Fletcher Factor Deficiency

A DIMINISHED RATE OF HAGEMAN FACTOR ACTIVATION CAUSED BY ABSENCE OF PREKALLIKREIN WITH ABNORMALITIES OF COAGULATION, FIBRINOLYSIS, CHEMOTACTIC ACTIVITY, AND KININ GENERATION

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ABSTRACT Fletcher factor-deficient plasma is deficient in prekallikrein and therefore generates no bradykinin upon activation with kaolin. It also possesses a diminished rate of kaolin-activable coagulation and fibrinolysis and possesses a defect in kaolin-activable chemotactic activity. These abnormalities are also corrected by reconstitution with purified prekallikrein. Addition of intact activated Hageman factor corrected the coagulation, fibrinolytic, and chemotactic defects and addition of Hageman factor fragments corrected the fibrinolytic defect and partially corrected the chemotactic defect; neither of these corrected the kinin-generating defect. Although the Hageman factor-dependent pathways appear to be initiated by contact activation of Hageman factor, the kallikrein generated activates more Hageman factor; this feedback is necessary for the Hageman factor-dependent pathways to proceed at a normal rate. It is the absence of this feedback in Fletcher factor-deficient plasma that accounts for the diminished rate of activation of Hageman factor and therefore a diminished rate of activation of the coagulation and fibrinolytic pathways. The ability of prekallikrein to correct the coagulation, fibrinolytic, kinin-generating, and chemotactic defects of Fletcher factor-deficient plasma is consistent with the identity of the Fletcher factor and prekallikrein.

INTRODUCTION

Fletcher factor deficiency, originally described by Hathaway, Belhasen, and Hathaway (1), is characterized by an abnormality of the intrinsic coagulation pathway in which the 2-min kaolin-activated partial thromboplastin time is prolonged, but progressively shortens when the duration of incubation with kaolin is increased (2). The plasma is not deficient in any of the known constituents of the intrinsic coagulation pathway. A factor was subsequently isolated from normal plasma that corrects this coagulation defect and was designated "Fletcher factor" (3). Recently Wuepper has shown that incubation of Fletcher factor-deficient plasma with kaolin generates no bradykinin because it is deficient in prekallikrein (4), and Kaplan, Goetzl, and Austen have shown that prekallikrein cannot be isolated from Fletcher factor-deficient plasma (5). In this communication we report that Fletcher factor-deficient plasma not only possesses abnormalities of coagulation and kinin-generation but also of kaolin-activated fibrinolytic activity and chemotactic activity, each of which is corrected by reconstitution with prekallikrein. The mechanism by which prekallikrein corrects each of these defects is shown to be attributable to the known functions of kallikrein as a kinin-generating enzyme (6), chemotactic factor (7), and activator of Hageman factor (8).

METHODS

Bradykinin triacetate (Sandoz Pharmaceuticals Ltd., Basel, Switzerland) was used as the standard for native bradykinin. Hexadimethrine bromide (Aldrich Chemical Co., Inc., Milwaukee, Wisc.); enzo-diffusion fibrin plates (Hyland Division, Travenol Laboratories, Inc., Costa Mesa, Calif.); chromium-51 (^{51}Cr) (Amersham/Searle Corp., Arlington Heights, Ill.); micropore filters (Millipore Corp., Bedford, Mass.) Gey's medium (Microbiological Associates, Inc., Bethesda, Md.), streptokinase (Hyland Division, Travenol Laboratories, Inc.), and hemostatic phosphatide (cephalin) (Nutritional Biochemical Corporation, Cleveland, Ohio) were obtained as indicated.

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antecedent (PTA)[1]-deficient plasma, and plasma thromboplastin component (PTC)-deficient plasmas collected in sodium citrate were obtained from Sera-Tec Biological, New Brunswick, N. J. Fletcher factor-deficient plasma was a gift from Dr. Charles Abildgaard (University of California, Davis, Calif.). Goat antibody to the fifth (C5) component of complement was supplied by Dr. Leonard Altman (National Institutes of Health, Bethesda, Md.).

Preparation of plasma proteins

Serum was processed for the isolation of the Hageman factor prealbumin fragments (9) while fresh plasma (7, 10) was utilized as the source of unactivated or activated Hageman factor, prekallikrein, precursor PTA (pre-PTA), plasminogen proactivator, plasminogen, and α_2 macroglobulin. For the preparation of plasma, blood was collected in plastic tubes containing 9 mg of EDTA and 3.6 mg of Polybrene in 0.1 ml of 0.15 M saline for each 10 ml of blood drawn. The tubes were immediately centrifuged at 900*g* for 20 min at 4°C and the plasma separated with plastic pipettes. Plastic columns and test tubes were utilized throughout all chromatographic procedures to minimize contact activation of Hageman factor. Samples were concentrated by ultrafiltration through a UM-10 membrane (Amicon Corp., Lexington, Mass.). Gel filtration on Sephadex G-100 (9), G-150 (7), G-200 (11) and 6B Sepharose (11), alkaline disc gel electrophoresis (9), and isoelectric focusing in polyacrylamide gels (7) were performed as previously described. Protein quantitation on a weight basis was performed by micro-Kjeldahl analysis.

Unactivated Hageman factor. Unactivated Hageman factor was isolated by a modification of a procedure previously described (10). 500 ml of human plasma was dialyzed for 8 h against three changes of 0.003 M PO_4 buffer, pH 8, centrifuged at 2,500 rpm for 15 min to remove any precipitate that had formed, and was applied to a 10 × 100-cm column of quaternary aminoethyl (QAE) Sephadex. The column was washed with 6 liters of equilibrating buffer to collect the effluent containing the proenzymes prekallikrein, pre-PTA, and plasminogen proactivator. Unactivated Hageman factor was then eluted by washing the column with 6 liters of 0.003 M PO_4 buffer containing 0.06 M NaCl. The unactivated Hageman factor peak was concentrated and the pH brought to 6.0 by addition of 1 N HCl before chromatography on a 5 × 60-cm column of sulfopropyl (SP) Sephadex equilibrated with 0.003 M PO_4 buffer, pH 6.0. The column was washed with 1 liter of the equilibrating buffer and then with 1 liter of equilibrating buffer containing 0.09 M NaCl. A linear gradient containing 4 liters of 0.003 M PO_4 buffer with 0.09 M NaCl and 4 liters 0.003 M PO_4 buffer with 0.5 M NaCl was then applied. The unactivated Hageman factor peak eluting between 0.15 and 0.20 M NaCl was pooled, concentrated to 2.5 ml, and fractionated on a 2.5 × 150-cm column of Sephadex G-150. The unactivated Hageman factor peak obtained was dialyzed against 0.003 M PO_4 buffer pH 8 and chromatographed on a 3.8 × 42-cm column of QAE Sephadex equilibrated with the same buffer. The column was washed with 500 ml equilibrating buffer and the unactivated Hageman factor was eluted with a linear gradient of 2 liters 0.003 M PO_4 buffer, pH 8.0, vs. 0.003 M PO_4 buffer, pH 8.0, containing 0.2 M NaCl. The unactivated Hageman factor peak was pooled, concentrated to 3 ml, and, when assessed by alkaline disc gel electrophoresis, revealed a single band (Fig. 1). Elution of a sliced, unstained, disc gel run simultaneously revealed that this band represented unactivated Hageman factor. No identifiable contaminants were seen upon immunoelectrophoresis against anti-whole human serum or after isoelectric focusing in polyacrylamide gels. The final preparation at a concentration of 10 μg/ml corrected the partial thromboplastin time (PTT) of Hageman factor-deficient plasma from a control time of 30 min to 2.5 min. This represents 0.7 U/ml when the Hageman factor activity of a pool of 10 normal plasmas is arbitrarily set at 1.0 U/ml. It contained approximately 10% activated Hageman factor, since it shortened the PTT of Hageman factor-deficient plasma to 8 min in the absence of kaolin. The preparation contained no detectable pre-PTA, prekallikrein, plasminogen proactivator, or plasminogen.

