Defective Activation of Clotting, Fibrinolytic, and Permeability-Enhancing Systems in Human Fletcher Trait Plasma

By Hidehiko Saito, Oscar D. Ratnoff, and Virginia H. Donaldson

ABSTRACT

When normal plasma is exposed to foreign surfaces, Hageman factor (HF, factor XII) is activated; under appropriate circumstances, it then initiates reactions leading to coagulation, fibrinolysis, increased vascular permeability, esterolytic activity, and kinin formation. However, coagulation, fibrinolysis, and increased vascular permeability were impaired in plasma from an individual with Fletcher trait, despite a normal concentration of HF that is functionally and immunologically indistinguishable from that in normal plasma. This defective clotting is completely repaired by the addition of small amounts of normal plasma, HF-deficient plasma, plasma thromboplastin antecedent (PTA)-deficient plasma, or activated PTA but only partially repaired by the addition of activated HF. Defective fibrinolysis and permeability-enhancing activity were partially corrected by the addition of small amounts of normal plasma, HF-deficient plasma, PTA-deficient plasma, or activated HF. A preparation of partially purified plasma kallikrein largely repaired defective coagulation and fibrinolysis in Fletcher trait plasma in the presence of kaolin. In immunodiffusion studies, no precipitin line formed between Fletcher trait plasma and monospecific antikallikrein serum. Fletcher trait plasma appeared to be deficient in a plasma prekallikrein, which most probably participates in the functioning of activated HF. These studies emphasize the intimate relationships among clotting, fibrinolysis, and enhancement of vascular permeability.

KEY WORDS Fletcher factor deficiency plasma prekallikrein

In 1965, Hathaway et al. (1) described an asymptomatic clotting defect in the offspring of a consanguineous union; the defect was called Fletcher factor deficiency after the affected kindred. Attempts to demonstrate a deficiency in any recognized clotting factor failed, but prolonged exposure of the abnormal plasma to glass, Celite, or kaolin repaired the clotting defect. At least three additional unrelated individuals with this defect have been described (2). Hathaway and Alsever (3) have suggested that the Fletcher factor participates in the clotting sequence before the Hageman factor does. Saito et al. (4) have reported that the Fletcher trait is associated with an inhibitory substance in plasma that blocks the activation of normal plasma by glasslike surfaces. These data suggest that the Fletcher trait, whether due to the absence of a factor or the presence of an inhibitor, is characterized by an abnormality in the initial stages of blood clotting. A common pathway has been described (5) which, under appropriate conditions, initiates clotting, fibrinolysis, enhancement of vascular permeability, esterolysis, and kinin formation when plasma is exposed to foreign surfaces. The present study shows that surface-mediated fibrinolysis, increased vascular permeability, and clotting are all defective in Fletcher trait plasma. Defective kinin release and impaired development of arginine esterase activity have also been demonstrated in such plasma (6, 7). These findings support the view expressed by Wuepper (8) that Fletcher trait plasma is deficient in a plasma prekallikrein.

Methods

MATERIALS

Plasma was obtained as described previously (9) from normal individuals and from individuals with deficiencies in Hageman factor (HF, factor XII), plasma thromboplastin antecedent (PTA, factor XI), or Christmas factor (factor IX); coagulation was prevented with citrate, and the plasma was never in contact with glass. A standard pool of 25 normal human plasma samples was prepared by a method described previously (10). Fletcher trait plasma was prepared from blood containing 0.13 M citrate buffer (pH 5.0)
(1:10 v/v) or 0.1M sodium oxalate (1:10 v/v). This plasma was obtained from patient 3 in the study of Hattersley and Hayse (2); he has also been the subject of studies by other investigators (8, 11). The plasma contained 0.86 units/ml of HF and 1 unit/ml of PTA. (One unit of HF or PTA activity is arbitrarily defined as that amount found in 1 ml of a standard pool of normal citrated plasma.) Unless otherwise noted, citrated Fletcher trait plasma was used throughout the present study.

Kaolin, Gliddex-P, and kaolin-Gliddex, a mixture of 50 mg of kaolin suspended in 5 ml of 0.1% Gliddex-P, were prepared as described previously (12).

Ellagic acid was used at a concentration of 10^{-4} M in barbitral-saline buffer after homogenization and centrifugation.

