Demonstration of Kallikrein-Like Protease Activity in Nonactivated Plasma of Patients With Cooley’s Anemia

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Routine evaluation of 12 children with Cooley’s anemia revealed that each one had a prolonged partial thromboplastin time (PTT). However, prothrombin time (PT) and thrombin time (TT) were within the normal range. Specific assays demonstrated low levels of the four contact factors: factors XI, XII, prekallikrein, and high molecular weight kininogen. Further investigation revealed activity against para-nitroanilide peptide substrates in unactivated plasma from all 12 patients. Following gel filtration on Sephadex G200, the activity emerged in one peak in the void volume, indicating a molecular weight of greater than 500,000. Activity was greatest against H-o-Pro-Phe-Arg-pNA, the substrate for plasma kallikrein, and was inhibited by diisopropyl fluorophosphate and trasylol. It was unaffected by hirudin, soy bean trypsin inhibitor, and lima bean trypsin inhibitor. It was destroyed by heating at 56°C. Specific antisera against human prekallikrein and human α2-macroglobulin did not reduce the activity. It is concluded that a high molecular weight kallikrein-like protease, is present in the plasma of these patients. It is postulated that it is released into the circulation from tissue as a result of damage due to iron overload. It is further postulated that this protease brings about in vivo activation of the contact factors, resulting in a fall in their circulating levels.

Rout ine Evaluation of 12 children with Cooley's anemia revealed that each one had a prolonged partial thromboplastin time (PTT). However, prothrombin time (PT) and thrombin time (TT) were within the normal range. Specific assays demonstrated low levels of the four contact factors: factors XI, XII, prekallikrein (PK), and high molecular weight kininogen (HMW-K). It appeared unlikely that these decreased levels were simply a reflection of impaired synthesis due to liver damage, because other coagulation factors (factors II, V, VII, VIII, IX, X, and fibrinogen) were within the normal range. Other responsible mechanisms considered were (1) that the contact factors were being synthesized with impaired biologic activity or (2) these factors were being activated in vivo followed by inactivation of the active forms by plasma inhibitors. In the course of investigating these possibilities, a protease was demonstrated in the unactivated plasma of these patients. In this article, the characterization of this protease is described.

Materials and Methods

Study Population

Twelve children, aged 2-17 yr, with β-thalassemia major were studied. Informed parental consent was obtained, and the study was approved by the Board of Research Associates of New York University Medical Center. The children were on a hypertransfusion protocol designed to maintain a pretransfusion hematocrit of greater than 30% and posttransfusion hematocrit greater than 40%. Transfusions consisted of frozen-thawed packed red blood cells suspended in saline administered at approximately 4-wk intervals. All but the youngest child were receiving subcutaneous Desferal (CIBA Pharmaceutical Company, Summit, N.J.) for 8 hr each day. The 7 oldest children had undergone splenectomy. The general health of all the children was good; all participated in normal activities including school and sports. SGPT and SGOT were moderately elevated in all children. Five milliliters of freely flowing blood was drawn from an antecubital vein into a plastic syringe. The blood was immediately transferred to a polycarbonate tube containing 3.8% sodium citrate and 0.1 M ε-aminocaproic acid (EACA) (1 part citrate-EACA to 9 parts blood). The blood was immediately centrifuged (4°C, 3000 g) and the platelet-poor plasma removed without glass contact. Plasma samples were stored in polycarbonate tubes at −70°C and were studied within 2 mo of obtaining the specimen.

Biologic Assays of Coagulation Factors

Samples were obtained from each patient on at least three different occasions and PTT, PT, TT performed. The biologic activity of factors XII, XI, PK, HMW-K, VIII, and IX were determined by methods based on the kaolin-activated partial thromboplastin time utilizing substrate plasma from congenitally deficient donors. Factors II, V, VII, and X were determined by methods based on the prothrombin time. Prekallikrein was also measured with the chromogenic substrate H-o-Pro-Phe-Arg-pNA ($2302 Ortho Diagnostics, Raritan, N.J.) following activation with dextran sulphate at 4°C. The results were expressed as a percentage of pooled plasma from 15 normal adults. Fibrinogen was measured by a heat precipitation method.

