PREKALLIKREIN DEFICIENCY IN MAN*

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Inherited deficiencies or functional abnormalities of plasma substances that participate in blood coagulation have provided a powerful tool for identification and analysis of these plasma proteins on a molecular basis. A new deficiency associated with prolonged blood thromboplastin formation was described by Hathaway, Belhasen, and Hathaway in 1965 (1). The abnormality was inherited as an autosomal recessive and was identified in four of eleven siblings. The responsible factor differed from other proteins of the intrinsic coagulation pathway since mutual correction occurred when this plasma was mixed with plasma obtained from patients with known coagulation factor deficiencies. The missing principle was identified by the surname of the propositus family and called Fletcher factor.

Recently, studies carried out with highly purified proteins of the plasma kinin-forming system led to an unexpected observation. The protein prekallikrein developed, upon incubation with active Hageman factor, potent clot-accelerating activity in a partial thromboplastin test when rabbit plasma was used as substrate (2). A role for kallikrein in coagulation was neither anticipated nor easily explained. When the opportunity became available to study blood plasma from an individual with Fletcher factor deficiency, several abnormalities, in addition to prolonged thromboplastin formation, were identified in this plasma and each was corrected by addition of the kallikrein precursor.

Materials and Methods

Blood Plasma.—Platelet-poor plasmas from normal individuals or from persons with established coagulation deficiencies were collected in acid citrate dextrose anticoagulant and stored at −70°C in 1-ml aliquots until used. Dr. C. Abildgaard kindly supplied plasma from the patient with Fletcher factor deficiency. Dr. S. I. Rapaport generously gave plasma from patients with plasma thromboplastin antecedent (PTA)† and plasma thromboplastin com-

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Abbreviations used in this paper: BAEe, benzoyl-L-arginine ethyl ester; PTA, plasma thromboplastin antecedent.
ponent (PTC) deficiencies. Mrs. Gunda Hiatt, deficient in Hageman factor, donated her blood for these studies.

**Coagulation Tests.**—The kaolin-activated partial thromboplastin test performed in plastic 10 × 75 mm tubes was used throughout. All dilutions were made in 0.15 M NaCl buffered with 0.01 M tris(hydroxymethyl)aminomethane (Tris-saline), pH 7.5. Correction of deficient plasmas by normal plasma was studied by performing twofold dilutions of normal plasma with the deficient plasma as diluent. Calibrated capillary pipettes (Scientific Products Div., McGraw Park, Ill.) used for dilutions were siliconized (Siliclad; Clay Adams, Parsippany, N.J.). To 50 μl of buffer was added 50 μl of plasma and 50 μl of a mixture that contained in final concentration kaolin 5 mg/ml and cephalin 1:10 (Bell and Alton cephalin; Sigma Chemical Co., St. Louis, Mo.). After incubation for 5 min, the mixture was recalcified with 0.05 M CaCl₂ 50 μl in Tris-saline. The time required for a clot to form was then determined. All incubations were performed at 37°C.

**Purified Proteins.**—The precursor of the enzyme kallikrein was prepared in highly purified form by methods described elsewhere (3) for rabbit prekallikrein.

Human prekallikrein purification followed the same procedure with minor modifications. After chromatography on DEAE-Sephadex A-50 and carboxymethyl Sephadex C-50, the pool of prekallikrein was concentrated by ultrafiltration and electrophoresed in Pevikon (Pharmacia, Uppsala, Sweden) at 10 V/cm for 22 h at 4°C. Before use, the Pevikon was rinsed with hexadimethrine bromide 50 μg/ml (Polybrene; Aldrich Chemical Co., Inc., Milwaukee, Wis.) and washed with distilled water on a Büchner funnel. The supporting granules were finally suspended in the electrophoresis buffer of citrate-phosphate, pH 6.2, ionic strength 0.04 relative salt concentration. The only protein known to contaminate the preparation of prekallikrein was β2-glycoprotein (I), determined by a specific antiserum obtained from Behring Diagnostics (Woodbury, N.Y.). The two substances were resolved by gradient ultracentrifugation in sucrose 5–25% in Tris-saline buffer.

**Immunochromatographic Studies.**—Antiserum to human prekallikrein was a gift of Behringwerke AG, Marburg-Lahn, West Germany. The antiserum was allowed to diffuse in agarose against highly purified prekallikrein. The antiserum was also absorbed with an equal volume of normal plasma or Fletcher factor-deficient plasma and tested similarly.

