A Bleeding Disorder Due to Deficiency of \( \alpha_2 \)-Antiplasmin

By Lindsey A. Miles, Edward F. Plow, Kathleen J. Donnelly, Cecil Hougie, and John H. Griffin

A deficiency of \( \alpha_2 \)-antiplasmin has been identified in a female patient with severe and frequent bleeding episodes. Routine coagulation and platelet assays of the patient’s plasma were within normal limits. However, abnormally rapid whole blood or dilute plasma clot lysis times and an abnormal FXIII test in which clots were lysed in the presence of urea or saline suggested an abnormal fibrinolytic system. Analysis of \( \alpha_2 \)-antiplasmin levels by radioimmunoassay revealed \(< 1.0 \mu g/ml \alpha_2 \)-antiplasmin. Functional assays indicated an \( \alpha_2 \)-antiplasmin level \( \leq 10\% \) of normal. Addition of purified \( \alpha_2 \)-antiplasmin to the patient’s plasma restored its ability to inhibit plasmin in vitro assays, and mixtures of patient plasma with normal plasma did not interfere with the antiplasmin activity of the normal plasma.

CONTROL AND REGULATION of the fibrinolytic system is essential for maintenance of hemostasis. In plasma, fibrinolysis is regulated by a balance between plasminogen activation and inhibition of plasminogen activation and plasmin activity. The major inhibitor of plasmin in plasma is \( \alpha_2 \)-antiplasmin, a glycoprotein with a molecular weight of approximately 67,000. \(^1\) The inhibitor reacts very rapidly to form a stable 1:1 complex with plasmin. \(^2\) Two families, one from Japan and one from the Netherlands, with a deficiency of \( \alpha_2 \)-antiplasmin have recently been described. \(^10,12\) The propositus in each family has a severe hemorrhagic diathesis. This article reports studies of a 35-yr-old female with a severe bleeding diathesis associated with minimal plasma antiplasmin functional activity and no detectable \( \alpha_2 \)-antiplasmin antigen. In addition, functional and antigenic levels of \( \alpha_2 \)-antiplasmin in family members of the propositus are presented that are consistent with an autosomal recessive inheritance. A notable feature of this American family was the bleeding diathesis present in two of the heterozygote patients.

MATERIALS AND METHODS

Proteins

Plasminogen was isolated from fresh plasma by affinity chromatography on lysine-Sepharose, followed by gel filtration on ultragel ACA 44. \(^{13}\) Plasmin (Kabi, Greenwich, Conn.) was dissolved in 50% 2 m HCl, 5 g/liter polyethylene glycol, and 50% glycerol to give a concentration of 14 \( \mu g/ml \) of active plasmin. This value was determined by comparison of its activity on the tripeptide substrate S-2251 (o-Val-Leu-Lys-paranitroanilide, Kabi) with plasmin that had been titrated with the active site titrant para-nitrobenzyl-guanidino benzoate. \(^{14}\)

\( \alpha_2 \)-Antiplasmin was purified by a modification of the methods of Wiman, \(^{15}\) Wiman and Colleen, \(^{16}\) and Moroi and Aoki. \(^2\) Human plasma was dialyzed against 40 mM Tris-HCl, 10 mM succinic acid, pH 8.2, and chromatographed on DEAE-Sephadex-A-50. \(^{11,17}\) Fractions containing \( \alpha_2 \)-antiplasmin were pooled and applied to a 2.5 \( \times \) 45 cm lysine-Sepharose affinity column equilibrated with 0.1 M Na phosphate, pH 7.4, 2 mM EDTA, 0.02% NaN\(_3\), and stored in aliquots at \(-80\)\(^\circ\)C.

Functional Assays

Functional \( \alpha_2 \)-antiplasmin activity was measured by testing the ability of purified \( \alpha_2 \)-antiplasmin or plasma samples to inhibit the amidolytic activity of plasmin on the tripeptide substrate S-2251 according to the manufacturer’s instructions and the method of Naito and Aoki. \(^{18}\) Plasma samples were initially diluted 25-fold in 50 mM Tris-HCl, pH 7.4, 0.11 M NaCl, 0.02% NaN\(_3\). The sample was applied to a 2.5 \( \times \) 1.7 cm column containing plasminogen lysine-binding-site I-Sepharose, \(^{19}\) equilibrated with the same buffer. The column was washed with 2 liters of this buffer and then fractions were step eluted with 0.25 M \( \epsilon \)-aminocaproic acid. Fractions containing \( \alpha_2 \)-antiplasmin were dialyzed against two changes of 2 liters distilled H\(_2\)O at 4\(^\circ\)C, centrifuged and then dialyzed against two changes of 0.04 M Na phosphate, pH 7.0, 0.15 M NaCl, 0.02% NaN\(_3\), and stored in aliquots at \(-80\)\(^\circ\)C.