Immunologic Assay of Factor XII

Factor XII was measured immunologically in 10 patients and the level obtained compared to biologic activity. Factor XII was purified from human plasma as described by Silverberg and coworkers. A protein with factor XII biologic
activity (28 U/mg protein) was obtained and yielded a single band on polyacrylamide gel electrophoresis. An antibody was raised in rabbits and partially purified with ammonium sulphate. This antisem to factor XII gave a single precipitin line on immunodiffusion with human plasma and purified factor XII, but no precipitin line against plasma congenitally deficient in factor XII. On crossed immunoelectrophoresis, a single peak was observed against human plasma. Factor XII levels in the patients were then determined by electroimmunoassay as described by Laurell. The coefficient of variation for the same sample assayed on eight occasions was 5%.

**Demonstration of Activity Against Chromogenic Substrates**

To test for the presence of active proteases, the following chromogenic substrates (Ortho Diagnostics) were used: ImM H-τ-Phe-Pip-Arg-pNA (S2238), 6 mM H-d-Pro-Phe-Arg-pNA (S2302), 1.6 mM N-benzoyl-l-lys-gluc-Arg-pNA (S2222), 3.5 mM H-d-Val-Leu-Lys-pNA (S2251), and 1.5 mM d-Val-Leu-Arg-pNA (S2266). Studies were conducted at 37°C with a Gilford recording spectrophotometer using semi-micro plastic cuvettes (Fisher Scientific Co., Springfield, N.J.). Reaction rate was determined by the increase in absorbance at 405 nm. The reaction mixture for all substrates, except S2302, consisted of 0.2 ml of uncontacted plasma (patient or control), 0.5 ml of a Tris/HCl buffer (0.5 M, pH 8.3) containing sodium chloride (1 - 0.15), and 0.4 ml of substrate. For S2302, 0.1 ml of plasma was diluted in 3.9 ml of buffer (Tris 50 mmole/liter, NaCl 12 0.15 M mmole/liter, pH 7.8). One milliliter of the dilution was mixed with 0.4 ml of S2302 (6 mM).

**Gel Filtration of Plasma on Sephadex G200**

A siliconized glass column 1.5 x 90 cm (Pharmacia K 15/90) of Sephadex G-200 was equilibrated with a 0.01 M Tris, 0.15 M NaCl buffer (pH 7.0) at 4°C. Two-millilitre samples of patient’s pooled plasma or normal pooled plasma were applied, the elution rate with downward flow was 6 ml/hr, and 0.66 ml fractions were collected in polypropylene tubes. Standard markers of known molecular weight obtained from Pharmacia, Fine Chemicals Division of Pharmacia Inc., Piscataway, N.J., were used to calibrate the column. These were ribonuclease A (13,200 daltons), ovalbumin (43,000), adiolase (158,000), and ferritin (440,000). The 0.66-mI fractions eluted from the column were analyzed for protein concentration (absorbance at 280 nm) and for activity against S2238 as described above for plasma.

**Effect of Inhibitors on Protease Activity**

The following inhibitors were evaluated in the final concentrations shown. Diisopropyl fluorophosphate (DFP) (Alrich Chemical Co. Inc., Milwaukee, Wisc.) 10 M, Hirudin (Sigma Chemical Co., St. Louis, Mo.) 2 IU; trasylosl (Sigma) 500 IU; soy bean trypsin inhibitor (SBTI) (Sigma) 100 μg/ml; and lima bean trypsin inhibitor (LBTI) (Sigma) 1 mg/ml. The effect of these inhibitors was tested by incubating 0.2 ml plasma, 0.1 ml inhibitor, and 0.4 ml Tris-HCl buffer (pH 8.3) together for 30 min at room temperature. Thereafter, 0.4 ml of S2238 (1 mM) prewarmed to 37°C was added and the reaction rate read as described above.