Antiserum to human Hageman factor was supplied by Dr. C. G. Cochrane. It was absorbed with human Hageman factor-deficient plasma before use.

**Bradykinin Generation.**—Measurement of kinin generation in plasma was performed with a uterine horn of a 150 g Sprague Dawley rat injected 48 h earlier with 75 μg of stilbestrol in mineral oil. To 50 μl of plasma in plastic tubes was added 50 μl of Tris-saline buffer and 50 μl of kaolin, 10 mg/ml. The optimal time for measurement of kinin generation by a 50 μl aliquot of this mixture occurred 3 min after addition of kaolin. In an attempt to replace the deficiency in Fletcher factor plasma, known quantities of prekallikrein in Tris-saline were substituted for buffer.

**Formation of Permeability Factor PF/dil.**—Tests for PF/dil formation followed the description of Ratnoff and Miles (4). 20 μl of plasma was diluted to 2 ml in sterile saline in a new glass tube, covered with parafilm, and inverted 40 times each minute on a Lab Tek aliquot mixer (Miles Laboratories Inc., Kankakee, Ill.) for 15 min. A volume of 0.1 ml was injected into the skin of rabbits that had received intravenous injections of 10 mg of chlorpheniramine maleate (Schering Diagnostics, Port Reading, N.J.) and 3 ml of a 2.5% solution of Evans blue dye in saline. The reactions that developed were maximal at 10–15 min. The animal was sacrificed 30 min after the injections were begun and the diameter of bluing on the undersurface of the skin of the back was measured. Duplicate injections were performed from rump to neck or neck to rump to offset regional variability that occurs in some animals.

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**Plasmin Activation by Kaolin.**—A method for generation of fibrinolytic activity in dilute acidified plasma in the presence of kaolin has been described (5). Tris-saline buffer was used throughout. Briefly, 0.5 ml of plasma to which 0.25 ml of kaolin 8 mg/ml was added was brought to 10 ml with 0.01 M acetate buffer, pH 4.8. This resulted in the formation of a euglobulin precipitate. After incubation 60 min at 37°C, the kaolin and euglobulin were sedimented by centrifugation. They were dispersed in 0.5 ml of buffer and 0.2-ml aliquots were taken for duplicate tests and placed in an ice bath. Human fibrinogen (Nutritional Biochemicals Corp., Cleveland, Ohio) 4 mg/ml and 0.1 ml bovine thrombin (Parke, Davis and Co., Detroit, Mich.) 50 NIH U/ml were added. The tubes were transferred to an incubation bath at 37°C and inspected each minute for lysis.

**RESULTS**

**Abnormal Thromboplastin Formation and Its Correction with Plasma or Prekallikrein.**—When Fletcher factor-deficient plasma was incubated with kaolin and cephalin for 3 min and recalcified, coagulation was abnormally prolonged. Normal plasma, mixed with Fletcher factor-deficient plasma, fully corrected the prolonged clotting when added in final concentration of 2% or greater (Fig. 1). The response was dose dependent between 0.1 and 2% of normal plasma. 2% final concentration of Hageman factor-deficient plasma or plasma thromboplastin antecedent-deficient plasma fully corrected the abnormality of Fletcher factor-deficient plasma.

When highly purified human or rabbit prekallikrein was added to normal plasma or plasma deficient in factor XII, factor XI, factor IX, or Fletcher factor-deficient plasma and tested by the kaolin-activated partial thromboplastin test, only the Fletcher factor-deficient plasma was corrected (Fig. 2). Reconstitution of Fletcher factor-deficient plasma by prekallikrein was dose dependent between 0.1 and 3 μg.

The final step in purification of rabbit prekallikrein was block electrophoresis in Pevikon. When aliquots of fractions were tested for their ability to correct the abnormal thromboplastin test, a peak of activity was readily identified. This peak corresponded exactly to fractions that developed the ability to release bradykinin from plasma or hydrolyze benzoyl-L-arginine ethyl ester.

![Fig. 1. Correction of abnormal thromboplastin formation of Fletcher factor-deficient plasma by normal plasma. The correction of Hageman factor-deficient or FTA-deficient plasma by normal plasma is shown for comparison.](image)
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Fig. 2. Reconstitution by rabbit prekallikrein of abnormal thromboplastin formation in Fletcher factor (FF)-deficient plasma. Plasmas deficient in Hageman factor (XII), plasma thromboplastin antecedent (XI), Christmas factor (IX), or normal plasma (N) were unaffected by addition of prekallikrein.

