: On the specific interaction between the lysine-binding sites in plasmin and complementary sites in $\alpha_2$-antiplasmin and in fibrinogen. Biochim Biophys Acta 579:142, 1979
10. Vogelaar EF, Brummelhuis HGJ, Krijnen HW: Contribution to the optimal use of human blood III large scale preparation of
human CI-esterase inhibitor concentrate for clinical use. Vox Sang 26:118, 1974
22. Fearnley GR, Tweed JM: Evidence of a active fibrinolytic enzyme in the plasma of normal people with observations on inhibition associated with the presence of calcium. Chin Sci 12:81, 1953
A familial hemorrhagic diathesis in a Dutch family: an inherited deficiency of alpha 2-antiplasmin

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