**Studies With Specific Antisera**

Purified IgG prepared from sheep antiserum against human prekallikrein (a gift from Dr. Allen Kaplan, SUNY at Stony Brook, N.Y.) was incubated with an equal volume of patients’ plasma for 1 hr at room temperature. Ninety-seven percent of the prekallikrein was removed from the patients’ plasma as assessed by an assay based on PTT using plasma from a patient with Fletcher trait as substrate. Levels of factors XI and XII were also measured and were not depleted. Following incubation with the antisem, activity of the plasma against S2238 was measured before and after treatment with the antisem.

The globulin fraction from a rabbit antiserum against human α2-macroglobulin (α2m) (Calbiochem-Behring Corp., San Diego, Calif.) was partially purified by ammonium sulphate precipitation. Alpha-2-macroglobulin was removed from the patient’s plasma by incubation with an equal volume of globulin fraction at room temperature for 2 hr. The antisem plasma mixture was then tested for activity against S2238 as described above. The α2m was measured in the patient’s plasma before and after incubation with the antisem by an immunologic method.

**Statistical Methods**

Results are expressed as mean ± standard error of the mean. Differences between mean values of the patients with Cooley’s anemia and normal controls were analyzed by Student’s nonpaired two-tailed t test, while the paired t test was used to compare observations within the same group. p Values of <0.05 were considered to be significant.

**RESULTS**

**Coagulation Tests**

Mean value for the PTT was 58 ± 2 sec for the 12 patients studied compared to 43 ± 1 sec for 12 age-matched healthy controls (p < 0.01). Mean levels of the four contact factors (XII, XI, PK, HMW-K) were below the normal range (Table 1), while all other factors measured II, V, VII, VIII, IX, X, and fibrinogen were in the normal range (Table 1).

**Immunologic Assay of Factor XII**

Immunologic factor XII levels were greater than biologic activity for 9 of 10 patients studied (mean 68 ± 8 versus 54 ± 6, p < 0.05). However, this did not adequately explain the low biologic activity of factor XII, as the immunologic levels of the patients were significantly less than those of 8 normal controls (mean 68% ± 8% versus 86% ± 7%, p < 0.05).

| Table 1. Levels of Coagulation Factors in 12 Patients With Cooley's Anemia |
|---------------------------------|-----------------|
| Coagulation Factor          | Level (%)*       |
| XII                           | 51 ± 7          |
| XI                            | 56 ± 6          |
| Prekallikrein                 | 59 ± 6          |
| High molecular weight kinogen | 67 ± 5          |
| II                            | 80 ± 5          |
| V                             | 96 ± 6          |
| VII and X                     | 110 ± 6         |
| VIII                          | 98 ± 9          |
| IX                            | 74 ± 4          |
| Fibrinogen                    | 182 ± 4         |

*Values are mean ± SEM. Normal range for all factors except fibrinogen is 70%-150%. Normal range for fibrinogen 150-300 mg/dl.
Activity Against Chromogenic Substrates

In all initial studies, S2238 was used to screen plasma for protease activity. High levels of activity were present in nonactivated plasma from patients with Cooley's anemia. These levels of activity were significantly greater than the activity in plasma from both adult and age-matched controls ($p < 0.001$) (Fig. 1). While most studies were performed on samples obtained just before transfusion, equal amounts of activity were present in posttransfusion samples.

Patients' plasma was tested on five different chromogenic substrates (Fig. 2). The greatest activity was observed on S2302, which is preferentially cleaved by plasma kallikrein. There was minimal activity on the substrates most specific for Xa (S2222), glandular kallikrein (S2266), and plasmin (S2251).

Gel Filtration on Sephadex G-200

The protease activity was located solely in one peak in the void volume, indicating a molecular weight of greater than 500,000. Normal pooled plasma treated

![Graph showing protease activity against chromogenic substrates](image1)

**Fig. 1.** The effect of plasma from patients with Cooley's anemia on the chromogenic substrate S2238. Reaction mixture consisted of 0.2 ml uncontacted plasma, 0.5 ml Tris/HCl buffer (0.5 M, pH 83), and 0.4 ml S2238. Studies were conducted at 37°C in a recording spectrophotometer using plastic cuvettes, and results are expressed as nmole pNA released/ml/min, mean ± SEM. Determinations were carried out on 72 different samples of plasma from the 12 patients. Differences between mean activity in patient's plasma and normal or age-matched controls are significant ($p < 0.001$).