Fig. 3. Correction of Fletcher factor-deficient plasma by aliquots of fractions containing prekallikrein recovered from Pevikon block electrophoresis. Prekallikrein was identified by its ability to generate kinin (data not shown) or hydrolyze the synthetic substrate BAEe after activation (BAEe) upon incubation with active Hageman factor or trypsin (Fig. 3). Such fractions contained a single protein band when subjected to analytical disk-gel electrophoresis at pH 9.3.

Human prekallikrein, similarly highly purified, behaved in an identical manner. After the fourth step in purification, block electrophoresis in Pevikon, human prekallikrein was contaminated only by β2-glycoprotein (type I) (Fig. 4). When the two substances were resolved by sucrose gradient ultracentri-
FIG. 4. Electrophoresis of human prekallikrein in 10% acrylamide gels containing sodium dodecyl sulfate after block electrophoresis in Pevikon or after sucrose gradient ultracentrifugation.

...fugation, only fractions containing prekallikrein corrected the abnormality in Fletcher factor-deficient plasma.

Attempts to Generate Kinin in Fletcher Factor-Deficient Plasma.—If prekallikrein were the protein absent from Fletcher factor-deficient plasma, such plasma might be expected to be abnormal in tests for kinin generation. Accordingly, plasma was incubated briefly with kaolin and an aliquot was added to a rat uterine preparation in a Schultz Dale apparatus. Normal plasma or PTA-deficient plasma readily released a kinin upon such treatment. Fletcher factor-deficient plasma, like Hageman factor-deficient plasma, failed to generate kinin (Fig. 5). These two plasmas gave mutual correction, however, when they were mixed. When 4 μg of highly purified human prekallikrein was added to the Fletcher factor-deficient plasma, the defect was corrected. Hageman factor-deficient plasma was unaffected by the addition of this plasma protein (Fig. 5).

Immunochemical Studies.—An antiserum to human prekallikrein precipitated with the highly purified protein by double diffusion in agar. Normal plasma absorbed out this antibody, whereas Fletcher factor-deficient plasma did not absorb the antibody to prekallikrein (Fig. 6). An antiserum to human Hageman factor gave a line of identity with antigens present in normal plasma or Fletcher factor-deficient plasma. The antigen in Hageman factor-deficient plasma was absent.

PF/dil.—Normal or coagulation factor-deficient plasma was diluted, incubated briefly in glass vessels, and injected into the skin of rabbits. PF/dil was not formed in Fletcher factor-deficient plasma or in Hageman factor-deficient plasma (Table I).

In an attempt to determine whether the presence of small quantities of Hageman factor or prekallikrein would allow one to assign the function of
FIG. 5. Bioassay of kinin formation by human plasma in the presence of kaolin. Abbreviations: B, bradykinin in nanograms; NP, normal plasma; XII, Hageman factor-deficient plasma; FF, Fletcher factor-deficient plasma; XI, PTA-deficient plasma; PK, human prekallikrein 4 ng; k, kaolin.

FIG. 6. Absence of prekallikrein antigen in Fletcher factor-deficient plasma. Wells 1 and 2 contain human prekallikrein. Well b contains rabbit antibody to prekallikrein. This antibody was absorbed with normal plasma (well a) or Fletcher factor-deficient plasma (well c).

PF/dil to one of these proteins, mixtures of the two plasmas, each deficient in one of these proteins, were made and tested for PF/dil formation. As little as 2% of either plasma gave nearly complete correction of the other (Table II).

Plasmin Generation.—Studies were conducted with dilute, acidified plasma in the presence of kaolin to learn whether prekallikrein deficiency would affect the generation of a fibrinolytic enzyme. Prekallikrein-deficient plasma, like Hageman factor-deficient plasma, was abnormal in this regard (Table III). Mixtures of the two plasmas resulted in full correction by as little as 5% of either plasma (Table IV). The addition of 1.7 ng of human prekallikrein to Fletcher factor-deficient plasma corrected the abnormally long lysis time of
TABLE I

Formation of PF/dil in Defined Plasmas

| Plasma source                  | Permeability diameter* |
|--------------------------------|------------------------|
| Normal plasma                  | 12, 12, 11, 9.5        |
| Hageman factor deficient        | 3(f), 4(f), 5          |
| Fletcher factor deficient       | 0, 3(f), 4.5           |
| PTA deficient                   | 11, 11.5, 8            |
| Christmas factor deficient      | 10, 10.5, 9.5          |
| Saline                          | 3(f), 5                |

* f refers to faint bluing.