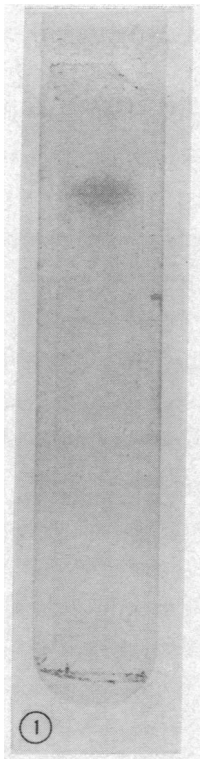

FIGURE 1 Alkaline disc gel electrophoresis of 25 μg unactivated Hageman factor at pH 9.3 in 7.5% acrylamide. The hazy broad band observed was shown to represent unactivated Hageman factor by elution and assay of a sliced replicate disc gel.

Activated Hageman factor. Activated Hageman factor was prepared as described above, with the substitution of carboxymethyl (CM) cellulose and DEAE cellulose for SP Sephadex and the final QAE Sephadex step, respectively. The initial QAE Sephadex effluent was concentrated, stirred in a glass vessel overnight, and then fractionated by CM cellulose, Sephadex G-150, and DEAE cellulose. The preparation was active upon all three Hageman factor sub-

[1] *Abbreviations used in this paper:* CM, carboxymethyl; cor cpm LF, corrected cpm lower filter; PTA, plasma thromboplastin antecedent; PTC, plasma thromboplastin component; PTT, partial thromboplastin time; QAE, quaternary aminoethyl; SP, sulfopropyl.

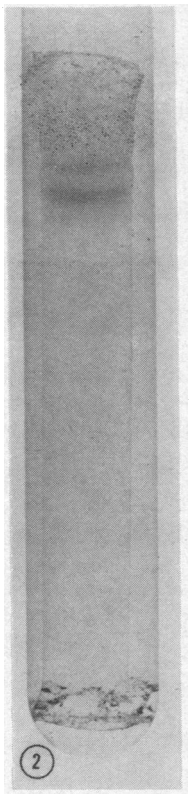

FIGURE 2 Alkaline disc gel electrophoresis of 25 μg purified prekallikrein at pH 9.3 in 7.5% acrylamide. The uppermost band seen is at the interface of the stacking and running gels and represents material that did not penetrate the gel. The single band that penetrated the running gel was shown to represent prekallikrein by elution and assay of a sliced replicate disc gel. Disc gel electrophoresis of purified kallikrein reveals a single band indistinguishable from the one shown.

strates, pre-PTA, prekallikrein, and plasminogen proactivator. When utilized at a concentration of 12 μg/ml, it corrected the coagulation defect of Hageman factor-deficient plasma from a control time of 30 min to 1 min 30 s (0.9 U/ml) in the presence or absence of kaolin, indicating that the preparation was completely activated. It had no effect upon PTA-deficient plasma.

Hageman factor fragments. The Hageman factor prealbumin fragments were purified by chromatography of serum on QAE Sephadex, Sephadex G-100, SP Sephadex, and eluation from alkaline disc gels after electrophoresis, as previously described (9). After dialysis the fragments were concentrated by ultrafiltration and contained no contaminants upon re-electrophoresis on alkaline disc gels. The fragments were routinely utilized at 25 μg/ml. When assessed functionally, 5 μl of Hageman factor fragment generated 100 ng bradykinin after incubation with 0.2 ml fresh plasma for 2 min at 37°C. There was no detectable contamination with any of the Hageman factor substrates, plasminogen, or plasmin.

Hageman factor substrates. Pre-PTA, prekallikrein, and plasminogen proactivator were isolated from the QAE Sephadex effluent obtained from 500 ml of plasma as described above. The proenzyme mixture was concentrated to 100 ml, the pH brought to 6.0 with 1 N HCl, and each proenzyme was isolated by sequential chromatography on SP Sephadex, twice on Sephadex G-150 and passage over an anti-IgG immunoadsorbent as previously described (12). The prekallikrein was utilized in these studies at 10 μg/ml and revealed a single band upon alkaline disc gel electrophoresis (Fig. 2) or after isoelectric focusing in polyacrylamide gels. Its isoelectric point was 8.75. 15 μl of this preparation after incubation with 15 μl Hageman factor fragments (25 μg/ml) generated 150 ng bradykinin from 0.2 ml heat-inactivated plasma. For studies in which the active enzyme kallikrein was used, the prekallikrein prepared through the QAE Sephadex, and SP Sephadex, and the first Sephadex G-150 step was concentrated to 2.5 ml and activated with 0.05 μg Hageman factor fragment by incubation for 24 h at 4°C. The preparation was then found to be fully activated and was fractionated on Sephadex G-150, which separated kallikrein from the Hageman factor fragments. The kallikrein preparation was then passed over the anti-IgG immunoadsorbent. 15 μl of a 10 μg/ml preparation generated 130 ng bradykinin after a 2-min incubation with heat-inactivated plasma (kininogen) (9), a value comparable to the activity generated after activation of prekallikrein at the same protein concentration.

α_2 Macroglobulin. α_2 macroglobulin was isolated by the method utilized by Schreiber, Kaplan, and Austen (11).

Plasminogen. Plasminogen was prepared by affinity chromatography of 100 ml of plasma utilizing a lysine-Sepharose column and epsilon-aminocaproic acid elution as described by Deutsch and Mertz (13). The plasminogen was then concentrated, dialyzed overnight at 4°C against 0.003 M PO_4 buffer, pH 8.0, containing 0.15 M NaCl, and further fractionated by Sephadex G-100 gel filtration. The plasminogen peak was pooled, concentrated, and adjusted to 200 μg/ml for routine use.