Partially purified human HF was prepared from human oxalated plasma by an adaptation of previously described methods (13, 14). A 500-ml volume of oxalated plasma containing 0.001M ethylenediaminetetraacetic acid (EDTA) and 0.05 mg hexadimethrine bromide/ml was adsorbed with tricalcium phosphate, (7 mg/ml) for 10 minutes at room temperature. After centrifugation, the fraction of the supernatant plasma that was soluble at 20% saturation with neutral ammonium sulfate and insoluble at 60% saturation was dialyzed against 0.025M Tris buffer (pH 8.0) containing 0.05M NaCl, 0.001M EDTA, and 0.05 mg hexadimethrine bromide/ml and applied to a QAE-Sephadex A-50 column (5 x 53 cm) equilibrated with the same buffer. The protein was eluted by steps using 0.05, 0.15, and 0.3M NaCl in 0.025M Tris buffer (pH 8.0) containing EDTA and hexadimethrine bromide. HF, which is present in the 0.3M NaCl fraction (13), was concentrated by the addition of solid ammonium sulfate to 60% saturation. The precipitate was dissolved and dialyzed in 0.01M sodium phosphate buffer (pH 7.7) containing EDTA and hexadimethrine bromide, applied to a DEAE-Sephadex A-50 column (2.5 x 35 cm) equilibrated with the same buffer, and eluted with linear gradient of 1 liter of the starting buffer and 1 liter of 0.15M NaH_2PO_4 containing 0.2M NaCl, EDTA, and hexadimethrine bromide (14). The eluted HF fraction was concentrated by precipitation with solid ammonium sulfate at 60% saturation; the precipitate was then dissolved and dialyzed in 0.025M Tris buffer (pH 8.0) containing 0.15M NaCl, EDTA, and hexadimethrine bromide. The dialyzed solution (approximately 5 ml) was filtered through a 2.5 x 93-cm column of Sephadex G150 that had been preswollen in the same buffer; 4-ml fractions were collected at a flow rate of 25 ml/hour. The HF-rich fraction (usually in tubes 49-60) was pooled, and solid ammonium sulfate was added to 60% saturation. The resultant precipitate was dissolved and dialyzed against 0.05M sodium acetate buffer (pH 5.2) containing 0.15M NaCl and 0.0001M EDTA; at this step and at subsequent steps, no hexadimethrine bromide was added. The dialyzed solution was then applied to a 1.5 x 23-cm column of Sephadex C-50 equilibrated with the same buffer, and it was eluted with a linear salt gradient of 150 ml each of the starting buffer and 0.05M sodium acetate buffer (pH 5.2) containing 0.5M NaCl and EDTA. HF was eluted at concentrations of NaCl between 0.32M and 0.35M. HF prepared by this method had a specific activity of 31-52 units/mg protein. Virtually all of this HF was in the precursor form; the activated form accounted for less than 0.2% of the preparation. No measurable amount of other clotting or fibrinolytic factors were present. Barium carbonate-activated HF that was free of particulate matter was prepared as described previously (15).

Partially purified human PTA (specific activity 82 units/mg protein) and PTA activated by Eznitryptsin (0.4 units/mg) were prepared as described previously (13).

Partially purified human plasma kallikrein was prepared as described previously (13) and was further purified by chromatography on a Sephadex G150 column. The preparation released 5.5 μM methanol/ml hour^{-1} (specific activity 132 μM methanol/ml protein hour^{-1}) in a p-toluenesulfonyl-l-arginine methyl esterase assay and, on the average, it released 330 ng bradykinin/ml in a rat uterus assay. It contained traces of activated PTA activity (less than 0.01 units/ml) and HF-cofactor; however, no measurable amounts of other clotting factors or α_{2}-macroglobulin, as tested by immunodiffusion against monospecific goat antiserum, were present. In experiments on the effect of kallikrein on purified HF, a kallikrein preparation which released 7.7 μM methanol/ml hour^{-1} was used.

Insoluble kallikrein was prepared from partially purified kallikrein by attachment to cyanogen bromide-activated Sepharose 4B (16). This kallikrein preparation released 26.4 μM methanol/ml hour^{-1} (specific activity 755 μM methanol/ml protein hour^{-1}) and contained no detectable amounts of clotting factors, plasmin, or HF-cofactor except for traces of activated PTA activity (< 0.002 units/ml). Insoluble kallikrein released 24.2 μM methanol/ml hour^{-1} in a p-toluenesulfonyl-l-arginine methyl esterase assay and 180 ng bradykinin/ml 30 min^{-1} in a rat uterus assay.

Rabbit antiserum to human HF was prepared and treated as reported earlier (17). Rabbit antiserum to human kallikrein was prepared by immunization of New Zealand albino female rabbits with partially purified kallikrein (specific activity 755 μM methanol/ml hour^{-1}). On immunodiffusion in 0.9% agarose, the unabsorbed antiserum gave three precipitin lines with normal, HF-deficient, or PTA-deficient plasma but only two lines with Fletcher trait plasma. After absorption with Fletcher trait plasma, the antiserum formed an apparent single precipitin line of identity against normal, HF-deficient or PTA-deficient plasma, purified prekallikrein, or kallikrein; however, it did not form a line against Fletcher trait plasma. The absorbed antiserum retained the ability to neutralize the kinin-generating activity of kallikrein. Rabbit antisera to human fibrin and human plasminogen were synthesized by Dr. James Cram, Case Western Reserve University.

The Fletcher trait plasma was generously supplied by Dr. Charles Abildgaard and Dr. Paul Hattersley, University of California at Davis.
obtained from Hyland Laboratories and Behringwerke AG, respectively. Goat antiserum to human α-macroglobulin was obtained from Hyland Laboratories.

Streptokinase ("high purity") was the gift of Led-erle Laboratories, American Cyanamid Company, Pearl River, New York.

α-Casein, bovine fibrinogen, and crude bovine thrombin were obtained from Worthington Biochemical Corp., Nutritional Biochemical Co., and Parke Davis & Co., respectively.

Euglobulin precipitates were prepared by diluting 0.5 ml of plasma with 9.5 ml of 0.01M sodium acetate buffer (pH 4.8) in 17 x 100-mm polystyrene tubes. After incubation at 4°C for 10 minutes, the precipitates were separated by centrifugation at 1,300 g.