TABLE II

Reconstitution of PF/dil Activity in Hageman Factor or Fletcher Factor-Deficient Plasmas by Limiting Quantities of the Other Plasma

| Plasma in mixture* | Permeability diameter† § |
|--------------------|--------------------------|
| XII dp             | FF dp                    |
| %                  |                          |
| 100                | 0                        | f, f, 3(f), 4(f)       |
| 98                 | 2                        | 10, 9.5, 9.9           |
| 75                 | 25                       | 11.5, 12, 12.5, 11.5   |
| 50                 | 50                       | 12, 10.5, 12, 12.      |
| 25                 | 75                       | 12.5, 12, 13, 11.5     |
| 2                  | 98                       | 10, 9.5, 10, 9         |
| 0                  | 100                      | f, f, f, 3(f)          |
| Saline             |                          | f, 3(f)                |

* Plasmas deficient in Hageman factor (XII dp) or Fletcher factor (FF dp) were mixed in proper proportion in plastic tubes, diluted 1:100 in sterile saline in glass tubes, inverted 15 min, and 0.1 ml injected.
† The injections were made from rump to neck on the right and from neck to rump on the left side of the animal. Results were read at 30 min on the reflected skin. The results of duplicate injections recorded by two observers are given.
§ f means faint reaction with indistinct margins; 3(f) means a 3 mm faint reaction.

78 min to normal, 11 min. The ability of Hageman factor-deficient plasma to form plasmin was unaffected by addition of the same quantity of prekallikrein.

DISCUSSION

Blood plasma from a person with Fletcher factor deficiency was found to be abnormal in tests for thromboplastin formation, kinin generation, evolution of a permeability globulin (PF/dil), or plasmin formation. Each of these abnormalities has, at a prior time, been reported for individuals with Hageman trait (4, 6–8). In the studies reported here, Fletcher factor-deficient plasma
TABLE III
Kao lin-Induced Euglobulin Lysis of Normal or Coagulation Factor-Deficient Plasmas

| Plasma source                  | Lysis time* |
|-------------------------------|-------------|
| Normal                        | 12 min      |
| Hageman factor deficient      | >125 min    |
| Fletcher factor deficient     | 78 min      |
| PTA deficient                 | 16 min      |
| Christmas factor deficient    | 13 min      |
| Buffer control                | >125 min    |

* Mean of duplicate determinations, estimated to the nearest minute.

TABLE IV
Correction of Abnormal Plasmin Formation of Fletcher Factor-Deficient Plasma by Mixing with Hageman Factor-Deficient Plasma

| Plasma in mixture*            | Lysis time† |
|-------------------------------|-------------|
| XII dp FF dp                  | min         |
| 100 0                         | >125        |
| 98 2                          | 38          |
| 95 5                          | 16          |
| 50 50                         | 11          |
| 5 95                          | 12          |
| 2 98                          | 36          |
| 0 100                         | 78          |

* Plasmas deficient in Hageman factor (XII dp) or Fletcher factor (FF dp) were mixed in correct proportion and 0.5 ml used as described in Materials and Methods.
† Mean of duplicate determinations, estimated to the nearest minute.

(but not Hageman factor-deficient plasma) was corrected by addition of highly purified prekallikrein from human or rabbit sources or by small quantities of Hageman factor-deficient plasma. The results argue strongly against an inhibitory substance as the cause of the multiple abnormalities in Fletcher factor-deficient plasma. The role that is played by Hageman factor or prekallikrein is worthy of discussion.

Three lines of evidence support the conclusion that the abnormality in Fletcher factor-deficient plasma represents an inherited deficiency of the plasma protein prekallikrein. First, addition of prekallikrein corrected in a dose-dependent fashion the abnormally prolonged formation of blood thromboplastin in Fletcher factor deficiency. Control plasmas deficient in Hageman factor, PTA, or PTC were not reconstituted by addition of prekallikrein. Secondly, bradykinin was not generated when Fletcher factor-deficient plasma was incubated with kaolin and tested by bioassay. This abnormality was also
reconstituted by addition of prekallikrein. Finally, Fletcher factor-deficient plasma appeared to lack prekallikrein antigen that was present in normal plasma. The abnormal responses of Hageman factor-deficient plasma in tests for thromboplastin generation or kinin formation were not affected by addition of prekallikrein.