Assay procedures

Hageman factor. Unactivated Hageman factor was assayed by determining its ability to shorten the PTT of Hageman factor-deficient plasma. 50 μl Hageman factor source was incubated for 2 min at 37°C with 50 μl "kaolin-cephalin" reagent (20 mg kaolin plus 20 μl cephalin [6 mg/ml] in 2 ml phosphate-buffered saline) and 50 μl Hageman factor-deficient plasma in 10 × 75-mm plastic tubes. 50 μl 0.05 M $CaCl_2$ was added and the clotting time determined at room temperature. The tubes were tilted each minute and the end point defined as the time interval required for the clot to adhere to the plastic tube.

Activated Hageman factor was assayed in the same manner with the cephalin reagent without any kaolin (14).

The percentage of activated Hageman factor in the unactivated Hageman factor preparation was determined in the following manner. The activated Hageman factor preparation was diluted so that the PTT in the absence of kaolin was identical to that of the unactivated Hageman factor preparation (2.5 min). The PTT of twofold falling dilutions of the activated Hageman factor preparation was determined and a standard curve obtained by plotting the log PTT vs. percent activated Hageman factor, the undiluted material being 100%. The percent of activated Hageman factor in the precursor preparation was then obtained by determining the PTT of this preparation in the absence of kaolin (8 min) and reading the percentage from the graph (10%).

Hageman factor fragments were assayed by incubation of 0.1 ml sample with 0.2 ml fresh plasma anticoagulated with

EDTA for 2 min at 37°C and the bradykinin generated was determined by bioassay.

Kallikrein and prekallikrein. Kallikrein was determined by incubation of 0.2 ml enzyme source with 0.2 ml heat-inactivated plasma prepared as previously described (9) for 2 min at 37°C and the bradykinin generated was determined by bioassay.

Prekallikrein was assessed by incubation of 0.1 ml prekallikrein source with 0.1 ml Hageman factor fragments (25 μg/ml) for 5 min at 37°C. 0.2 ml heat-inactivated plasma was then added, the mixture incubated for 2 min at 37°C and the bradykinin generated determined.

Pre-PTA and activated PTA. Pre-PTA and activated PTA were assessed by determining their ability to correct the PTT of PTA-deficient plasma with and without kaolin as previously described (7).

Plasmin and plasminogen. Plasmin was assayed with Hyland fibrin plates as previously described (12). A standard curve relating ring diameter to plasmin concentration was obtained by activating 0.2 ml of a reference preparation of purified plasminogen (12) containing 250 μg/ml with 140 U of streptokinase for 30 min at 37°C; a linear plot relating log concentration plasmin to ring diameter was obtained between 3 and 250 μg plasmin/ml.

Plasminogen was assayed by incubating 0.2 ml of plasminogen source with 140 U of streptokinase for 30 min at 37°C, and the plasmin generated was determined by the fibrin plate assay.

Plasminogen activator and plasminogen proactivator. Plasminogen activator was assayed by incubating 20 μl of plasminogen activator source with 20 μl plasminogen (200 μg/ml) for 1 h at 37°C and the plasmin generated determined by fibrin plate.

Plasminogen proactivator was assayed by incubation of 10 μl plasminogen proactivator with 10 μl Hageman factor fragments (25 μg/ml) for 10 min at 37°C. 20 μl plasminogen (200 μg/ml) was added, the mixture incubated for 1 h at 37°C, and the plasmin generated determined.

Kaolin-activated PTT. The rate of kaolin-activated coagulant activity of Fletcher factor-deficient plasma was assessed by incubating 75 μl Fletcher factor-deficient plasma with 75 μl cephalin-kaolin reagent (20 mg kaolin plus 20 μl cephalin [6 mg/ml] in 2 ml normal saline) from intervals of 2–10 min at 37°C in 10 × 75-mm plastic tubes. 75 μl 0.05 M calcium chloride was added and the end point taken as the time required for formation of a fibrin clot. When the effect of purified proteins upon the kaolin-activated PTT was examined, 50 μl of the sample was added to 50 μl Fletcher factor-deficient plasma. 100 μl of the cephalin-kaolin reagent was then added and the mixture incubated for either 2 min or 10 min at 37°C. 100 μl 0.05 M calcium chloride was added and the clotting time determined.

Kaolin-activated fibrinolytic activity. The rate of generation of kaolin-activated fibrinolytic activity of plasma was assessed by a modification of the procedure described by Ogston, Bennett, Ogston, and Ratnoff (15). To 0.2 ml plasma or 0.1 ml plasma and 0.1 ml test sample were added 0.1 ml kaolin (4 mg/ml in saline) and 3.7 ml 0.01 M sodium acetate buffer, pH 4.8. After incubation at 37°C, the samples were centrifuged at 2,000 rpm for 10 min at 4°C, the supernate was aspirated, and the precipitate was washed with 2.0 ml 0.01 M sodium acetate buffer and again centrifuged at 2,000 rpm for 10 min at 4°C. The precipitate obtained was dissolved in 0.1 ml 0.01 M PO_4 buffer, pH 7.5, containing 0.15 M NaCl, and centrifuged at 2,000 rpm for 5 min to sediment the kaolin, and the supernate was assayed for plasmin by the fibrin plate assay previously described (12).

Kaolin-activable chemotactic activity. Kaolin-activable chemotactic activity was assayed by the method of Gallin, Clark, and Kimball (16) utilizing ^{51}Cr-labeled neutrophils and a double-filter Boyden chamber. Human granulocytes were obtained from normal volunteers. The chemotactic factor was generated by incubation of 0.5 ml serum and either 0.25 ml buffer or test sample with 25 μl kaolin (20 mg/ml) for 10 min at 37°. The sample was centrifuged at 2,000 rpm for 10 min at 4°C and assayed for chemotactic activity. 0.2 ml of the chemotactic stimulus in 2.0 ml Hanks' solution was placed in the lower compartment and the ^{51}Cr-labeled cells suspended at a concentration of 2.3×10^6 granulocytes/ml were placed in the upper compartment. The chemotactic chambers were then incubated for 3 h in 100% humidity and 5% CO_2 in air. The kinetic studies reported previously showed that the number of granulocytes migrating through the upper filter and incorporated into the lower filter is proportional to the radioactivity in the lower of the two micropore filters (16). After adjusting for variable specific activity and incorporation of the ^{51}Cr by the granulocytes, chemotaxis was expressed as corrected counts per minute lower filter (cor cpm LF) (16). The chemotactic response for each experimental condition was the average of three chambers. Student's *t* test was used to compare the means of different studies.