![Graph showing gel filtration on Sephadex G-200](image2)

**Fig. 2.** The effect of plasma from patients with Cooley's anemia on different chromogenic substrates. Reaction mixture for all substrates except S2302 consisted of 0.2 ml of uncontacted plasma, 0.5 ml buffer, and 0.4 ml substrate. The reaction mixture for S2302 was 1 ml of a 1/10 dilution of plasma and 0.4 ml of substrate. Substrate concentrations were: S2238 1 mM, S2302 6 mM, S2222 1.6 mM, S2251 3.5 mM, and S2266 1.5 mM. Studies were conducted at 37°C in a recording spectrophotometer using plastic cuvettes. Results, expressed as nmole pNA released/ml/min, are the mean ± SEM of samples obtained from 8 different patients. The protease with the greatest affinity for each substrate is indicated beneath each bar graph.

![Graph showing gel filtration of plasma](image3)

**Fig. 3.** Gel filtration of plasma from patients with Cooley's anemia on Sephadex G-200. Two-milliliter samples of pooled patients' plasma or normal pooled plasma were applied. Fractions were tested for protease activity against S2238. (●–●) = activity against S2238 in patient's plasma, (○–○) = activity against S2238 in normal plasma.
in an identical manner had a minimal amount of activity also detected in the void volume (Fig. 3).

Effect of Inhibitors on Protease Activity

The activity in patients' plasma was readily inhibited by DFP and Trasylol ($p < 0.001$) but not by hirudin, LBTI, or SBTI (Fig. 4). Identical findings were obtained when inhibitors were tested against the void volume fraction of patient's plasma. Heating at 56°C for half an hour destroyed all activity.

Studies With Specific Antisera

Activity against S2238 was unaffected by incubation of patient's plasma with the antiserum against human PK (2 out of 2 experiments), although 97% of prekallikrein was removed from the plasma. Similarly, an antibody against α₂m did not reduce activity of patient's plasma against S2238 (4 out of 4 experiments); 75% of α₂m was removed by the antibody.

DISCUSSION

We have demonstrated that patients with Cooley's anemia have low biologic activity of factors XI, XII, prekallikrein, and high molecular weight kinogen. Caocci and coworkers had previously reported decreased levels of XI and XII in a similar group of patients.\(^\text{12}\)

The purpose of these studies was to investigate the possible mechanisms contributing to the low biologic activity of these factors. Our data indicate that depressed protein synthesis is unlikely in view of the normal values for all other coagulation factors synthesized by the liver (Table I). Although immunologic levels of factor XII in these patients were higher than their biologic levels ($p < 0.05$), immunologic levels were still considerably lower than normal controls ($p < 0.05$). To test for the presence of active forms of the contact factors, the unactivated plasma from these patients was placed on a chromogenic substrate. Significantly greater amounts of protease activity was present in the patients' plasma than in the control plasmas ($p < 0.001$). Protease activity in unactivated plasma has only previously been reported in patients with disseminated intravascular coagulation, carcinoid syndrome, acute pancreatitis, and E. coli bacteremia.\(^\text{13,14}\)

The protease was characterized as a serine protease because it was inhibited by DFP.\(^\text{15}\) The known plasma serine proteases include the activated forms of coagulation factors (II, VII, IX, X, XI, XII, and PK) plasmin, plasminogen activator, trypsin, glandular kallikrein, and some components of the complement system. To investigate which, if any, of these serine proteases was involved, we utilized substrate affinity, pattern of response to protease inhibitors, and estimation of molecular weight.