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and utilized to develop double antibody radioimmunoassays as
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collection in accordance with the Helsinki Agreement. Blood was
episodes. Informed consent was obtained from the patients for blood
obtain platelet-poor plasma.

(0.5%
detergent-soluble extracts
quantitated from the inhibition curve produced by the purified
in rabbits
nonlabeled protein. Antisera to the proteins were elicited
containing soya bean trypsin inhibitor and thrombin for assays of
and prothrombin consumption assays. Blood was collected into tubes
plasma being a
I /20 dilution. In each assay, a normal plasma pool
radiolabeled by a modified
SDS-polyacrylamide gels with a mobility unaltered from that of the.
tCi/zg. Each ligand yielded a single band of radioactivity on
Plasminogen-Related Antigens

Diagnostics, Inc., Aguada, Puerto Rico) and Automated APTI
Medical Industries, St. Louis Mo.) using Ihromboplastin-C (Dade
Prothrombin times and activated partial thromboplastin times
Fibrinogen assay22 (Lancer Fibrinogen Analyzer, Sherwood
Plasminogen and
radiolyzer, with a modification of the APTT system, using
a synthetic fluorometric substrate assay (Protopath, Dade Division,
dilutions in duplicate were tested. Functional a2-antiplasmin activity
ability of plasma samples
activity was also determined by testing the
dilution curve and the normal
pool dilution curve. a2-Antiplasmin

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I

normal donors. Plasma dilutions of
amidolytic activity of plasmin on the tripeptide

Calcium-modified fibrinogen (Simplastin-A,
General Diagnostics, Morris Plains, N.J.).
Whole blood was placed in clean glass test tubes for clot formation
bleeding decreased significantly with birth control pills
and was hospitalized for 2 mo because of anemia. Her
bleeded excessively following dental extractions, and at
head. The cut appeared to heal but bleeding recurred 2
easy bruising. At the age of 2 yr she fell and cut her

0.025
canada and
added and the mixture was placed in a plastic disposable cuvette
The bleeder time was performed using the Simplate Method
The prothrombin consumption time was performed by allowing
Whole blood clots were kept at 37#{176}C and observed for lysis. Plasma
bled excessively following dental extractions, and at

I .8 ml of blood to clot for 1 hr at 37#{176}C, adding 0.2 ml of 0.1

M

CaCl2 and were suspended
in saline and
observed for lysis.

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M

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in saline and
observed for lysis.
bleed that lasted for 36 hr. More recently, she knocked her right leg against the door of her car. The back of her knee then became swollen and a hematoma extended up into her thigh and down her leg. She was treated with multiple transfusions of plasma and was on crutches for 2 mo following the accident. She has had apparently spontaneous hemarthroses of both ankles, especially in the summer months.

**Laboratory Studies**

All coagulation tests and platelet function tests on the patient's plasma were within normal limits (Table 1). Platelet aggregation studies with ADP, collagen, ristocetin, and epinephrine were normal. Abnormal findings included an abnormal factor XIII assay in which plasma clots suspended in urea were partially lysed, while those suspended in saline were completely lysed at 24 hr. This initial finding suggested that the patient might have an abnormal fibrinolytic system, and it is noted that in order to assay factor XIII in this patient, an immunologic assay would be most useful.

**Fibrinolytic Studies**

Analysis of the fibrinolytic system of the patient is summarized in Table 2. The patient had normal levels of plasminogen and fibrinogen. Whole blood clots showed lysis beginning at 2 hr, while normal control clots showed no evidence of lysis up to 24 hr. Dilute clot lysis occurred at 5 hr, while controls had not lysed at 24 hr. α2-Antiplasmin antigen was undetectable at a sensitivity of 1 μg/ml and was confirmed on three separate bleedings of the patient. Functional α2-antiplasmin levels were ≤10% of normal, the lower limit of detection of our assay system using S–2251, and less than 1% using D-Val-Leu-Lys-5-amidoiso-phthalic acid, dimethyl ester. No fibrin split products were detected, and euglobulin lysis times were normal.

