Complexes Between C1-Inhhibitor, Kallikrein, High Molecular Weight Kininogen, Plasma Thromboplastin Antecedent, and Plasmin in Normal Human Plasma and Hereditary Angioneurotic Edema Plasmas Containing Dysmorphic C1-Inhibitors: Role of Cold Activation

By Virginia H. Donaldson and Richard A. Harrison

When normal human plasma was adsorbed at 4°C with monospecific antibody globulins to human C1-INH inhibitor (anti-C1-INH), C1-INH was removed from the plasma in proportion to the amount of antibody globulins used. Clotting activity attributable to Fletcher factor (prekallikrein), high molecular weight kininogen (HMW-K), and irreguarly, plasma thromboplastin antecedent (PTA, factor XI) were also removed by these antibody globulins, and the removal of PTA and HMW-K was dependent on the presence of the prekallikrein. Plasma kallikrein and HMW-K antigen concentrations were also reduced by adsorption with anti-C1-INH. Neither prekallikrein, high molecular weight kininogen, nor PTA were removed from plasma of persons with hereditary angioneurotic edema containing any of three different types of dysmorphic C1-INH proteins. Hageman factor was not removed from normal plasma by this immune adsorption procedure, even after the plasma was activated with glass or ellagic acid, but it was required for immune depletion of kallikrein and HMW-K, for neither was reduced by adsorption of Hageman trait plasma. In the presence of Hageman factor, prekallikrein was apparently activated at 4°C, the kallikrein formed complexes with C1-INH, and this complex was then removed by the anti-C1-INH. Plasminogen was not removed from normal plasma by adsorption with anti-C1-INH globulins, but plasmin was removed once generated by treating plasma with streptokinase. Similarly, after exposure of hereditary angioneurotic edema plasmas containing dysmorphic C1-INH proteins to streptokinase, the plasminogen concentration was reduced and some plasmin was removed by the anti-C1-INH.

MATERIALS AND METHODS

Platelet-deficient plasma was obtained by centrifugation of blood drawn into 1/50 volume of buffered 0.5 M sodium citrate, pH 5.2, in silicone-coated vessels (GE-Dri-Film, SC-87, General Electric, Watertown, N.Y.) and recentrifugation at 20,000 g in polycarbonate tubes coated with silicone oil (General Electric, Silicone Oil, 200 centipoids viscosity). Citrated plasma from persons with severe inherited deficiencies of Hageman factor (factor XII), Fletcher factor (prekallikrein), and plasma thromboplastin antecedent (PTA, factor XI) were purchased from George King Biologies, Kansas City, Mo. Plasma deficient in both high and low molecular weight kininogens was prepared in this laboratory. This subject and other normal persons provided blood after giving informed consent in accordance with the Helsinki Declaration. Plasma in citrate or EDTA from persons with hereditary angioneurotic edema having dysmorphic C1-INH proteins of 3 different electrophoretic mobilities had been obtained from patients referred for diagnostic study and stored at -70°C. One plasma had been thawed previously.

Antigens were quantified by electroimmunodiffusion using the procedure of Laurell.5,6,7 Agarose (L’Industrie Biologique Francaise, Clichy, Paris, from Fisher Scientific Co., Cincinnati, Ohio) was used in a concentration of 1% on running plates and electrophoresis was carried out at pH 8.6 using a 0.075 M barbital buffer. After washing plates in 0.15 M sodium chloride and drying, antigen–antibody precipitin reactions were stained with 0.25% Coomassie brilliant blue (w/v) dissolved in a solution of 3:1:9 parts methanol, glacial acetic acid, and water, and destained in the methanol, acetic acid, and water solution. In some experiments, a semiquantitative estima-
tion of the depletion of plasma of CT-INH antigens with anti-CT-INH globulins was made using an Ouchterlony procedure in which serial dilutions of test plasmas were reacted with monospecific anti-CT-INH. This antisera gave a precipitin reaction with normal plasma and serum through a dilution of 1/32; the normal serum and plasma contained 20 mg CT-INH/dl when quantified in electroimmunodiffusion.