The protease activity was not due to plasmin or activated factor X or glandular kallikrein, as there was minimal activity on the substrates most specific for these enzymes (S2251, S2222, S2266) (Fig. 2). Trypsin is also excluded as it has considerable affinity for S2222. It was also unlikely to be activated factor XII, as Silverberg and coworkers have reported that activated factor XII has greater activity on S2222 than S2238;\(^\text{6}\) plasma of the patients reported here demonstrated the reverse activity (Fig. 2). The pattern of substrate affinity shown in Fig. 2 is consistent with the presence of plasma kallikrein.

The inhibitor studies excluded thrombin, as hirudin (a thrombin inhibitor) did not affect the activity. Similarly, two trypsin inhibitors, SBTI and LBTI, did not reduce the activity (Fig. 4). Two observations, the preferential affinity for S2302 (the substrate for plasma kallikrein) and the 80% reduction in activity by trasylol (a potent kallikrein inhibitor) suggested that the protease activity in the patients' plasma might be plasma kallikrein.
The molecular weight of plasma kallikrein is approximately 100,000, however at least 80% of PK, and therefore, its active form kallikrein, circulates bound to high molecular weight kininogen (HMW-K) in a one-to-one ratio, forming a complex with a molecular weight of 285,000. Using biologic assays for both PK and HMW-K, the PK–HMW-K complex elutes from the column at a molecular weight of 290,000. Since the protease in the patients’ plasma elutes in the void volume, indicating a molecular weight greater than 500,000, it is unlikely that a kallikrein–HMW-K complex is responsible for this activity. We therefore hypothesized that the material was plasma kallikrein complexed to a large protein probably α2-macroglobulin (α2m). α2m has a molecular weight of 725,000 and inactivates serine proteases without binding to the active site of the protease. Enzymes, including kallikrein, when bound to α2m, retain their ability to cleave small peptides such as chromogenic substrates. This ability is inhibited by low molecular weight inhibitors (<8000) such as DFP and trasylol, while larger molecular weight inhibitors such as SBTI, LBTI, and hirudin do not.

To determine whether plasma kallikrein was the responsible protease, patients’ plasma was treated with a specific antiserum against human prekallikrein. However, protease activity remained unaffected. The possibility was then considered that α2m might impede the antigenic sites of the kallikrein that it is bound to, thus preventing its removal by the antibody. However, the failure to remove protease activity with an antiserum specific for human α2m excludes the hypothesis of kallikrein bound to α2m.

The possibility remains that the activity in the plasma of these patients is due to a kallikrein released into the circulation from parenchymal tissue. Kallikrein is a term used for designation of serine proteases capable of generating peptides called kinin from protein substrates. In addition to the kallikrein generated from plasma prekallikrein, kallikrein-like proteases have been described in urine, glandular tissue, leukocytes, and basophils. There are similarities between the protease in the plasma of the patients with Cooley’s anemia and the basophil kallikrein-like enzyme described by Newball and coworkers. Specifically, the basophil protease had a substrate specificity similar to that of plasma kallikrein, high molecular weight (1.2 million), was destroyed by heating at 56°C, and was inhibited DFP and trasylol but minimally by soy bean trypsin inhibitor. Newball obtained this protease from the basophils in response to challenge with anti-IgE. It is conceivable that a similar kallikrein-like protease is being released from a tissue into the circulation of the patients with Cooley’s anemia as a result of iron overload. In these patients, all tissues are loaded with iron in its storage forms, resulting in parenchymal damage.

Newball and coworkers also demonstrated that the basophil kallikrein-like enzyme is active in the proteolytic cleavage of Hageman factor. The circulating protease in the children reported here may also cleave this factor, resulting in activation of the contact system within the circulation. The activated forms would then be inactivated by the naturally occurring plasma protease inhibitors (α2m, C, esterase inhibitor, antithrombin III, and α1-antitrypsin). This would explain the low levels of these clotting factors.

Thus, a kallikrein-like protease has been demonstrated in plasma of patients with Cooley’s anemia. This may be released from parenchymal tissue as a result of damage due to iron overload. This protease may activate the contact factors within the circulation leading to their depletion.

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