Crude Celite eluates were prepared with Celite 512 from citrated normal plasma, Fletcher trait plasma, or HF-deficient plasma as described previously (18).

Glass-adsorbed plasma was prepared from normal human citrated plasma as described previously (19); it was deficient in HF-cofactor but contained normal amounts of HF (0.98 units/ml) in the precursor form, plasminogen (720 μg acid-soluble tyrosinelike materials released/ml hour), and plasma components required for the function of HF-cofactor (19).

Hirudin (4080 antithrombin units/ml protein) and p-toluenesulfonyl-L-arginine methyl ester were obtained from Veb Arzneimittelwerk and Nutritional Biochemical Corp., respectively.

The barbital-saline buffer consisted of 0.025M barbital in 0.125M NaCl (pH 7.5).

PROCEDURE

Assays for HF, PTA, Christmas factor, antihemophilic factor (AHF), Stuart factor, factor VII, proaccelerin, prothrombin, thrombin, and fibrinogen were performed by techniques described previously (20). Plasma plasminogen concentrations were assayed in euglobulin precipitates by a modification (21) of the method of Remmert and Cohen (22). p-Toluenesulfonyl-L-arginine methyl esterase activity was assayed by a previously described modification (23) of Siegelman’s method (24). HF-cofactor was assayed by a method described previously (25). Immunodiffusion was performed in 0.9% agarose gel in 0.05M barbital buffer (pH 8.6) for 2 days at room temperature.

The coagulative abnormality in Fletcher trait plasma was studied by measuring the partial thromboplastin time. The effect of addition of normal plasma, HF-deficient plasma, or PTA-deficient plasma on the kaolin partial thromboplastin time of Fletcher trait plasma was tested in mixtures of different proportions of Fletcher trait plasma and normal, HF-deficient, or PTA-deficient plasma in 10 x 75-mm polystyrene tubes; 0.1 ml of each mixture was transferred to a 10 x 75-mm glass tube containing 0.1 ml of kaolin-Gliddex. After a 1-minute incubation at 37°C, 0.1 ml of 0.025M CaCl₂ was added, and the clotting time was determined. The effect of the addition of activated PTA on the partial thromboplastin time of Fletcher trait plasma was tested by adding 0.1 ml of activated PTA to 0.1 ml of Gliddex and 0.1 ml of Fletcher trait plasma in a 10 x 75-mm polystyrene tube. After a 1-minute incubation at 37°C, 0.1 ml of 0.025M CaCl₂ was added, and the clotting time was measured. The effect of the addition of partially purified kallikrein on normal human HF was studied by incubating equal amounts of purified HF (5 units/ml, 52 units/mg protein) and kallikrein (7.7 μM methanol/ml hour⁻¹) in 10 x 75-mm polystyrene tubes at 37°C. At intervals, samples were tested for HF-like clot-promoting properties with or without kaolin, using human HF-deficient plasma as the substrate. The effect of insoluble kallikrein on purified human HF was studied by incubating equal amounts of purified HF (3.2 units/ml, 52 units/mg protein) and insoluble kallikrein or Sepharose 4B in silicone-coated 10 x 75-mm polystyrene tubes at 37°C with continuous stirring. At 1-, 2-, and 3-hour intervals, 0.2-ml samples were centrifuged, and the supernatant fluids were tested for HF-like clot-promoting properties as described above. Other modifications of the partial thromboplastin time used in additional experiments are described in the legends to Tables 1-3 and Figure 1.

Measurement of kaolin-induced generation of fibrinolytic activity in plasma was performed by a method described previously (19). The effect of the addition of partially purified plasma kallikrein on the kaolin-induced fibrinolysis of Fletcher trait plasma was studied in 17 x 100-mm polystyrene tubes by adding in order (1) 0.25 ml of kaolin suspension (8 mg/ml barbital-saline buffer), (2) 0.1 ml of partially purified plasma kallikrein (5.5 μM methanol/ml hour⁻¹), (3) 0.5 ml of test plasma, and (4) 9.0 ml of sodium acetate buffer (0.01M, pH 4.8). The tubes were incubated at 37°C for 60 minutes and the fibrinolytic activity that was generated was assayed by the method described. The techniques used in additional studies of fibrinolysis are described in the legends to Tables 4-6.

Vascular permeability was measured by the method of Miles and Wilhelm (26) who injected the solutions to be tested (0.1 ml, intracutaneously) into the depilated backs of albino guinea pigs that had been injected with a 5% solution of pontamine sky blue 6X in 0.075M NaCl (1.2 ml/kg body weight, iv). Fifteen minutes after the last injection, the size of the blue spots that appeared at the injection sites was estimated by averaging the largest diameter and its perpendicular; the blue spots were a consequence of the vascular permeability-enhancing properties of the material injected. The assay was performed in four guinea pigs, and the data were averaged. The techniques used in the studies of the development of permeability-enhancing properties in human plasma are described in the legends to Tables 7 and 8.

Protein was determined by the method of Lowry et al. (27).