Antiserum to purified CT-Inhibitor (CT-INH) was prepared in rabbits or goats, and after adsorption with serum from patients with hereditary angioneurotic edema who were markedly deficient in both the functional and antigenic properties of CT-INH, each antisera was monospecific. Monospecificity was designated by immunoelectrophoretic analysis using the technique of Grabar and Williams, in which a single precipitin arc was found when the antisera was reacted with plasma from normal persons, but none in plasma from patients with hereditary angioneurotic edema markedly deficient in both properties of CT-INH. Goat and rabbit antisera gave reactions of complete identity with each other when they were reacted against normal human serum in double agarose diffusion. CT-Inhibitor used to immunize rabbits was prepared according to the method of Pensky et al. and that used to immunize goats was prepared by a method recently reported by Harrison and Rosen and was a single band of protein in analytical polyacrylamide disc gel electrophoresis (Fig. 1), but revealed two closely associated bands of different molecular weights in polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) (Fig. 1), as reported earlier. The antibody preparations used gave no precipitin reactions in double agarose diffusion against partially purified preparations of plasma kallikrein, Hageman factor, high molecular weight kininogen, PTA, or plasminogen. Rabbit antibody to kallikrein purified according to the methods of Saitott and Nagase and Barrett was monospecific.

To produce antibody specifically directed against human HMW-K, a preparation of HMW-K purified according to the method of Kerbiriou and Griffin, which gave a single band in SDS gel electrophoresis, was digested with streptokinase-activated plasmin at 37°C for 25 min and the mixture depleted of plasmin by mixing with lysine-sepharose. These digests were reduced with 0.05 M dithiothreitol and alkylated in 0.12 M iodoacetamide at room temperature for 45 min. After dialysis against pH 5.8, 0.05 M sodium acetate buffer containing 0.05 M sodium chloride and 0.01 M benzamidine, the digests were applied to a 6 × 1.6-cm column of SP-Sephadex C-50 equilibrated with the same buffer. The column was washed with 2 volumes of equilibrating buffer and a linear gradient to 0.6 M sodium chloride in the same buffer was applied. A fraction eluted in 0.38 M sodium chloride containing HMW-K clotting activity and specific HMW-K antigens detected in a hemagglutination inhibition assay, but which failed to give a precipitin reaction in agarose diffusion against an antibody that reacts with antigenic determinants shared by HMW-K and LMW-K, was lyophilized and injected into rabbits in complete Freund's adjuvant. This antibody gave a single precipitin arc when reacted with normal plasma, whereas antibody to both HMW-K and LMW-K gave a double precipitin arc of immunologic identity in immunoelectrophoresis with normal human plasma. The anti-HMW-K gave a single precipitin band when reacted with normal plasma in agarose, did not react with plasma deficient in HMW-K but containing LMW-K (Fitzgerald trait), but gave a single precipitin band after purified HMW-K was added to the plasma; it gave no precipitin reaction with plasma deficient in both HMW-K and LMW-K. Therefore, this antibody reacts with antigenic determinants of HMW-K, but not with LMW-K or other plasma proteins. Antibody to Hageman factor raised in a goat using material purified to electrophoretic homogeneity according to a published method was also monospecific.
nol (Eastman Kodak, CO, Rochester, N.Y.) as a substrate. The method and units of activity were defined by Levy and Lepow. Streptokinase (Lederle Laboratories, Pearl River, N.Y.) was used to activate plasminogen in plasma and euglobulins separated from plasma in a concentration of 1000 U/ml of 0.15 M NaCl. Heparin-Ultragel was obtained from L'Institut Biologique Francaise, Clichy, Paris, France. It was washed 5 times in 10 vol of barbital-saline buffer, sedimented, and 2 vol of normal plasma were incubated with the heparin-Ultragel at 37°C for an hour with intermittent mixing by inversion, and at 4°C overnight. After removing the insoluble substances, the residual Hageman factor in the fluid phase was quantified. Plasma that had been exposed to 10 M ellagic acid for 0, 10, and 80 min was tested in this manner.

To quantify Hageman factor (factor XII), PTA (factor XI), high molecular weight kininogen (HMW-K), and prekallikrein (Fletcher factor) in plasmas, the ability of several dilutions of test samples to shorten the activated partial thromboplastin time was measured. Plasma from an individual with a severe inherited deficiency of the clotting factor in question was used. Percentage activity in test samples was calculated by comparing the activated partial thromboplastin times of plasma from normal persons added to the deficient plasma.

Ellagic acid (4,4', 5,5', 6,6-hexahydroxydiphenic acid 2,6, 2'6' dilactone) was synthesized by the method of Perkin and Nierenstein by oxidative coupling of gallic acid, as reported earlier. It was dissolved in barbital-saline buffer in a concentration of 10 M and used as a soluble activator of Hageman factor in final concentrations of 10 M in plasma. The ellagic acid solution was homogenized and centrifuged at 15,000 g for 10 min before use to remove any insoluble particles.