The α2-antiplasmin antigen level in detergent extracts of washed platelets of the propositus was 20.8 ng/10⁹ platelets, while that of platelets from a normal donor processed in parallel was 68 ng/10⁹ platelets. The mean level of platelet α2-antiplasmin in 10 normal donors was determined to be 62 ± 24 ng/10⁹ platelets. Thus, the platelet α2-antiplasmin of the propositus was significantly reduced.

In order to test whether the low levels of α2-antiplasmin function and antigen were due to mole-

<table>
<thead>
<tr>
<th>Table 1. Coagulation Assays*</th>
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<tbody>
<tr>
<td>Assay</td>
</tr>
<tr>
<td>Prothrombin time (sec)</td>
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<tr>
<td>APTT (sec)</td>
</tr>
<tr>
<td>Bleeding time (min)</td>
</tr>
<tr>
<td>Prothrombin consumption time (sec)</td>
</tr>
<tr>
<td>Fibrin monomer paracoagulation</td>
</tr>
<tr>
<td>Factor VIII (%)</td>
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<tr>
<td>Factor IX (%)</td>
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<td>Factor XI (%)</td>
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<tr>
<td>Factor XII (%)</td>
</tr>
<tr>
<td>Factor XIII</td>
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<tr>
<td>Platelet count/μl</td>
</tr>
<tr>
<td>vWD antigen (%)</td>
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<tr>
<td>vWF–ristocetin cofactor (% activity)</td>
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<tr>
<td>Platelet aggregation studies</td>
</tr>
<tr>
<td>Epinephrine</td>
</tr>
<tr>
<td>Ristocetin</td>
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<td>Connective tissue</td>
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*Assays were performed as described in Materials and Methods.
†Casein units as defined by the Committee on Thrombolytic Agents (CTA).

<table>
<thead>
<tr>
<th>Table 2. Fibrinolytic Studies*</th>
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<tbody>
<tr>
<td>Assay</td>
</tr>
<tr>
<td>α2-Antiplasmin</td>
</tr>
<tr>
<td>S–2251</td>
</tr>
<tr>
<td>Fluorometric substrate (Dade)</td>
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<tr>
<td>Antigen (μg/ml)</td>
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<tr>
<td>Antigen (ng/10⁹ platelets)</td>
</tr>
<tr>
<td>Whole blood clot lysis (hr)</td>
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<tr>
<td>Dilute plasma clot lysis (hr)</td>
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<tr>
<td>Euglobulin lysis time (min)</td>
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<td>FDP (μg/ml)</td>
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<tr>
<td>Plasminogen</td>
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<tr>
<td>CTA (U/ml)†</td>
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<tr>
<td>Fibrinogen (mg/100 ml)</td>
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</tbody>
</table>

*Assays were performed as described in Materials and Methods.
†Casein units as defined by the Committee on Thrombolytic Agents (CTA).

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molecules in the patient's plasma that might interfere with the expression of α2-antiplasmin, the patient's plasma was diluted 31-fold and mixed with an equal volume of varying concentrations of purified α2-antiplasmin and incubated at 37°C for 2 min. The mixture was then assayed for functional α2-antiplasmin activity as described in Materials and Methods. Addition of purified α2-antiplasmin restored the ability of the patient's plasma to inhibit plasmin to that of the normal plasma pool. Moreover, when a mixture of 50% normal plasma and 50% plasma of the propositus was incubated at 37°C for 30 min and placed on ice for 30 min or incubated on ice for 60 min, the ability of the mixture to inhibit plasmin in an assay for functional α2-antiplasmin was not less than the ability of 50% normal plasma and 50% buffer to inhibit plasmin. When a 50:50 mixture of normal plasma and propositus plasma was incubated at 37°C for 30 min and placed on ice for 30 min the ΔA_405/min was 0.293, while that of the 50:50 mixture of plasma and buffer was 0.338. These experiments indicate that the patient's plasma does not contain molecules that interfere with the expression of α2-antiplasmin activity.