RESULTS

Abnormalities of coagulation, kinin generation, and fibrinolysis in fletcher factor-deficient plasma. The kaolin-activated PTT of Fletcher factor-deficient plasma was examined by varying the incubation time with kaolin before recalcification. As shown in Fig. 3, as the incubation time with kaolin was increased from 2 to 10 min, the PTT progressively diminished to normal levels. Mixtures of 60 μl Fletcher factor-deficient plasma with

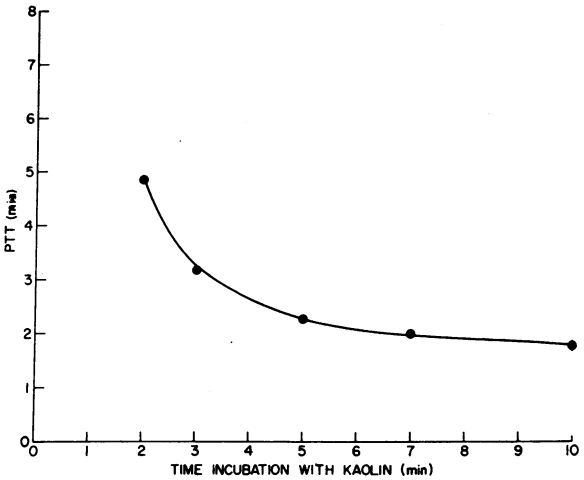

FIGURE 3 Determination of the PTT of Fletcher factor-deficient plasma as the incubation time with kaolin is increased.

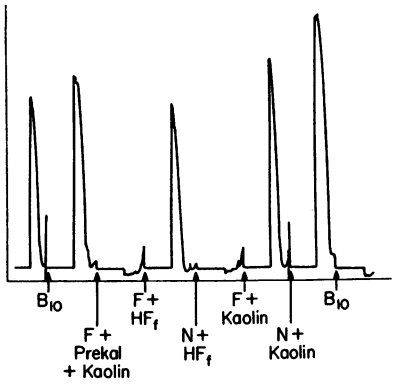

FIGURE 4 Bioassay recording of the contractions of the guinea pig ileum comparing the ability of normal plasma and Fletcher factor-deficient plasma to generate bradykinin after activation with kaolin. The arrows indicate the time of application of the sample to the bioassay. From right to left, the samples tested are: A 10-ng bradykinin standard (B 10), kaolin activation of normal plasma (N + kaolin), kaolin activation of Fletcher factor-deficient plasma (F + kaolin), normal plasma incubated with Hageman factor fragments (N + HF_f), Fletcher factor-deficient plasma incubated with Hageman factor fragments (F + HF_f), kaolin activation of Fletcher factor-deficient plasma after reconstitution with prekallikrein (F + prekallikrein + kaolin), and a 10-ng bradykinin standard (B_{10}).

15 μl of plasmas deficient in Hageman factor, PTA, PTC, and antihemophilic globulin gave mutual correction of their respective coagulation defects, indicating that the Fletcher factor does not correspond to any of these known coagulation factors.

The kinin-generating capacity of Fletcher factor-deficient plasma was next evaluated, as shown in Fig. 4. Incubation of 0.2 ml Fletcher factor-deficient plasma with 200 μg kaolin for 2 min at 37°C did not generate any detectable bradykinin (F + kaolin), whereas similar treatment of normal plasma yielded a contraction equivalent to 8 ng/ml bradykinin (N + kaolin). A further increase in incubation time of Fletcher factor-deficient plasma with kaolin did not yield any bradykinin. Incubation of 0.2 ml of Fletcher factor-deficient plasma with 2.5 μg Hageman factor fragments for 2 min at 37°C also failed to yield bradykinin (F + HF_f) suggesting a deficiency of either prekallikrein or kininogen. Addition of 1 ml prekallikrein (10 μg/ml) to 1 ml of Fletcher factor-deficient plasma followed by activation of 0.4 ml of the mixture with 200 μg kaolin for 2 min at 37°C resulted in kinin generation equivalent to that of normal plasma. The prekallikrein preparation was free of kallikrein, unactivated Hageman factor, or activated Hageman factor. Thus no bradykinin was generated after incubation of kaolin with prekallikrein in the absence of additional plasma or after incubation of the prekallikrein with Fletcher factor-deficient plasma in the absence of kaolin. The data therefore indicate that Fletcher factor-deficient plasma is deficient in prekallikrein and possesses both Hageman factor and kininogen.

The possibility that Fletcher factor-deficient plasma might also reveal a fibrinolytic defect was evaluated by determining the rate of plasmin generation by kaolin activation of diluted, acidified plasma. The Fletcher factor-deficient plasma was compared with normal plasma and with Hageman factor-deficient plasma. As shown in Fig. 5, Hageman factor-deficient plasma did not generate any detectable plasmin during a period of incubation with kaolin for 90 min, whereas normal plasma rapidly generated plasmin activity. Fletcher factor-deficient plasma gave an intermediate result in which the generation of fibrinolytic activity was slow but gradually reached normal levels. Further incubation of either normal plasma or Fletcher factor-deficient plasma for periods extending to 4 h revealed progressive diminution of detectable plasmin presumably secondary to the action of plasmin inhibitors.

Correction of the coagulation and fibrinolytic defects in Fletcher factor-deficient plasma by reconstitution with prekallikrein. Since the kinin-generating defect of Fletcher factor-deficient plasma appeared to be caused by a deficiency of the Hageman factor substrate prekallikrein, the relationship of prekallikrein and the other Hageman factor substrates, pre-PTA, and plasminogen proactivator to the observed coagulation and fibrinolytic defects was next examined. The QAE Sephadex effluent obtained from 500 ml plasma containing prekallikrein, pre-PTA, and plasminogen proactivator was fractionated on SP Sephadex and assayed for each proenzyme as shown in Fig. 6A. The various fractions were then assayed for their ability to correct the coagulation and fibrinolytic defects of Fletcher factor-deficient plasma. As shown in Fig. 6B, both defects were corrected by those fractions containing prekallikrein and plasminogen proactivator but not by those containing pre-PTA. The mixture of prekallikrein and plasminogen proacti-

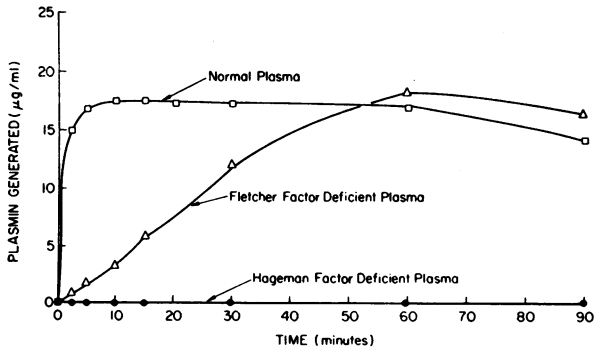

FIGURE 5 A time course of generation of kaolin-activable fibrinolytic activity in normal plasma, Fletcher factor-deficient plasma, and Hageman factor-deficient plasma.