Results

STUDIES ON THE CLOTTING DEFECT OF FLETCHER TRAIT PLASMA

As Hathaway and Alsever (3) have reported, the prolonged partial thromboplastin time characteristic of Fletcher trait plasma shortened pro-
Fletcher trait plasma was more evident when exposure to surfaces was short. When trace amounts (5%) of HF-deficient plasma were added to Fletcher trait plasma, the shortening of the kaolin partial thromboplastin time with time was similar to that of normal plasma (Fig. 1). Also, the addition of small amounts of normal plasma or PTA-deficient plasma corrected the defect in Fletcher trait plasma; this finding agrees with that of Hathaway et al. (1). For example, after 1 minute of activation, the kaolin partial thromboplastin time of Fletcher trait plasma alone was 364 seconds, but that of a mixture of 95% Fletcher trait plasma and 5% normal plasma was 73 seconds and that of normal plasma alone was 59 seconds. As anticipated, the defect in HF-deficient plasma was corrected by the addition of purified HF.

These experiments imply that Fletcher trait plasma may be lacking in components necessary for normal contact activation of clotting. Since small amounts of normal plasma repair this defect, it cannot be explained by the presence of an inhibitor. A preparation of Enzite-trypsin-activated PTA shortened the partial thromboplastin time of Fletcher trait plasma, PTA-deficient plasma, and HF-deficient plasma to the same degree, i.e., to 93, 90, and 88 seconds, respectively, but it did not affect the Christmas factor-deficient plasma. In the absence of activated PTA, the partial thromboplastin time of each of the test plasmas was greater than 300 seconds. Thus, the defect in Fletcher trait plasma affects clotting progressively to the normal range after prolonged shaking with kaolin, but that of HF-deficient plasma did not change significantly under the same conditions (Fig. 1). Thus, the defect in

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**TABLE 1**

**Effect of Addition of a Kallikrein Preparation on the Partial Thromboplastin Time of Fletcher Trait Plasma**

<table>
<thead>
<tr>
<th>Substrate plasma</th>
<th>Kallikrein-kaolin* (seconds)</th>
<th>Albumin-kaolin* (seconds)</th>
<th>Kallikrein alone† (seconds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fletcher trait</td>
<td>88.2</td>
<td>&gt; 300</td>
<td>&gt; 300</td>
</tr>
<tr>
<td>HF-deficient</td>
<td>242.4</td>
<td>&gt; 300</td>
<td>&gt; 300</td>
</tr>
<tr>
<td>PTA-deficient</td>
<td>173.8</td>
<td>230.2</td>
<td>&gt; 300</td>
</tr>
<tr>
<td>Christmas factor-deficient</td>
<td>&gt; 250</td>
<td>&lt; 1</td>
<td>&gt; 250</td>
</tr>
</tbody>
</table>

* A tenth of a ml of partially purified kallikrein (5.5 μmol methanol/ml hour) or of albumin was added to 0.1 ml of kaolin-Gliddex and 0.1 ml of substrate plasma in 10 × 75-mm glass tubes. After a 1-minute incubation at 37°C, 0.1 ml of 0.025M CaCl₂ was added, and the clotting time was determined.

† A tenth of a ml of partially purified kallikrein (5.5 μmol methanol/ml hour) was added to 0.1 ml of Gliddex and 0.1 ml of substrate plasma in 10 × 75-mm polystyrene tubes. After a 1-minute incubation at 37°C, 0.1 ml of 0.025M CaCl₂ was added, and the clotting time was determined.

† Percent of clot-promoting activity was estimated by testing serial dilutions of purified kallikrein in the same assay system using Fletcher trait plasma as a substrate. A linear relationship existed between the logarithm of the concentration of kallikrein and the logarithm of the kaolin partial thromboplastin time.

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at a point earlier than that at which activated PTA participates, and this plasma possesses no abnormal inhibitory activity directed against either activated PTA or any clotting factor acting subsequent to PTA.

Wuepper (8) has shown that the clotting defect of Fletcher trait plasma is corrected by the addition of prekallikrein, which is the precursor of plasma kallikrein. He concluded that the defect in Fletcher trait plasma is a deficiency in prekallikrein. In agreement with this theory, kallikrein could not be demonstrated by immunodiffusion of Fletcher trait plasma with monospecific antibody against human plasma kallikrein, but a precipitin line between normal, HF-deficient, or PTA-deficient plasma and the same antibody was observed. Wuepper (8) has made somewhat similar observations. Furthermore, the addition of a preparation of human plasma kallikrein significantly shortened the kaolin partial thromboplastin time of Fletcher trait plasma but did not appreciably affect that of HF-deficient plasma, PTA-deficient plasma, or Christmas factor–deficient plasma (Table 1). The effect of the kallikrein preparation could not be attributed to contamination by the activated forms of HF, PTA, or Christmas factor. Its corrective action on Fletcher trait plasma was noted only in the presence of kaolin.

The site of action of the clot-promoting property in the preparation of plasma kallikrein on Fletcher trait plasma was studied in several ways. The addition of partially purified, unactivated PTA shortened the prolonged kaolin partial thromboplastin time of Fletcher trait plasma but not that of HF-deficient plasma, PTA-deficient plasma, or Christmas factor–deficient plasma (Table 1). The effect of the kallikrein preparation could not be attributed to contamination by the activated forms of HF, PTA, or Christmas factor. Its corrective action on Fletcher trait plasma was noted only in the presence of kaolin.