Plasminogen concentrations in plasma samples were measured in duplicate euglobulin fractions that were precipitated by adding 1 vol of plasma to 19 vol of 0.01 M sodium acetate, pH 5.2. After 15 min at room temperature, precipitates were separated by centrifugation and dissolved in a volume of barbital-saline buffer equal to the starting volume of test plasma. One volume of streptokinase (High Purity, Lederle Corp., Pearl River, N.Y.), 100 Christensen U/ml was mixed with 3 vol of euglobulins and incubated at room temperature for 10 min to allow activation. The mixture was then transferred to a 2°C bath and 0.3 ml of bovine fibrinogen, 3 mg/ml (General Diagnostics Branch of Warner Lambert, Morris Plains, N.J.), was mixed with 0.1 ml of the streptokinase activated euglobulins and clotted with 0.1 ml of bovine thrombin (Parke-Davis Co., Detroit, Mich.), 50 U/ml. After duplicates of each mixture were mixed thoroughly with thrombin in 10 x 75 mm Pyrex tubes, the mixtures were transferred to a 37°C bath, and the time required for complete dissolution of the fibrin in each tube was measured. Barbitral-saline buffer, pH 7.5, contained 2.76 g of barbital, 2.06 g of sodium barbital, and 7.3 g sodium chloride per liter.

**RESULTS**

When normal human plasma was adsorbed at 37°C for 1 hr and 4°C overnight with various volumes of anti-Cl-INH, the amounts of Cl-INH activity removed from plasma were proportional to the volume of antibody added (Table 1, column 3). In addition, the plasma was depleted of clot-promoting activity attributable to PTA, prekallikrein, and high molecular weight kininogen in proportion to the amounts of antibody globulin used (Table 1, columns 5–7). Hageman factor activity was not removed by anti-Cl-INH (Table 1, column 4), and the depletion of plasma PTA was irregularly observed in these experiments. Serum was subjected to these procedures, the same coagulants and Cl-INH were removed in parallel fashion by immune adsorption (not shown). Neither buffer nor nonimmune rabbit globulin depleted the plasmas of Cl-INH or of their coagulant activities. In addition, when normal plasma was treated with an equal volume of rabbit antibody globulins directed against fibrinogen, or goat antibody globulins directed against antihemophilic factor (factor VIII), Cl-INH, prekallikrein, PTA, and high molecular weight kininogen concentrations were not decreased. Therefore, the depletion in plasma of these coagulants was merely due to the effect of an immune complex.

The apparent immune depletion of kallikrein, HMW-K, and PTA required exposure to 4°C as well as Hageman factor. Neither prekallikrein nor HMW-

<table>
<thead>
<tr>
<th>Sample</th>
<th>Volume Anti-Cl-INH</th>
<th>Residual Activities (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>or Other</td>
<td>Cl-INH</td>
</tr>
<tr>
<td>Normal human plasma + anti-Cl-INH</td>
<td>0.5 ml</td>
<td>0 U/ml</td>
</tr>
<tr>
<td>Normal human plasma + anti-Cl-INH</td>
<td>0.25 ml</td>
<td>0.4 U/ml</td>
</tr>
<tr>
<td>Normal human plasma + anti-Cl-INH</td>
<td>0.1 ml</td>
<td>1.6 U/ml</td>
</tr>
<tr>
<td>Normal human plasma + buffer</td>
<td>0.5 ml</td>
<td>5.9 U/ml</td>
</tr>
<tr>
<td>Normal human plasma + buffer</td>
<td>0.25 ml</td>
<td>6.4 U/ml</td>
</tr>
<tr>
<td>Normal human plasma + buffer</td>
<td>0.1 ml</td>
<td>7.8 U/ml</td>
</tr>
<tr>
<td>Normal human plasma + nonimmune rabbit globulin</td>
<td>0.5 ml</td>
<td>11.6 U/ml</td>
</tr>
</tbody>
</table>