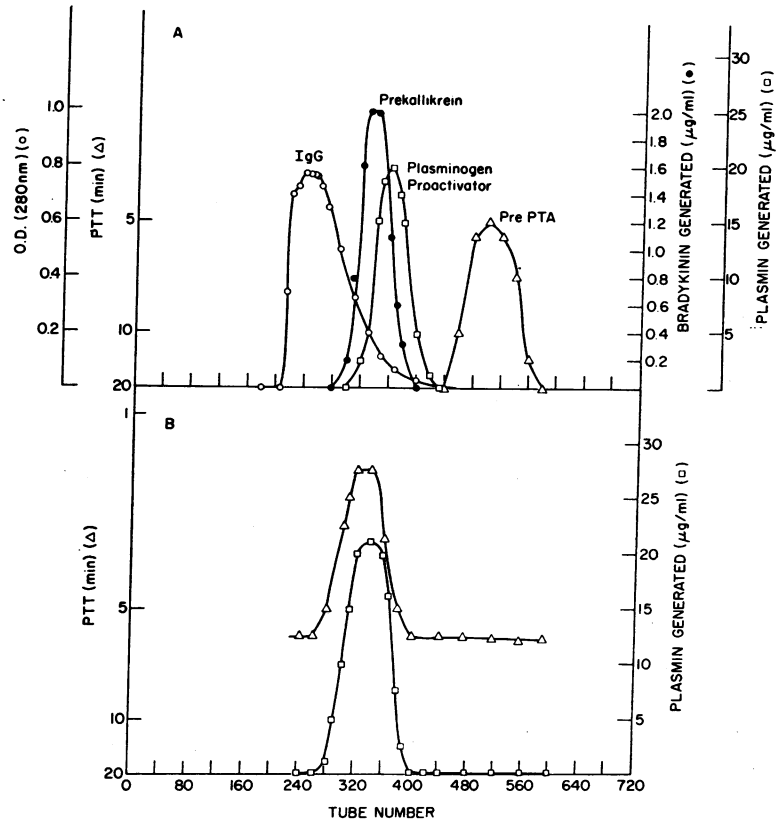

FIGURE 6 SP Sephadex chromatography (A) of the Hageman factor substrates obtained from the QAE Sephadex effluent of 500 ml of plasma. Below (B) is shown the 2-min PTT and the 10-min kaolin-activable fibrinolytic activity determined after addition of equal volumes of the various column fractions to Fletcher factor-deficient plasma.

vator was then fractionated on Sephadex G-150, which resulted in further resolution of prekallikrein and plasminogen proactivator (Fig. 7A). When these fractions were assessed for their ability to correct the coagulation and fibrinolytic defects of Fletcher factor-deficient plasma, both defects were corrected by the prekallikrein-containing fractions (Fig. 7B). Five different normal plasmas were processed in the above fashion; in each instance the prekallikrein-containing fractions corrected both the coagulation and fibrinolytic defects of Fletcher factor-deficient plasma.

A chemotactic defect in Fletcher factor-deficient serum. When prekallikrein and plasminogen proactivator are activated by Hageman factor, both the kallikrein and plasminogen activator generated are chemotactic for human neutrophils (5). To evaluate a possible chemotactic defect in Fletcher factor-deficient serum, normal serum and Fletcher factor-deficient serum were assayed for chemotactic activity before and after incubation with kaolin. As shown in Fig. 8, a prominent increase in chemotactic activity was observed in the normal serum after a 15-min incubation with kaolin ($P < 0.001$). However, no increase in chemotactic activity was observed when Fletcher factor-deficient serum was assayed under the same conditions ($P > 0.05$). Reconstitution of Fletcher factor-deficient serum to yield a final concentration of 3 μg prekallikrein/ml did not yield an increase in chemotactic activity in the absence of kaolin ($P > 0.05$). Kaolin activation of Fletcher factor-deficient serum reconstituted to 3 μg prekallikrein/ml yielded chemotactic activity comparable to that observed after kaolin treatment of normal serum ($P > 0.05$) and significantly greater than kaolin-activated Fletcher factor-deficient serum without prekallikrein ($P < 0.001$). A mixture of prekallikrein and kaolin alone had no chemotactic activity (mean 183±26 cor cpm LF). The magnitude of the chemotactic activity generated by kaolin was comparable to that observed with immune complex or endotoxin activation of normal serum (17). The kaolin was free of endotoxin as assessed by the limulus assay (18). The chemotactic activity could be generated in the presence of 5 mM EDTA, which prevents the generation of complement-dependent chemotactic factors (19), and was not inhibited by goat antibody to

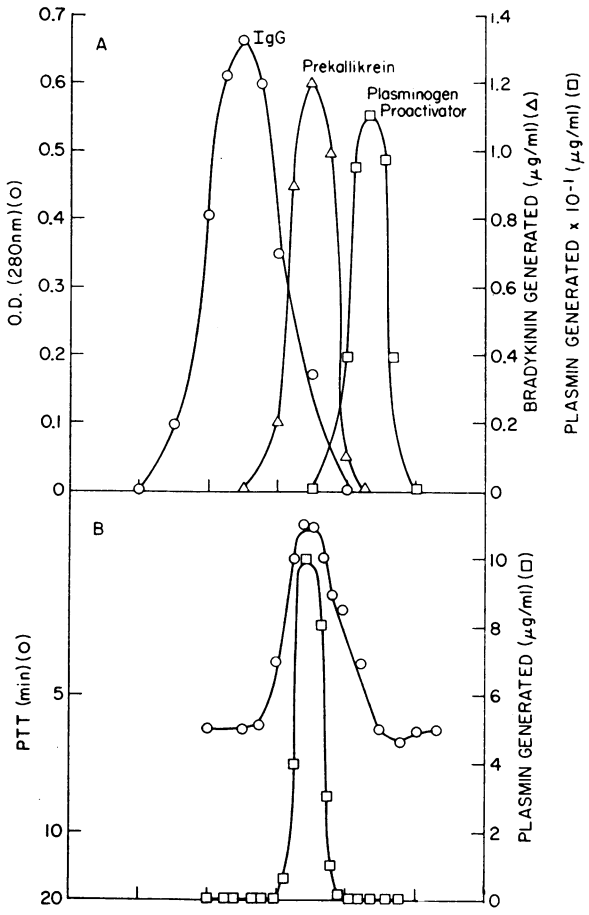

FIGURE 7 Sephadex G-150 chromatography (A) of a mixture of prekallikrein and plasminogen proactivator obtained from SP Sephadex (Fig. 6). Below (B) is shown the 2-min PTT and 10-min kaolin-activable fibrinolytic activity determined after addition of an equal volume of the various column fractions to Fletcher factor-deficient plasma.

C5, which was previously shown to inactivate C5a, the major complement-derived chemotactic factor resulting from in vitro activation of serum (17).