The partial thromboplastin time was measured (Table 2). The partial thromboplastin time of the mixture containing HF-deficient plasma was considerably shorter than that of the mixture containing Fletcher trait plasma; the same experiment with three other HF-deficient plasma samples gave similar results. This experiment localized the defect in Fletcher trait plasma to a point after the interaction of HF and kaolin. However, in these experiments kaolin was transferred to the substrate plasma before the partial thromboplastin time was measured. To avoid this problem, activated HF that was free of particulate matter was prepared by adsorption of purified HF onto barium carbonate, which was then dissolved in weak acid. When this activated HF was added to HF-deficient plasma and tested immediately, the partial thromboplastin time of the mixture was 180 seconds; this time was noticeably shorter than the partial thromboplastin time after activated HF was added to Fletcher trait plasma (293 seconds). When the partial thromboplastin time was measured after activated HF had been incubated with plasma for 4 minutes, the difference was less noticeable; the clotting time with HF-deficient plasma was 99 seconds, and that with Fletcher trait plasma was 127 seconds. The activated HF had no effect on PTA-deficient plasma.
The preparation of barium carbonate-activated HF contained a large proportion of unactivated HF. No evidence that this unactivated fraction was activated by exposure to polystyrene during the experiment was obtained. Conceivably the unactivated HF could have been activated by kallikrein derived from a prekallikrein in the HF-deficient plasma as a consequence of the addition of the activated HF. This newly formed activated HF could then have been responsible for the shorter partial thromboplastin time observed when HF-deficient plasma was the substrate. This explanation seems unlikely, because partially purified kallikrein did not induce clot-promoting activity when it was incubated with partially purified, unactivated HF in the absence of kaolin (Table 3). The same negative result was obtained when insoluble kallikrein was incubated with the preparation of HF. These experiments suggest that the clot-promoting effect of kallikrein preparations is exerted after the interaction of HF and kaolin. This view agrees with the experiment described in the preceding paragraph in which purified kallikrein accelerated clotting of Fletcher trait plasma only if kaolin was present.

In summary, impaired clotting in Fletcher trait plasma was corrected by a preparation of kallikrein if kaolin was present. The site of action of the kallikrein preparation appeared to be localized to a step after the interaction of HF and kaolin (or similar agents) and before the activation of PTA.

### STUDIES ON THE FIBRINOLYTIC DEFECT OF FLETCHER TRAIT PLASMA

The generation of fibrinolytic activity in the incubation mixture of kaolin and diluted, acidified normal plasma depends on the presence of HF (28). Ogston et al. (19) and Kaplan and Austen (29) have shown that at least one or two additional components besides HF and plasminogen are necessary. One of these components has been designated as the HF-cofactor (19) or plasminogen proactivator (29). Together with Herbert et al. (30), these investigators have provided evidence that HF-cofactor or plasminogen proactivator may be different from plasma kallikrein.

The generation of fibrinolytic activity was defective in Fletcher trait plasma and HF-deficient plasma. Thus, the lysis time of diluted, acidified normal plasma, incubated with kaolin for 60 minutes before testing, was 7 minutes, but the lysis

### TABLE 3

Effect of Addition of Partially Purified Kallikrein on Purified HF

<table>
<thead>
<tr>
<th>Incubation time (minutes)</th>
<th>HF Activity (seconds)</th>
<th>With kaolin</th>
<th>Without kaolin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>49.6</td>
<td>255.2</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>51.1</td>
<td>228.5</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>55.0</td>
<td>219.1</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>49.8</td>
<td>255.8</td>
<td></td>
</tr>
</tbody>
</table>

HF (0.15 ml, 5 units/ml, 52 units/mg protein) was incubated with an equal amount of kallikrein (7.7 μM methanol/ml hour⁻¹) in 10 x 75-mm polystyrene tubes. At intervals, 0.1-ml samples were tested for clot-promoting activity in the presence or absence of kaolin using a substrate of human HF-deficient plasma.

The addition of 5% of normal, HF-deficient, or PTA-deficient plasma to Fletcher trait plasma shortened the prolonged clot lysis time significantly.

### TABLE 4

Kaolin-Induced Generation of Fibrinolytic Activity of Fletcher Trait Plasma: Effects of Adding Small Amounts of Normal, HF-Deficient, or PTA-Deficient Plasma or of Adding Glass-Adsorbed Plasma

<table>
<thead>
<tr>
<th>Test plasma</th>
<th>Lysis time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fletcher trait</td>
<td>200</td>
</tr>
<tr>
<td>95% Fletcher trait + 5% normal</td>
<td>35</td>
</tr>
<tr>
<td>95% Fletcher trait + 5% HF-deficient</td>
<td>19</td>
</tr>
<tr>
<td>95% Fletcher trait + 5% PTA-deficient</td>
<td>31</td>
</tr>
<tr>
<td>Normal</td>
<td>7</td>
</tr>
<tr>
<td>HF-deficient</td>
<td>120</td>
</tr>
<tr>
<td>PTA-deficient</td>
<td>10</td>
</tr>
<tr>
<td>Fletcher trait</td>
<td>240</td>
</tr>
<tr>
<td>HF-deficient</td>
<td>120</td>
</tr>
<tr>
<td>Glass-adsorbed</td>
<td>122</td>
</tr>
<tr>
<td>50% Fletcher trait + 50% HF-deficient</td>
<td>10</td>
</tr>
<tr>
<td>50% Fletcher trait + 50% glass-adsorbed</td>
<td>110</td>
</tr>
<tr>
<td>50% HF-deficient + 50% glass-adsorbed</td>
<td>16</td>
</tr>
</tbody>
</table>