Studies of Family Members

The functional and antigenic levels of α2-antiplasmin were tested, and fibrinolytic assays were performed on plasma of the members of the patient's family (Table 3). The parents and three siblings were found to have α2-antiplasmin levels less than half of normal as determined by radioimmunoassay. The average α2-antiplasmin concentration in the plasma of these family members was 29.6 ± 4.6 μg/ml, while that of the normal plasma pools was 75.4 μg/ml. These family members were considered to be heterozygotes for the deficiency of α2-antiplasmin (Fig. 1). No siblings with normal levels of α2-antiplasmin were found. One brother (II-5) had undetectable antigen levels. The functional α2-antiplasmin levels of the parents and four siblings were tested by measuring the ability of the test plasma to inhibit the cleavage of a tripeptide substrate as described in Materials and Methods. The parents and three siblings had average α2-antiplasmin functional activities of 48% ± 12%. No family members had α2-antiplasmin functional levels above 57%. The brother (II-5) who had no detectable α2-antiplasmin by radioimmunoassay had 12% functional α2-antiplasmin activity. He was considered to be a homozygote for the deficiency of α2-antiplasmin. When functional α2-antiplasmin activity of family members and normals was plotted versus α2-antiplasmin antigen levels, the slope of the line was 1.15 with a correlation of 0.96 (Fig. 2). Plasminogen antigen levels as determined by radioimmunoassay of all family members were within normal limits. The mechanism of inheritance of the deficiency of α2-antiplasmin is con-

Table 3. α2-Antiplasmin Levels in Plasma of Family Members*

<table>
<thead>
<tr>
<th></th>
<th>α2-Antiplasmin Functional Activity (%)</th>
<th>α2-Antiplasmin Antigen (μg/ml)</th>
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<tbody>
<tr>
<td></td>
<td>S-2251 Fluorometric Substrate IDade) lpg/ml</td>
<td>Antigen (μg/ml)</td>
</tr>
<tr>
<td>Propositus (II-3)†</td>
<td>10 &lt; 1</td>
<td>&lt; 1.0</td>
</tr>
<tr>
<td>Brother (II-5)</td>
<td>12</td>
<td>&lt; 1.0</td>
</tr>
<tr>
<td>Sister (II-1)</td>
<td>57 30</td>
<td>22.9</td>
</tr>
<tr>
<td>Sister (II-2)</td>
<td>52 30</td>
<td>28.1</td>
</tr>
<tr>
<td>Brother (II-4)</td>
<td>57</td>
<td>30.6</td>
</tr>
<tr>
<td>Father (I-2)</td>
<td>30</td>
<td>30.9</td>
</tr>
<tr>
<td>Mother (I-1)</td>
<td>42</td>
<td>35.4</td>
</tr>
<tr>
<td>Normal plasma pool</td>
<td>100 100</td>
<td>75.4</td>
</tr>
</tbody>
</table>

*α2-Antiplasmin levels were determined as described in Materials and Methods.
†Family tree is given in Fig. 1.
sistent with an autosomal recessive mechanism, as in
the previously described families (Fig. 1).

The brother (II-5) who lacks detectable antigen also
has a remarkable bleeding history similar to that of the
propositus. The other brother (II-4) has not had
remarkable bleeding problems, but one sister (II-2)
bruised easily and the other (II-1) has bled excessively
with tooth extractions but has not been hospitalized
and has had no hemarthroses.

DISCUSSION

Studies of a patient with an unexplained severe
hemorrhagic diathesis were undertaken. Fibrinolytic
tests of the patient’s plasma showed normal fibrinogen
and plasminogen levels, but whole and dilute clot lysis
times were shorter than normal. α₂-Antiplasmin func-
tional activity was 10% using one spectrophotometric
assay and less than 1% using a different fluorometric
tripptide assay. Addition of purified α₂-antiplasmin to
the patient’s plasma restored the ability to inhibit
plasmin to that of normal plasma in vitro assays.
Mixtures of normal plasma and the patient’s plasma
prolonged the dilute clot lysis time to normal values.
These studies indicate that the functional deficiency of
α₂-antiplasmin is not due to molecules in the patient’s
plasma that affect expression of α₂-antiplasmin anti-
gen or function. Corroborating the functional deficien-
cies of the inhibitor was the absence of detectable
α₂-antiplasmin antigen (less than or equal to 1%) in
her plasma. Two patients with immunologically unde-
tectable levels of α₂-antiplasmin and less than or equal
to 3% immediate plasmin inhibitory activity have been
previously described in families from Japan and
Holland. Both of these deficient patients had severe
bleeding diatheses. Thus, the bleeding problem in this
American patient as well as the Japanese and Dutch
deficient patients can most likely be attributed to the absence of
α₂-antiplasmin. The only routine coagulation test that
was abnormal in this patient was a factor XIII assay in
which clots were rapidly lysed in the presence of saline
or urea. This initial finding indicated a possible abnor-
ality in the fibrinolytic system of the patient. It is
noted that in order to assay factor XIII in this patient,
an immunologic assay would be most useful.