Correction of the coagulation, fibrinolytic, and chemotactic defects by reconstitution with intact activated Hageman factor or Hageman factor fragments. The mechanism by which the Fletcher factor was thought to correct the coagulation defect in Fletcher factor-deficient plasma was by interacting with Hageman factor or PTA or both (3). However, the observation that Fletcher factor-deficient plasma also possesses a fibrinolytic defect that is corrected by addition of prekallikrein suggests that it might act upon Hageman factor, the one factor common to both the intrinsic coagulation and fibrinolytic pathways. Reconstitution of Fletcher factor-deficient plasma with either intact activated Hageman factor or Hageman factor fragments was therefore attempted in order to determine whether prekallikrein might act at the Hageman factor step. Fletcher factor-deficient plasma was reconstituted so that it contained 5 μg/ml of either purified prekallikrein, intact activated Hageman factor, or Hageman factor fragments and assayed for kaolin-activated coagulation and fibrinolytic activities. Each assay was performed in quadruplicate. The results are reported as the mean of four determinations and the standard errors are indicated in each figure. As shown in Fig. 9, after a 10-min incubation with kaolin, the PTT was 2 min and was not further increased when reconstituted with activated Hageman factor or Hageman factor fragments. However, a 2-min incubation with kaolin revealed a prolonged PTT of 8 min, which was corrected to 2 min by addition of intact activated Hageman factor but not by addition of Hageman factor fragments. When the 2-min PTT of the Fletcher factor plasma alone (7.9 min) minus its 10-min PTT control (1.4 min) is compared with the 2-min PTT upon addition of intact activated Hageman factor (1.9 min) minus its 10-min PTT control (1.5 min) the results are statistically significant with $P < 0.001$. Similarly the comparison of the 2-min PTT after addition of Hageman factor fragments (7.2 min) minus its 10-min control (1.5 min) is different from the value obtained after subtracting the 2-min PTT value obtained after addition of intact activated Hageman factor minus

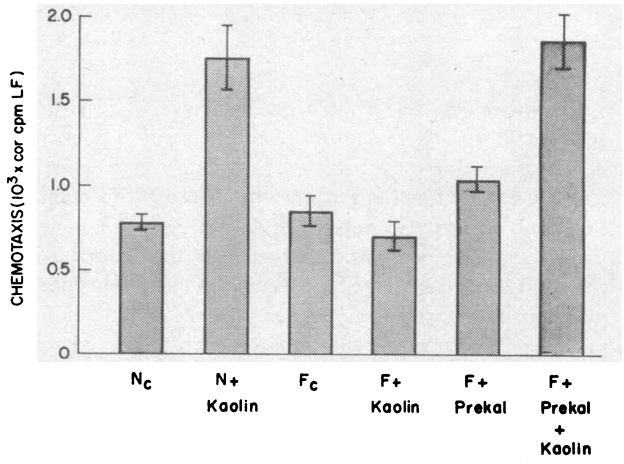

FIGURE 8 Comparison of kaolin-activable chemotactic activity generated in normal serum and Fletcher factor-deficient serum. The bars indicate the mean±SE (three determinations) of the chemotactic activity of normal control serum (Nc), normal serum activated with kaolin (N + kaolin), Fletcher factor-deficient control serum (Fc), Fletcher factor-deficient serum activated with kaolin (F + kaolin), Fletcher factor-deficient serum reconstituted with prekallikrein (F + prekallikrein), and Fletcher factor-deficient serum activated with kaolin after reconstitution with prekallikrein (F + prekallikrein + kaolin).

its control ($P < 0.001$). A comparison of the 2-min PTT of the Fletcher factor-deficient plasma control (7.9 min) with the 2-min PTT obtained after addition of Hageman factor fragments (7.2 min) yields a small, yet significant difference, $P < 0.05$ but > 0.02. Reconstitution with 5 μg/ml *unactivated* Hageman factor did not accelerate the PTT after a 2-min incubation with kaolin.

The assay for kaolin-activated fibrinolytic activity utilized an incubation time of 10 min, at which time Fletcher factor-deficient plasma generates little plasmin activity (Fig. 5). As shown in Fig. 10, the normal plasma control generated 18 μg plasmin/ml after a 10-min incubation; however, Fletcher factor-deficient plasma generated only 4 μg plasmin/ml. The fibrinolytic defect was corrected by reconstitution of Fletcher factor-deficient plasma with either prekallikrein, intact activated Hageman factor, or Hageman factor prealbumin fragments ($P < 0.001$). Similarly, the chemotactic defect of Fletcher factor-deficient serum was evaluated after a 10-min incubation with kaolin before and after reconstitution to contain 5 μg/ml prekallikrein, intact activated Hageman factor, or Hageman factor prealbumin fragments (Fig. 11). Incubation of normal serum with kaolin significantly increased the chemotactic activity ($P < 0.001$), but kaolin treatment of Fletcher factor-deficient serum did not ($P > 0.05$). Reconstitution of Fletcher factor-deficient serum with prekallikrein or intact activated Hageman factor completely corrected the chemotactic defect ($P < 0.001$) whereas addition of Hageman factor fragments gave only partial correction compared to addition of either prekallikrein ($P < 0.05$) or intact activated Hageman factor ($P < 0.01$).

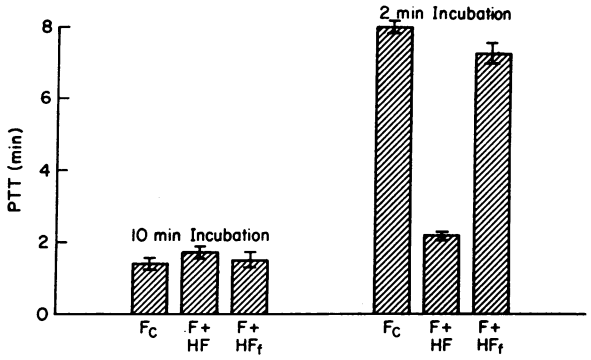

FIGURE 9 Comparison of the mean±SE (four experiments) of the 10-min and 2-min PTT of Fletcher factor-deficient plasma before and after reconstitution with 5 μg/ml of either intact activated Hageman factor or Hageman factor fragments. The control PTT of the Fletcher factor-deficient plasma (Fc) is indicated for either a 10-min or 2-min incubation with kaolin followed by the PTT of Fletcher factor-deficient plasma reconstituted with either intact activated Hageman factor (F + HF) or Hageman factor fragments (F + HF_f).

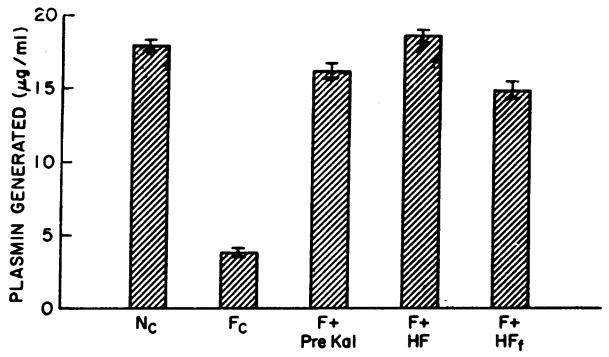

FIGURE 10 Reconstitution of the 10-min kaolin-activable fibrinolytic activity of Fletcher factor-deficient plasma after addition of prekallikrein, intact activated Hageman factor, or Hageman factor fragments. The bars indicate the mean ±SE (four experiments) of normal plasma (Nc), Fletcher factor-deficient plasma (Fc), and Fletcher factor-deficient plasma reconstituted with either prekallikrein (F + prekallikrein), intact activated Hageman factor (F + HF), or Hageman factor fragments (F + HF_f).