Test plasma (0.5 ml), kaolin (0.25 ml, 8 mg/ml), and sodium acetate buffer (0.5 ml, 0.01M, pH 4.8) were incubated together in 17 x 100-mm polystyrene tubes at 37°C for 60 minutes. The fibrinolytic activity generated was assayed as described in Methods.
TABLE 5

<table>
<thead>
<tr>
<th>Incubation time (minute)</th>
<th>Lysis time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fletcher Trait Plasma</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>HF-Deficient Plasma</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Normal Plasma</td>
</tr>
<tr>
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<td>0</td>
</tr>
</tbody>
</table>

Test plasma (0.5 ml) was shaken with kaolin suspension (0.25 ml, 8 mg/ml barbital-saline buffer) in a 17 × 100-mm polystyrene tube at 37°C for the time indicated. Sodium acetate buffer (9.25 ml, 0.01 M, pH 4.8) was then added and the tube was incubated at 37°C for 60 minutes. After centrifugation, the euglobulin precipitate containing kaolin was resuspended in 0.5 ml of barbital-saline buffer. The clot lysis time was measured after the addition of bovine fibrinogen and thrombin as described in Methods.

TABLE 6

<table>
<thead>
<tr>
<th>Celite eluate</th>
<th>Plasma</th>
<th>Lysis time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fletcher trait</td>
<td>Fletcher trait</td>
<td>6</td>
</tr>
<tr>
<td>Fletcher trait</td>
<td>HF-deficient</td>
<td>5</td>
</tr>
<tr>
<td>HF-deficient</td>
<td>Fletcher trait</td>
<td>6</td>
</tr>
<tr>
<td>HF-deficient</td>
<td>HF-deficient</td>
<td>74</td>
</tr>
<tr>
<td>Normal</td>
<td>Fletcher trait</td>
<td>7</td>
</tr>
<tr>
<td>Normal</td>
<td>HF-deficient</td>
<td>5</td>
</tr>
<tr>
<td>Barbital-saline buffer</td>
<td>Fletcher trait</td>
<td>&gt; 90</td>
</tr>
<tr>
<td>Barbital-saline buffer</td>
<td>HF-deficient</td>
<td>&gt; 90</td>
</tr>
</tbody>
</table>

In 17 × 100-mm polystyrene tubes, the following reagents were added successively: (1) 1 ml of Celite eluate of Fletcher trait, HF-deficient, or normal plasma or 1 ml of barbital-saline buffer, (2) 0.25 ml of kaolin suspension (8 mg/ml), (3) 0.5 ml of Fletcher trait or HF-deficient plasma, (4) 0.1 ml of hirudin (15 antithrombin units/ml), and (5) 9 ml of 0.01 M sodium acetate buffer (pH 4.8). The tubes were incubated at 37°C for 60 minutes and were then centrifuged at 1,300 g for 5 minutes. The euglobulin-kaolin precipitates were dissolved in 0.5 ml of barbital-saline buffer and the clot lysis time was measured as described in Methods.

In earlier studies, fibrinolytic activity did not develop on the addition of kaolin to plasma rich in HF and plasminogen but depleted of certain other agents by adsorption with glass (19). When glass-adsorbed plasma mixed with an equal amount of HF-deficient plasma was tested for kaolin-induced generation of fibrinolytic activity, the clot lysis time was short (Table 4). In contrast, the addition of glass-adsorbed plasma to Fletcher trait plasma did not correct its defective generation of fibrinolytic activity, suggesting that Fletcher trait plasma is deficient in components that are also absent from glass-adsorbed plasma. Although it is possible that the deficient agent was HF-cofactor, more than one plasma component might have been removed by the glass-adsorption procedure.

When Fletcher trait plasma was shaken at 37°C with kaolin for prolonged periods of time before acidification and dilution, the clot lysis time was progressively shortened; however, the clot lysis time of HF-deficient plasma was not changed by the same treatment (Table 5). Thus, prolonged exposure to kaolin repaired the defective fibrinolysis of Fletcher trait plasma in the same manner that prolonged exposure to kaolin corrected the long partial thromboplastin time of Fletcher trait plasma (3).

Celite eluates prepared from Fletcher trait plasma or HF-deficient plasma repaired the defective fibrinolysis of Fletcher trait plasma (Table 6). In contrast, the addition of Celite eluate derived from HF-deficient plasma did not shorten the long clot lysis time of HF-deficient plasma. These experiments suggest that Celite eluates, even those prepared from HF-deficient plasma, contain components that hasten the development of fibrinolytic activity but depend on the presence of HF for their action. Clearly, the Celite eluates derived from Fletcher trait plasma corrected the deficient generation of fibrinolysis in this plasma.