Fibrinolytic studies of members of the patient’s
family showed less than half normal levels of α₂-
antiplasmin antigen and function in three siblings and
in both parents. Therefore, these family members were
considered to be heterozygotes for the deficiency. One
sibling had undetectable α₂-antiplasmin antigen levels
and 12% α₂-antiplasmin functional activity near the
lower limit of detection according to the S-2251 sub-
strate and was not tested using the fluorometric sub-
strate assay. This sibling has a severe bleeding history
similar to that of the propositus. Two sisters, both
heterozygotes, have positive bleeding histories. This
finding is similar to a Dutch family with α₂-antiplas-
min deficiency in which six heterozygotes had mild
bleeding problems. However, in a deficient Japanese
family, the heterozygotes had no bleeding problems.
This suggests that under certain circumstances, half
normal levels of plasma α₂-antiplasmin do not suffi-
ciently protect clots in vivo from normally rapid
plasmic digestion. Differences in levels of plasminogen,
histidine-rich glycoprotein, and plasminogen activa-
tors could account for the observed bleeding in some
heterozygotes and not in others. The father and mother
of the propositus in this report are from a small town in
a rural mountainous region and the patient reports that
her great-great-grandparents were related. This con-
sanguinity in the family could have contributed to the
finding of homozygous descendents. The mode of
inheritance of the deficiency in this family is consistent
with an autosomal recessive mechanism, as reported
for the two previously studied families.

α₂-Antiplasmin is the major inhibitor of plasmin in
plasma. Thus, its absence could result in lack of
protection of formed clots from rapid plasmic diges-
tion. The nature of the bleeding episodes experienced
by the propositus is consistent with the concept that a
hemostatic plug is formed normally followed by pre-
mature lysis.

α₂-Antiplasmin has also been reported to inhibit the
procoagulant activity of activated factor XII, kalli-
crein, activated factor X, and activated factor X in in
vitro assays, and a role for the proteins of the contact
activation system in in vitro fibrinolytic assays has
been reported. If this inhibitor is physiologically
important for these enzymes, it is possible that its
deficiency allows an excess expression of the factor-
XII-dependent fibrinolytic pathway. However, no
fibrinogen degradation products were detected in the
patient’s plasma. This indicates that either the fibrino-
lytic system is not activated in vitro to cleave fibrino-
gen, or if the fibrinolytic system is activated, fibrinogen
is protected from plasmin degradation by inhibitors
such as α₂-macroglobulin. Thus, fibrinogenolysis
may be inhibited whereas fibrinolysis is not.

α₂-Antiplasmin antigen is found in platelets corre-
sponding to 0.05% of the total α₂-antiplasmin in blood
on a volume basis, and 87.5% of this material can be
released when platelets are stimulated by thrombin.
Detergent extracts of platelets of the propositus con-
tained 20.8 ng α₂-antiplasmin/10⁹ platelets, while the
mean of 10 normals was 62 ± 24 ng/10⁹ platelets in a
previous study. Thus, there is a significant difference
in α₂-antiplasmin levels in platelets of the propositus
compared to normal platelets. This significant level of
platelet antigen in comparison to her plasma levels could be due to separate loci for the production of platelet and plasma \( \alpha_2 \)-antiplasmin or to sequestration by platelets of any trace amounts of \( \alpha_2 \)-antiplasmin in her plasma. It seems noteworthy that thrombi formed in this patient will contain platelet \( \alpha_2 \)-antiplasmin, and it is possible that the platelet-derived inhibitor is laid down in the clot to provide some resistance to fibrinolysis, although platelet \( \alpha_2 \)-antiplasmin is apparently insufficient to prevent bleeding episodes due to trauma.

The identification of patients lacking \( \alpha_2 \)-antiplasmin who also have severe bleeding problems indicates the importance of plasma \( \alpha_2 \)-antiplasmin in protection of clots from abnormally rapid lysis in vivo. These studies also emphasize that a lack of regulation of the fibrinolytic system can result in a bleeding diathesis.

ACKNOWLEDGMENT

The authors wish to thank Dr. Nobuo Aoki for confirming the functional and antigenic deficiency of the inhibitor in the plasma of the propositus and Dr. Beshota, Department of Hematology, Appalachian Regional Hospital, South Williamson, Ky, for obtaining plasma samples from some family members. The skillful technical assistance of Erieh Biele is gratefully acknowledged.

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A bleeding disorder due to deficiency of alpha 2-antiplasmin

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