Normal human plasma samples were incubated with rabbit anti-human Cl-INH globulin or buffer, in the volumes noted (column 2). At 37°C for 60 min and 4°C overnight in 12 x 75 mm polystyrene tubes. After removal of immune precipitates by centrifugation at 2000 g for 30 min at 4°C, the residual Cl-INH activity was quantified in the esterolytic assay of Levy and Lepow and the Hageman factor (HF or factor XII), plasma thromboplastin antecedent (PTA or factor XI), Fletcher factor (prekallikrein), and high molecular weight kininogen (HMW-K) clotting activities were quantified in assays specific for each factor (see Materials and Methods). The residual activities are expressed as a percent of that present in the original plasma sample before dilution; no correction in titer was made for the dilution. Residual HF was not quantified in the control mixture containing nonimmune rabbit serum in this experiment, since antibody did not remove HF. Similar results were obtained in two other experiments using serum and plasma.
Table 2. Role of Temperature and Hageman Factor in Reduction of Prekallikrin and HMW-K by Anti-Cl-INH

<table>
<thead>
<tr>
<th>Factor Measured</th>
<th>Adsorbed Plasma</th>
<th>Incubation</th>
<th>Residual Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prekallikrin (Fletcher)</td>
<td>Normal 37° + 4°</td>
<td>37° + 4°</td>
<td>9</td>
</tr>
<tr>
<td>HMW-K</td>
<td>Normal 37° + 4°</td>
<td>37° + 4°</td>
<td>65</td>
</tr>
<tr>
<td>Prekallikrin</td>
<td>Normal 37°</td>
<td>&gt;100</td>
<td></td>
</tr>
<tr>
<td>HMW-K</td>
<td>Normal 37°</td>
<td>37° + 4°</td>
<td>82</td>
</tr>
<tr>
<td>Prekallikrin</td>
<td>HF-def</td>
<td>37° + 4°</td>
<td>100</td>
</tr>
<tr>
<td>HMW-K</td>
<td>HF-def</td>
<td>37° + 4°</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

A summary of representative experiments of the effect of chilling and of Hageman factor on immune depletion of plasmas with anti-Cl-INH. Equal volumes of plasma noted (second column) and anti-C1-INH, or plasma alone, were incubated at 37°C for 1 hr and 4°C overnight, or only at 37°C until immune precipitates formed (6 hr). Then, immune precipitates were removed and residual activities (%) were measured. Residual activities were corrected for dilution.

K concentration was reduced when Hageman trait plasma was adsorbed with anti-C1-INH, and normal plasma was not depleted if adsorption was carried out as 37°C (Table 2).

Immune depletion of normal plasma with anti-C1-INH globulins did not remove plasminogen from plasma (Table 3); the times required for complete lysis of a mixture of bovine fibrin and euglobulins separated from normal plasma before and after its adsorption with anti-Cl-INH were the same (Table 3, lines 1 and 2). Once plasminogen in plasma was activated with streptokinase, however, the residual plasminogen, detected by reexposure to streptokinase, was reduced by adsorption of the plasma with anti-Cl-INH globulins (Table 3B, lines 1 and 2), for the time required for clot lysis with added streptokinase was 7 min instead of 4. Moreover, most of the active plasmid that had formed in plasma exposed to streptokinase was removed by adsorption with anti-Cl-INH, for the lysis times of clots formed without adding streptokinase to assay mixtures was 330 min instead of 28 min (Table 3B, lines 3 and 4).

In another experiment, the possibility that Cl-INH was complexed with fibronectin (cold-insoluble globulin) was explored by adsorbing normal plasma with various volumes of gamma globulin fractions of rabbit and anti-human fibronectin serum or nonimmune rabbit serum. The concentration of Cl-INH activity was 6.2 U/ml in the normal plasma, which was adsorbed with an equal volume of antifibronectin globulin, and 5.8 U/ml after its adsorption with nonimmune rabbit globulins. The differences in these concentrations is insignificant, for the error in this method is 0.8 U/ml. Therefore, fibronectin and Cl-INH apparently do not form complexes in plasma.

Since activated Hageman factor preparations were inhibited by Cl-INH in earlier studies, it was possible that Hageman factor in plasma might form a complex with Cl-INH only when activated. To examine this possibility, plasma was shaken with 100-mesh acid-washed powdered Pyrex (20 mg/ml plasma) for 2 min and the Pyrex removed by centrifugation. The glass-activated plasma was then adsorbed with anti-C1-INH globulins. There was no reduction in the concentration of Hageman factor in glass-treated plasma, which was then treated with anti-Cl-INH, as compared to that incubated without anti-Cl-INH (Table 4, lines 1 and 2). Plasma that was incubated at 37°C with 10⁻⁵ M ellagic acid, a soluble activator of Hageman factor, for 10 min or 80 min and then adsorbed with anti-C1-INH globulins was not depleted of its Hageman factor activity (Table 4, lines 5 and 6). The recalcified silicone clotting time of the mixture of plasma and ellagic acid after 10 min of incubation was shortened from its control level of over 600 sec to 100 sec, but
Table 4. Hageman Factor and C1-INH in Plasma: Effect of Glass Activation