Earlier we showed that, in addition to HF-cofactor, a second component was necessary for the generation of fibrinolytic activity in kaolin-treated, diluted, acidified plasma (19). This second unnamed component was found in the supernatant fluid after separation of the euglobulin precipitate. The addition of the supernatant fraction of Fletcher trait plasma, HF-deficient plasma, and glass-adsorbed plasma to a normal

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euglobulin precipitate shortened the clot lysis time from 70 minutes to 13, 11, and 10 minutes, respectively, demonstrating that all of these plasmas contained this second agent.

When purified HF was first mixed with kaolin and then added to Fletcher trait plasma, the clot lysis time of Fletcher trait plasma decreased from 200 minutes to 21 minutes. The degree of shortening, however, was less than that observed when purified HF was added to HF-deficient plasma, i.e., from 120 minutes to 8 minutes. The action of purified HF was not due to contamination by HF-cofactor, since it did not affect glass-adsorbed plasma.

When partially purified plasma kallikrein was added to Fletcher trait plasma, the clot lysis time decreased from 200 minutes to 10 minutes. The kallikrein preparation also corrected the defective fibrinolysis of glass-adsorbed plasma but to a much lesser degree; it shortened the clot lysis time from 77 minutes to 40 minutes. The kallikrein preparation had no effect on HF-deficient plasma. Thus, this kallikrein preparation appeared to contain small amounts of HF-cofactor.

STUDIES ON THE DEFECT IN PERMEABILITY-ENHANCING ACTIVITY IN FLETCHER TRAIT PLASMA

Incubation of normal plasma with ellagic acid, a soluble activator of HF, induces the permeability-enhancing activity (31). Neither HF-deficient plasma nor Fletcher trait plasma acquired this property when they were diluted a hundredfold in 5 x 10^{-5} M ellagic acid and incubated at 37°C (Table 7). The addition of as little as 5% of HF-deficient plasma to Fletcher trait plasma partially corrected its defective generation of the permeability-enhancing activity, suggesting that Fletcher trait plasma was deficient in components required for the generation of permeability-enhancing activity distinct from HF.

The addition of small amounts of purified HF treated with ellagic acid induced the formation of permeability-enhancing activity in HF-deficient plasma (Table 8). The ellagic acid–HF mixture also induced permeability-enhancing activity in Fletcher trait plasma but at a slower rate and to a lesser degree.

TABLE 7
Effect of Ellagic Acid on the Development of Permeability-Enhancing Activity in Normal, HF-Deficient, or Fletcher Trait Plasma

<table>
<thead>
<tr>
<th>Plasma (diluted 1/100)</th>
<th>Diluent</th>
<th>0 minutes</th>
<th>6 minutes</th>
<th>12 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Buffer</td>
<td>1.0</td>
<td>1.0</td>
<td>0.9</td>
</tr>
<tr>
<td>Normal</td>
<td>Ellagic acid</td>
<td>3.9</td>
<td>7.0</td>
<td>7.0</td>
</tr>
<tr>
<td>HF-deficient</td>
<td>Buffer</td>
<td>1.1</td>
<td>1.2</td>
<td>0.8</td>
</tr>
<tr>
<td>HF-deficient</td>
<td>Ellagic acid</td>
<td>3.4</td>
<td>3.0</td>
<td>2.7</td>
</tr>
<tr>
<td>Fletcher trait</td>
<td>Buffer</td>
<td>0.9</td>
<td>1.1</td>
<td>1.2</td>
</tr>
<tr>
<td>Fletcher trait</td>
<td>Ellagic acid</td>
<td>3.7</td>
<td>3.1</td>
<td>3.1</td>
</tr>
<tr>
<td>95% Fletcher trait + 5% HF-deficient</td>
<td>Buffer</td>
<td>1.0</td>
<td>0.9</td>
<td>1.1</td>
</tr>
<tr>
<td>95% Fletcher trait + 5% HF-deficient</td>
<td>Ellagic acid</td>
<td>2.8</td>
<td>4.5</td>
<td>5.1</td>
</tr>
<tr>
<td>Buffer</td>
<td>Ellagic acid</td>
<td>3.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buffer</td>
<td>Buffer</td>
<td>1.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Plasma (0.05 ml) was diluted with 4.95 ml of 5 x 10^{-5} M ellagic acid or with barbital-saline buffer in 13 x 100-mm silicone-coated glass tubes. Each tube was incubated at 37°C and, at the time indicated, 0.1-ml samples were injected into each of four guinea pigs. The permeability-enhancing activity was measured as described in Methods as the mean diameter of blued spots.

Discussion

The clotting abnormality described by Hathaway and his associates (1) as Fletcher trait is characterized by an abnormally long kaolin partial thromboplastin time despite the presence of normal amounts of any recognized plasma clotting factor. The abnormal kaolin partial thromboplastin time was shortened by prolonged exposure of Fletcher trait plasma to agents such as glass, kaolin, or Celite. Hathaway and Alsever (3) have suggested that the defect in Fletcher trait plasma is localized to the steps preceding the participation of HF in the intrinsic pathway of coagulation. Recently, Wuepper (8) has reported that the addition of a plasma prekallikrein corrects the long kaolin partial thromboplastin time of Fletcher trait plasma. The present report used two different approaches to clarify the defect in Fletcher trait plasma. In the first ap-
proach, purified known clotting factors were used for the localization of the clotting defect. The addition of partially purified, activated PTA corrected the clotting defect of Fletcher trait plasma, but the addition of partially purified, activated HF only partially shortened its prolonged partial thromboplastin time. Therefore, the defect appears to involve impairment of the action of activated HF on PTA. However, the alternate possibility that kallikrein is required as a cofactor in the activation of HF kaolin cannot be excluded. In a second approach, the effect of the addition of purified kallikrein on Fletcher trait plasma was studied. Kallikrein corrected the clotting defect of Fletcher trait plasma in the presence of kaolin. Furthermore, plasma prekallikrein was not demonstrated by immunodiffusion in Fletcher trait plasma. Thus, in agreement with Wuepper (8), Fletcher trait plasma appears to be deficient in a kallikrein which might be required for the action of HF.