Activation of unactivated Hageman factor with kallikrein. The ability of intact activated Hageman factor to reconstitute the coagulation, fibrinolytic, and chemotactic defects of Fletcher factor-deficient plasma suggests that prekallikrein affects these functions by accelerating the conversion of unactivated Hageman factor to activated Hageman factor (20). However, activated Hageman factor or its fragments convert prekallikrein to kallikrein (9, 14, 21, 22). Thus a feedback

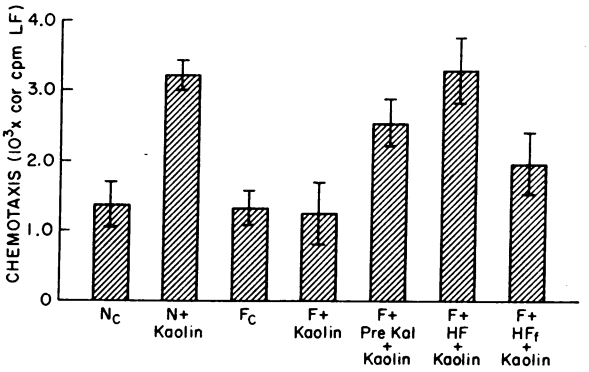

FIGURE 11 Reconstitution of the chemotactic activity of Fletcher factor-deficient serum after addition of prekallikrein, intact activated Hageman factor, or Hageman factor fragments. The bars indicate the mean±SE (three determinations) of the chemotactic activity after a 10-min incubation of kaolin with normal control serum plus saline (Nc), normal serum plus kaolin (N + kaolin), Fletcher factor-deficient control serum plus saline (Fc) Fletcher factor-deficient serum plus kaolin (F + kaolin), Fletcher factor-deficient serum plus kaolin after reconstitution with either prekallikrein (F + prekallikrein + kaolin), intact activated Hageman factor (F + HF + kaolin), or Hageman factor fragments (F + HF_f + kaolin).

TABLE I

*Activation of Hageman Factor by Kallikrein**

150 μl A		15 μl B		25 μl C	PTT of Hageman factor-deficient plasma‡	Generation of bradykinin from fresh plasma§	Activation of plasminogen proactivator‖
					min	*ng/ml*	*μg plasmin/ml*
Hageman factor	+	buffer	+	α_2M	8	500	6
Hageman factor	+	kallikrein	+	α_2M	2.5	1,200	20
Buffer	+	kallikrein	+	α_2M	23	0	0
Buffer	+	buffer	+	α_2M	35	0	0

* 150 μl Hageman factor (10 μg/ml) or 150 μl phosphate-buffered saline (A) was incubated with 15 μl of either kallikrein (0.2 μg/ml) or buffer (B) for 15 min at 37°C and then incubated with 25 μl α_2 macroglobulin (2.0 μg/ml) (C) for an additional 30 min at 37°C. The samples were then assayed for their ability to correct Hageman factor deficiency, generate bradykinin in fresh plasma, and activate plasminogen proactivator.

‡ The PTT was performed without additional kaolin in order to determine activated Hageman factor.

§ 20 μl of each sample was incubated with 200 μl fresh plasma for 2 min at 37°C and the bradykinin generated determined by bioassay utilizing the isolated guinea pig ileum.

‖ 10 μl of each sample was incubated with 10 μl plasminogen proactivator (15 μg/ml) for 10 min at 37°C and the mixture incubated with 20 μl plasminogen (200 μg/ml) for 1 h at 37°C. The plasmin generated was determined by digestion of fibrin incorporated into agar plates.

mechanism is suggested in which kallikrein activates unactivated Hageman factor as has been demonstrated by Cochrane, Revak, Aikin, and Wuepper (8). In order to assess the product of the interaction of unactivated Hageman factor with kallikrein, 150 μl of unactivated Hageman factor (10 μg/ml) was incubated either with 15 μl buffer or with 15 μl kallikrein (0.2 μg/ml) for 30 min at 37°C and then incubated with 25 μl of α_2 macroglobulin (2.0 μg/ml) for 30 min at 37° to inactivate the kallikrein. The mixture was then assayed for activated Hageman factor by its ability to initiate coagulation, kinin generation, and fibrinolysis. As shown in Table I, the preparation of unactivated Hageman factor was partially active; it corrected the PTT of Hageman factor-deficient plasma to 8 min in the absence of additional kaolin, generated 500 ng bradykinin/ml from fresh plasma, and generated 6 μg plasmin/ml after incubation with plasminogen proactivator and then with plasminogen. However, after interaction with kallikrein, the Hageman factor corrected the partial thromboplastin time of Hageman factor-deficient plasma to 2.5 min, the same value obtained when the Hageman factor preparation was first incubated with kaolin and then assessed for its coagulant activity. Thus, kallikrein had converted the unactivated Hageman factor in the preparation to activated Hageman factor. The small increment in coagulant activity noted for the kallikrein preparation (23 min) compared to the control time (35 min) is undoubtedly secondary to trace contamination with the Hageman factor fragments utilized in preparing the kallikrein, since it had no coagulant activity upon PTA-deficient plasma. When activated Hageman factor was diluted to give a PTT of 8 min when added to Hageman factor-deficient plasma, and the preparation then incubated in an identical fashion with the kallikrein preparation, the PTT was not altered. Thus unactivated Hageman factor was converted to an active enzyme by the kallikrein rather than the alternative possibility, that kallikrein enhanced the activity of the small quantity of activated Hageman factor in the precursor preparation. Similarly, the Hageman factor and kallikrein mixture generated 1,200 μg bradykinin from fresh plasma and after incubation with plasminogen proactivator generated 20 μg plasmin/ml from plasminogen, indicating that the activated Hageman factor resulting from the interaction of kallikrein and unactivated Hageman factor is also active upon prekallikrein and plasminogen proactivator.