This abnormality in Fletcher factor-deficient plasma presented an important opportunity to study the role of prekallikrein in the formation of PF/dil. Fletcher factor-deficient plasma was as abnormal in the development of this permeability agent as Hageman factor deficiency (4). Prekallikrein-deficient plasma had a normal amount of Hageman factor by the reference plasma method and Hageman factor antigen was readily demonstrated. Hageman factor per se acting alone in the absence of prekallikrein is therefore incapable of forming PF/dil. It was hoped that by restricting the quantity of Hageman factor or prekallikrein in mixtures of the two deficient plasmas that one might identify one of these proteins as PF/dil. The results suggested otherwise. Both proteins were clearly necessary for the formation of this permeability agent. Only 2% of their normal concentration in plasma was sufficient for PF/dil formation. The amount of Hageman factor or prekallikrein in such mixtures would be that present at a 1:5,000 dilution of plasma. Plasma at this concentration does not give reactions of permeability. Taken together, information at hand supports the conclusion that both Hageman factor and prekallikrein are necessary, both possibly not sufficient, for the formation of PF/dil.

Hageman factor has been known for some time to play a role in formation of a fibrinolytic substance (8, 10, 11). When prekallikrein-deficient plasma was tested for its ability to form plasmin, dissolution of the clot was abnormally prolonged. Small quantities of either Hageman factor-deficient plasma or prekallikrein-deficient plasma corrected the other plasma fully. Experience in this laboratory has failed to give evidence that active Hageman factor or kallikrein cause lysis of fibrin clots directly. Nor do these enzymes cause direct activation of plasminogen. This suggests that another plasma protein of the plasma fibrinolytic system is activated by one of these enzymes. Evidence for a plasma plasminogen proactivator that is activated by Hageman factor was recently described by Kaplan and Austen (12).

The clinical reports concerning patients with Fletcher factor deficiency reveal that these persons have no trouble with hemostasis (1). This is not unlike the situation with Hageman factor deficiency. In tests of coagulation in vitro, the role of prekallikrein must be placed earlier than the activation of plasma thromboplastin antecedent. When highly purified active PTA is tested in defined plasmas, it accelerates coagulation of Hageman factor, Fletcher factor, and PTA-deficient plasmas (13). It did not cause acceleration of coagulation with Christmas factor-deficient plasma (13). There is now
ample evidence that Hageman factor in its activated form is an enzyme. This enzyme has been shown to activate prekallikrein (3) or precursor plasma thromboplastin antecedent (13) by limited proteolysis. Recent evidence has been presented that catalytic amounts of kallikrein activate precursor Hageman factor (14). Taken together, these observations suggest that Hageman factor and prekallikrein become activated together in a cyclical or cocatalytic fashion. When kaolin serves as the stimulus to activation of this system, normal amounts of prekallikrein in the absence of Hageman factor (Hageman factor-deficient plasma) do not give normal coagulation after prolonged incubation with kaolin. On the other hand, normal amounts of Hageman factor in the absence of prekallikrein (Fletcher factor-deficient plasma) slowly correct the coagulation abnormality upon prolonged incubation with kaolin (9).

It is probably artificial to consider the plasma kinin-forming system and the intrinsic blood coagulation pathway separately. Since two of the three components of the kinin system, i.e. Hageman factor and prekallikrein, are now known to participate in normal blood thromboplastin formation, these two systems should be considered to be functionally and fundamentally united. Prekallikrein is a plasma protein whose presence is required for the normal function of the intrinsic coagulation pathway, the generation of bradykinin, the formation of PF/dil, and the activation of plasmin under defined in vitro conditions.

**SUMMARY**

Blood plasma obtained from an individual with abnormal thromboplastin formation, due to deficiency of Fletcher factor, was fully corrected by 2% of normal, Hageman factor- or PTA-deficient plasma. It was also reconstituted by addition of highly purified human or rabbit prekallikrein. The plasma failed to generate kinin upon exposure to kaolin, a defect which was also corrected by addition of prekallikrein. Prekallikrein antigen was not detectable in this plasma.

Fletcher factor-deficient plasma did not support the normal generation of PF/dil when dilute plasma was incubated in glass vessels and injected intracutaneously. Small quantities of Fletcher factor-deficient or Hageman factor-deficient plasma corrected the ability of the other to generate PF/dil.

The formation of plasmin in dilute, acidified plasma incubated with kaolin was also abnormal in Fletcher factor-deficient plasma. Plasmin generation was normalized by addition of prekallikrein or small quantities of Hageman factor-deficient plasma.

The data support the identity of Fletcher factor and prekallikrein.

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