How plasma kallikrein interacts with activated HF is not known. Since kallikrein does not activate PTA (13), it could serve as a cofactor for activated HF. Cochrane et al. (32) have recently reported that human plasma kallikrein activates purified human HF in the fluid phase, as assayed on a substrate of prekallikrein rather than in a clotting system. In contrast, in our experiments, human plasma kallikrein did not evoke clot-promoting activity when it was incubated with human HF; these results are similar to those obtained by Wuepper and Cochrane (33) who could not demonstrate shortening of the partial thromboplastin time of human plasma by human plasma kallikrein.

As Hathaway et al. (1) have demonstrated, the defect in coagulation in Fletcher trait plasma can be corrected by the addition of small amounts of normal plasma, suggesting that a functional deficiency of a normal plasma component is present. It has also been reported (4) that, under appropriate conditions, the plasma of individuals with Fletcher trait, like that of individuals with Hageman trait, inhibits the clot-promoting properties of glass. The nature of this inhibitory property is not yet clear. One likely explanation is that normal plasma contains an inhibitor of the activation of HF by glass. Perhaps the inhibitory activity is revealed only if activation of HF or its subsequent action is impeded through some other mechanism, e.g., a deficiency in HF. Evidence to support this view using plasma artificially depleted of HF or prekallikrein is available (34). Similarly, if an agent absent from the plasma of individuals with Fletcher trait is needed for the optimal functioning of HF, the effect of the inhibitor might be expressed.

Besides its clot-promoting function, HF participates in reactions leading to fibrinolysis (19, 28), enhancement of vascular permeability (31, 35), and activation of plasma prekallikrein (36–38). Therefore, it was not surprising to find that fibrinolysis and enhancement of vascular permeability were defective in Fletcher trait plasma. The impaired development of permeability-enhancing activity and of kaolin-activated fibrinolysis in Fletcher trait plasma has also been reported recently by Wuepper (8) and Weiss et al. (11), respectively. The defective fibrinolysis resembled the clotting defect in that it could be corrected by prolonged exposure of Fletcher

### TABLE 8

<table>
<thead>
<tr>
<th>Plasma (diluted 1/100)</th>
<th>Diluent</th>
<th>Permeability-enhancing activity (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 minutes</td>
</tr>
<tr>
<td>Normal</td>
<td>Ellagic acid-HF</td>
<td>7.5</td>
</tr>
<tr>
<td>HF-deficient</td>
<td>Ellagic acid-HF</td>
<td>7.4</td>
</tr>
<tr>
<td>Fletcher trait</td>
<td>Ellagic acid-HF</td>
<td>4.5</td>
</tr>
<tr>
<td>Buffer</td>
<td>Ellagic acid-HF</td>
<td>3.7</td>
</tr>
<tr>
<td>Buffer</td>
<td>Buffer</td>
<td>1.7</td>
</tr>
</tbody>
</table>

Ellagic acid (4.95 ml, 5 x 10⁻⁵M) containing purified HF (0.04 units/ml, 31 units/mg protein) was incubated at 37°C for 5 minutes in a 15 x 100-mm silicone-coated glass tube; 0.05 ml of plasma was added and the tube was incubated at 37°C. At the time indicated, 0.1-ml samples were injected into each of four guinea pigs. The permeability-enhancing activity was measured as described in Methods as the mean diameter of blued spots.
trait plasma to kaolin. Both defective fibrinolysis and defective permeability-enhancing activity were partially repaired by the addition of small amounts of HF-deficient plasma or activated HF.

Defective fibrinolysis in Fletcher trait plasma was not corrected by the addition of glass-adsorbed plasma, suggesting that at least one component was missing from both. Partially purified, kaolin-treated HF partially corrected the defect in Fletcher trait plasma but did not correct that of glass-adsorbed plasma. These data are consistent with the view that Fletcher trait plasma contains HF cofactor. Indeed, Celite eluates of Fletcher trait plasma had properties resembling this agent (Table 6). Similarly, the partial correction of defective generation of permeability-enhancing activity by the addition of HF treated with ellagic acid implies that the abnormality in Fletcher trait is not due to absence of the precursor of the agent causing permeability-enhancing activity.

We have emphasized that the abnormalities in assays testing the generation of clot-promoting, fibrinolytic, and permeability-enhancing activities were all only partially corrected by the addition of partially purified activated HF. The most appealing explanation is that a plasma kallikrein, which can be derived from normal plasma but not Fletcher trait plasma is needed for the optimal functioning of activated HF. An alternative possibility is that plasma kallikrein may be required as a cofactor in the activation of HF by surfaces (38).

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