DISCUSSION

Fletcher factor-deficient plasma is characterized by a diminished rate of kaolin activation of the intrinsic coagulation pathway, a criterion fulfilled by the plasma utilized in these studies. In agreement with the findings of Wuepper (4), this plasma contains no detectable prekallikrein and therefore generates no bradykinin when incubated with kaolin, regardless of the duration of incubation, unless the plasma is reconstituted with purified prekallikrein. The rate of activation of the Hageman factor-dependent fibrinolytic pathway was also di-

minished and, like the coagulation defect, gradually attained normal levels as the incubation time with kaolin was increased, suggesting that those proteins that are absolute requirements of these pathways were present. Thus although the coagulation and fibrinolytic defects were both corrected by prekallikrein, it appeared likely that prekallikrein affected the function of some other factor required for both the coagulation and fibrinolytic pathways. Wuepper has also observed that Fletcher factor-deficient plasma possesses a fibrinolytic defect correctable by prekallikrein (23) and suggested that prekallikrein and the fibrinolytic factor designated Hageman factor co-factor (15) are identical. Plasminogen proactivator (12), a Hageman factor substrate separable from prekallikrein by various chromatographic techniques has been shown to complete the Hageman factor-dependent pathway leading to the conversion of plasminogen to plasmin. The function of this substrate was thought to resemble that of the Hageman factor co-factor. However Fletcher factor-deficient plasma clearly possesses plasminogen proactivator, since it has been isolated from Fletcher factor-deficient plasma (5) in the absence of any detectable prekallikrein. Since plasma deficient in prekallikrein possesses a diminished rate of activation of the fibrinolytic pathway, it appears that both molecules are required for an optimal rate of fibrinolysis. The procedure utilized by Ogston et al. (15) to deplete plasma of its fibrinolytic capability renders it deficient in prekallikrein (23) and possibly plasminogen proactivator as well; thus, the phenomenon they observed is most likely attributable to a mixture of prekallikrein and plasminogen proactivator.

The observation that both kallikrein and plasminogen activator are chemotactic enzymes (5) suggested that Fletcher factor-deficient serum might also possess a chemotactic defect. The chemotactic activity generated by kaolin activation of normal serum represents the first demonstration of Hageman factor-dependent generation of chemotactic activity in whole serum and indicates that a serum pathway exits that generates chemotactic activity independent of complement. The generation of kaolin-activable chemotactic activity in Fletcher factor-deficient serum was found to be diminished, and the abnormality was also correctable by reconstitution with prekallikrein. However, the chemotactic defect could be corrected by addition of activated Hageman factor, suggesting that the absolute contribution of kallikrein to the chemotactic activity of kaolin-activated serum is small, the majority of this activity being secondary to plasminogen activator as well as to other as yet unidentified chemotactic factors.

The mechanism of activation of Hageman factor in human plasma is thought to be initiated by contact activation with surfaces bearing a negative charge. It is clear, however, that Hageman factor can also be activated enzymatically. Kaplan and Austen have previously shown that a preparation of "activated" Hageman factor, which was fully active upon pre-PTA but poorly reactive with prekallikrein, when interacted with streptokinase-activated plasminogen (plasmin) resulted in fragmentation of the Hageman factor and a prominent diminution of its clotting activity but complete activation with respect to its ability to act upon prekallikrein (12). Subsequently, a similar finding was demonstrated by Burrowes, Movat, and Soltay (24). Cochrane et al. have shown that by utilizing low concentrations of plasmin, Hageman factor can be activated so that it is fully active both in coagulation and kinin generation without necessarily resulting in fragmentation and that kallikrein can act in a similar fashion (8). Since prekallikrein corrected both the coagulation and fibrinolytic defects of Fletcher factor-deficient plasma, it appeared likely that prekallikrein was acting at Hageman factor, the only protein known to be common to both pathways. Correction of the coagulation and fibrinolytic defects of Fletcher factor-deficient plasma by addition of intact activated Hageman factor indicated that the requirement for prekallikrein in both these pathways had been bypassed and that prekallikrein does indeed contribute to Hageman factor activation in whole plasma. The ability of the Hageman factor prealbumin fragments to correct the fibrinolytic defect but not the coagulation defect is consistent with the observation that the fragments are fully active in the fibrinolytic pathway (12) but possess only 2–5% of the clotting activity of the parent molecule (10).

The pathways initiated by activated Hageman factor are shown diagrammatically in Fig. 12, indicating the positions of known enzymatic feedback and the functions derived from each pathway. Although plasmin is capable of converting unactivated Hageman factor to activated Hageman factor in the fluid phase, addition of 50 μg/ml of plasmin to Fletcher factor-deficient plasma gave minimal correction to its kaolin-activated PTT, whereas addition of 50 μg/ml plasmin plus 2 μg/ml kallikrein was fully corrective. However, less efficient fluid phase activation of Hageman factor by plasmin or other enzymes that possess a feedback capability may account for the gradual normalization of the coagulation and fibrinolytic defects of prekallikrein-deficient plasma when the incubation time is increased. Although the activation of Hageman factor in plasma is initiated by contact, the ability of kallikrein to convert unactivated Hageman factor to activated Hageman factor in the fluid phase is required for the Hageman factor-dependent pathways to proceed at a normal rate.

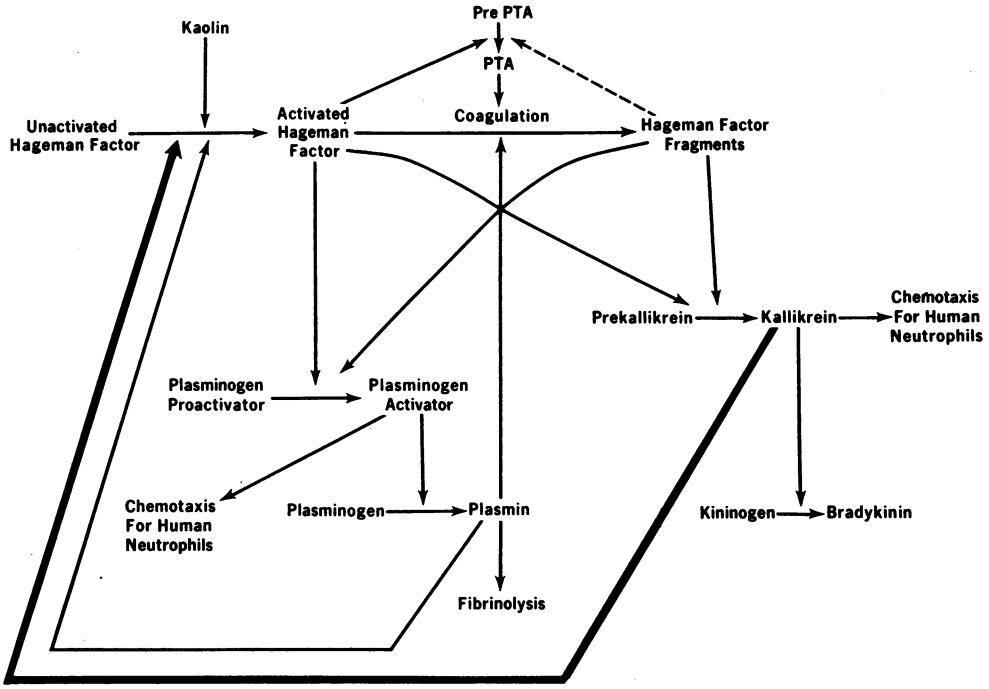

FIGURE 12 Diagram of the Hageman factor-dependent pathways indicating the functions derived and the positions of known enzymatic feedback.

The ability of prekallikrein to correct the coagulation, fibrinolytic, kinin-generating, and chemotactic defects of Fletcher factor-deficient plasma is consistent with the conclusion that the Fletcher factor is prekallikrein.

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