<table>
<thead>
<tr>
<th>Plasma Sample</th>
<th>Residual HF Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass-normal plasma + anti-C1-INH</td>
<td>91</td>
</tr>
<tr>
<td>Glass-normal plasma, incubated, undiluted</td>
<td>92</td>
</tr>
<tr>
<td>Normal plasma + 10^5 M ellagic acid + anti-C1-INH</td>
<td>100</td>
</tr>
<tr>
<td>Normal plasma + 10^5 M ellagic acid + buffer</td>
<td>100</td>
</tr>
<tr>
<td>Normal plasma + anti-C1-INH</td>
<td>91</td>
</tr>
<tr>
<td>Normal plasma, incubated, undiluted</td>
<td>100</td>
</tr>
</tbody>
</table>

1.0 ml of normal plasma was shaken at room temperature with powdered Pyrex in a polystyrene tube for 2 min. After the glass was sedimented, the plasma was mixed with an equal volume of anti-C1-INH globulins and incubated at 37°C for 60 min and 4°C overnight. After separation of immune precipitates, the Hageman factor clotting activity in the supernatant plasma was quantified in a specific assay (see Materials and Methods) (line 1). Control glass-treated plasma samples were incubated at 37°C and 4°C undiluted and then tested for their Hageman factor activity. Ellagic acid, 10^4 M, 0.05 ml, was incubated with 0.45 ml of silicone-citrate platelet-deficient normal plasma in silicone-coated 10 x 75 mm Pyrex tubes at 37°C for 180 min (lines 3 and 4). Then, 0.5 ml of anti-C1-INH globulin or buffer was added; these mixtures were incubated as above at 37°C and 4°C, and after the removal of immune precipitates by centrifugation, the residual Hageman factor in each mixture was quantified. The experimental mixture shown in lines 5 and 6 demonstrates the lack of effect of anti-C1-INH on unactivated normal plasma and of incubation of the untreated plasma samples alone; the untreated incubated plasma samples were used as the standard for comparison of the amounts of Hageman factor activity remaining in the test mixtures.

The percent activity remaining is corrected for dilution in each instance.

after 80 min of incubation, it was again over 600 sec, as if the activated Hageman factor had become inhibited.

It was possible that the activated Hageman factor in ellagic acid-treated plasma became inhibited by antithrombin III (AT-III). Therefore, ellagic acid treated and untreated normal plasma was exposed to washed heparin-Ultrogel, which would form a complex with AT-III. When the Hageman factor remaining in the untreated plasma, which was adsorbed with heparin-Ultrogel, was measured with a specific activated partial thromboplastin time, 88%–90% remained, but only 47% of that in plasma exposed to ellagic acid for 80 min before adsorption remained.

To determine if the depletion of normal plasma of its prekallikrein, HMW-K, and PTA activities by anti-C1-INH involved separate interactions of the inhibitor in plasma with each of these proteins or its involvement in multiple-component complexes, plasma from individuals with severe inherited deficiencies of each of these procoagulant proteins were adsorbed with an equal volume of anti-C1-INH globulin. After removal of the immune precipitates, the other two clotting factors removed from normal plasma by this maneuver were quantified. It is clear that prekallikrein was required for the immune depletion of plasma of HMW-K and PTA, for neither was removed from prekallikrein-deficient (Fletcher trait) plasma in such experiments (Table 5). The amounts of HMW-K and prekallikrein removed from PTA-deficient plasma, or of PTA and prekallikrein removed from kininogen-deficient plasma, varied over a wide range in different experiments (Table 5), but there was never any detectable loss of PTA or HMW-K from prekallikrein-deficient plasma.

Evidence of Immune-Depletion of Plasma of Kallikrein and HMW-K

The reduction of plasma concentrations of prekallikrein and HMW-K coagulant properties after adsorption with anti-C1-INH did not demonstrate the involvement of these coagulants with the C1-INH–anti-C1-INH complex. The residual plasma concentrations of prekallikrein, HMW-K, and C1-INH were

Table 5. C1-INH and Surface-Reactive Clotting Factors: Effect of Anti-C1-INH on Deficient Plasmas

<table>
<thead>
<tr>
<th>Deficient Plasma</th>
<th>Factor Measured</th>
<th>Percent Residual Activities (Average, 6 assays)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fletcher trait</td>
<td>PTA (XI)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Fletcher trait</td>
<td>HMW-K (Fitzgerald factor)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Kininogen-deficient</td>
<td>PTA</td>
<td>86.7</td>
<td>80–100</td>
</tr>
<tr>
<td>Kininogen-deficient</td>
<td>Prekallikrein (Fletcher factor)</td>
<td>52.3</td>
<td>26–96</td>
</tr>
<tr>
<td>PTA-deficient</td>
<td>HMW-K</td>
<td>57.3</td>
<td>16–100</td>
</tr>
<tr>
<td>PTA-deficient</td>
<td>Prekallikrein</td>
<td>36.3</td>
<td>13–63</td>
</tr>
</tbody>
</table>

Samples of 0.5 ml each of plasmas deficient in specific clotting factors were adsorbed with equal volumes of anti-C1-INH immune globulin at 37°C for 60 min and 4°C overnight. After removal of immune precipitates by centrifugation, the supernatant plasmas were tested for residual clotting factor activity, and residual activity was estimated by comparison of the clotting time of 2 dilutions of the samples to a double logarithmic plot of the clotting times of a series of dilutions of unadsorbed plasma controls. Control plasma mixtures containing buffer instead of anti-C1-INH were incubated as were test mixtures. The values shown represent the averages of clotting times of 2 dilutions tested in each of 6 experiments. The range of values of residual activities in all 6 experiments are shown in the last column; the residual values of 100% PTA and HMW-K are from a single experiment. Others demonstrated partial depletion of each factor.
therefore estimated with an immunodiffusion procedure. Antibody to PTA was not obtainable. When the concentration of prekallikrein was estimated in plasma after adsorption at 4°C, at least 75% of the antigenic properties had disappeared (Fig. 2, top pair), as occurred in the case of HMW-K (Fig. 2, center pair). Virtually all of the C1-INH antigens were removed by this procedure (Fig. 2, bottom pair). When plasma was incubated with anti-C1-INH at 37°C only, or if Hageman trait plasma was used, neither prekallikrein nor HMW-K antigen concentrations were reduced.

Attempts to identify coagulant or antigenic properties of kallikrein or HMW-K in the immune complexes formed failed. In fact, even after the immune complexes were dissolved and dissociated in 6 M guanidine HCl, 3 M sodium chloride, or at pH 3, neither the coagulant of antigenic properties of kallikrein, HMW-K, or even C1-INH could be identified. It was therefore not possible to account for the quantities of antigens lost from plasma in the immune complexes.

**Dysmorphic C1-INH in Hereditary Angioneurotic Edema (HANE) Plasmas and Hemostatic Proteins**

To determine if dysmorphic C1-INH proteins in plasmas from certain persons with HANE (CRM+*) could form complexes with surface-reactive procoagulants in plasma, CRM+ plasmas were adsorbed with an equal volume of anti-C1-INH globulins and after removing the immune precipitates, residual prekallikrein (Fletcher factor), high molecular weight kininogen, PTA, and Hageman factor were quantified. The CRM+ plasmas were from persons with 3 different types of nonfunctional C1-INH: 2 contained high concentrations of C1-INH antigens that demonstrated 2 separate bands of antigens following immunoelectrophoresis;8 2 contained nonfunctional C1-INH in the same concentration and electrophoretic mobility as the C1-INH in normal plasma, and 2 contained protein of more anodal electro-mobility than normal, but in normal concentration.8 Adsorption removed all of the C1-INH antigens from each CRM+ plasma shown by agarose-gel diffusion (Fig. 3). Even so, neither the prekallikrein, HMW-K, PTA, Hageman factor, nor plasminogen concentration was decreased.

When each of the CRM+ plasmas (numbered 1, 2 and 3, Table 6) was activated with streptokinase and then adsorbed with anti-C1-INH globulins, the residual streptokinase-activatable plasminogen was decreased, for lysis times were longer (lines 6 of each set) than those of samples that had been incubated with anti-C1-INH alone (line 2 of each set). Some of the plasmin generated by streptokinase was removed from plasma No. 3 by the anti-C1-INH, for the lysis time without added streptokinase of mixtures that had already been incubated with streptokinase and then anti-C1-INH globu-

---

*CRM+: nonfunctional C1-INH that contains normal concentrations of C1-INH antigens found in plasmas from certain persons with hereditary angioneurotic edema.*
plasminogen and plasmin. or of buffer to measure residual already active plasmmn.

appears to involve cold activation of plas-
experiments
depletion of plasma kallikrein and HMW-K in these
that of the original plasma sample, and each mixture tested for its fibrmnolytmc actmvmty against bovine fibrin
globulins or buffer, and euglobulins were prepared from the supernatant fluids. The euglobulins were dissolved in barbmtal-salmne buffer in a volume equal
to that of the original plasma sample, and each mixture tested for its fibrinolytic activity against bovine fibrin in the presence of SK to measure
plasminogen and plasmin, or of buffer to measure residual already active plasin.

Each of 3 different types of CRM-positive HANE plasmas was treated with streptokinase (SK) or buffer and then was adsorbed with anti-C1-INH
globulins or buffer, and euglobulins were prepared from the supernatant fluids. The euglobulins were dissolved in barbmtal-salmne buffer in a volume equal
to that of the original plasma sample, and each mixture tested for its fibrinolytic activity against bovine fibrin in the presence of SK to measure
plasminogen and plasmin, or of buffer to measure residual already active plasin.

CRM-positive (CRM⁺) plasma no. 1: normal electrophoretic mobility C1-INH protein with normal antigen concentration.

CRM⁺ plasma no. 2: anodal electrophoretic mobility C1-INH protein with normal antigen concentration.

CRM⁺ plasma no. 3: 2 anodal electrophoretic components C1-INH protein with 400% antigen concentration compared to normal plasma.

Of the procoagulants in normal plasma which, when purified and activated, are inhibited by C1-INH prepara-
tions, only kallikrein, PTA, and high molecular weight kiningogen appear able to form complexes with C1-INH in normal plasma or serum; monospecific antibody globulins to human C1-INH removed signif-
quant amounts of each of these clotting activities concur-
with immune depletion of C1-INH at 4°C. The anti-C1-INH did not, however, remove any of these procoagulants from CRM⁺ plasmas of persons with
HANE, as if these nonfunctional proteins failed to
form complexes with prekallikrein, high molecular weight kiningogen, or PTA. The mechanism of immune
depletion of plasma kallikrein and HMW-K in these
experiments appears to involve cold activation of plas-
ma, which requires Hageman factor and results in the
activation of prekallikrein, as originally reported by Gjønnaess.39 Neither factor was reduced in Hageman
trait plasma adsorbed at 4°C, nor in normal plasma
adsorbed at 37°C. Therefore, activation of the prekalli-
krein in a complex with HMW-K probably leads to the
interaction of this comlex with C1-INH and to its
removal from plasma with anti-C1-INH.

Hageman factor activity in normal plasma and serum was not removed with anti-C1-INH, even after
the plasma had been exposed to powdered glass or
eellagic acid to activate the Hageman factor. Neither
factor was reduced in Hageman trait plasma adsorbed at 4°C, nor in normal plasma
adsorbed at 37°C. Therefore, activation of the prekalli-
krein in a complex with HMW-K probably leads to the
interaction of this comlex with C1-INH and to its
removal from plasma with anti-C1-INH.

Of the procoagulants in normal plasma which, when
purified and activated, are inhibited by C1-INH prepara-
tions, only kallikrein, PTA, and high molecular

DISCUSSION

Of the procoagulants in normal plasma which, when
purified and activated, are inhibited by C1-INH prepara-
tions, only kallikrein, PTA, and high molecular
weight kiningogen appear able to form complexes with
C1-INH in normal plasma or serum; monospecific
antibody globulins to human C1-INH removed signif-
quant amounts of each of these clotting activities concur-
with immune depletion of C1-INH at 4°C. The anti-C1-INH did not, however, remove any of these procoagulants from CRM⁺ plasmas of persons with
HANE, as if these nonfunctional proteins failed to
form complexes with prekallikrein, high molecular
weight kiningogen, or PTA. The mechanism of immune
depletion of plasma kallikrein and HMW-K in these
experiments appears to involve cold activation of plas-
ma, which requires Hageman factor and results in the
activation of prekallikrein, as originally reported by Gjønnaess.39 Neither factor was reduced in Hageman
trait plasma adsorbed at 4°C, nor in normal plasma
adsorbed at 37°C. Therefore, activation of the prekalli-
krein in a complex with HMW-K probably leads to the
interaction of this comlex with C1-INH and to its
removal from plasma with anti-C1-INH.

Hageman factor activity in normal plasma and serum was not removed with anti-C1-INH, even after
the plasma had been exposed to powdered glass or
eellagic acid to activate the Hageman factor. Neither
factor was reduced in Hageman trait plasma adsorbed at 4°C, nor in normal plasma
adsorbed at 37°C. Therefore, activation of the prekalli-
krein in a complex with HMW-K probably leads to the
interaction of this comlex with C1-INH and to its
removal from plasma with anti-C1-INH.

These results might appear to disagree with the
report of Forbes and his associates,1 but they demon-
strated inhibition of activated Hageman factor that had been isolated from normal plasma and incubated with a preparation of CI-INH before being mixed with a substrate plasma in a clotting assay. The difference found in the present experiments may indicate that when Hageman factor is activated in unfractionated plasma, other substances prevent its interaction with CI-INH. After 80 min of incubation with ellagic acid, the recalcified clotting time of plasma was markedly prolonged as compared to the clotting time after only 10 min exposure before recalcification. Conceivably, the Hageman factor activates by ellagic acid was then inhibited by another plasma inhibitor during prolonged incubation. One possible candidate for this inhibition is antithrombin III. The fact that insolubilized heparin depleted normal plasma of some of its Hageman factor after treatment of the plasma with ellagic acid for 80 min, but not after 10 min, supports the possibility that the Hageman factor, which has been activated then interacted with AT-III, in turn bound to the insolubilized heparin. It was possible that some of the Hageman factor bound directly to the insoluble heparin, but the control samples (0 min and 10 min) were not depleted of Hageman factor by the heparin-Ultrogel.

The concurrent removal of HMW-K, kallikrein, and PTA when normal plasma was adsorbed with anti-CI-INH doubtless reflects the existence of fluid-phase complexes of these coagulants following cold-activation of plasma. The relationship between these coagulants and CI-INH was demonstrated when plasma deficient in one of them was adsorbed with anti-CI-INH and the residual concentrations of the other two were then measured (Table 5). Prekallikrein, or kallikrein, was clearly required for depletion of plasma of its PTA or HMW-K by immune adsorption with anti-CI-INH. This suggests that a large complex may form in normal plasma consisting of kallikrein, HMW-K, PTA, and CI-INH, but PTA and prekallikrein were complexed with separate HMW-K molecules in an earlier study. Complexes between kallikrein, HMW-K, and CI-INH, as well as between activated PTA, HMW-K, and CI-INH, probably form. These complexes may be important in physiologic reactions to injury within the vascular system. Although reductions in concentrations of kallikrein and HMW-K antigens in normal plasma roughly paralleled the loss of their coagulant properties from the plasma, neither functional nor antigenic properties could be identified in chemically dissociated and dialyzed immune complexes removed from plasma. In fact, CI-INH antigens could not be identified. The undissociated complexes actually had an anticoagulant effect on the specific assays used to measure kallikrein, HMW-K, PTA, and Hageman factor. Possibly, there is an excess of CI-INH removed that impairs clotting in these assays. Even so, one must postulate that involvement of kallikrein and HMW-K with CI-INH in the immune complexes obscures the antigenic determinants of all three substances, for after dissociation of the complexes, during which insoluble materials dissolved, gamma globulin antigens of the species from which the antibody was derived could be identified in gel diffusion, but no CI-INH, prekallikrein, or HMW-K antigens could be found.

Since there was no difference in the concentration of streptokinase-activatable plasminogen after immune adsorption or normal plasma with the anti-CI-INH globulins (Table 2), but plasmin was removed from streptokinase-activated plasma (Table 3) by anti-CI-INH, activation of plasminogen in plasma by streptokinase appears to occur before an interaction with CI-INH can take place, as reported earlier by Ratnoff and his associates and Schreiber et al. The dysmorphic CI-INH in several CRM HANE plasmas did seem to form complexes with streptokinase-activated plasmin generated from endogenous plasminogen, in contrast to their failure to form complexes with the four clotting factors that were examined. This does not mean that the dysmorphic proteins actually inhibited the plasmin that was generated, for each may have formed a complex with the enzyme without inhibiting it. The inhibitory effect of purified dysmorphic CI-INH proteins is being evaluated to assess their functional capacity.

ACKNOWLEDGMENT

The willing and expert technical assistance of Connie Wagner and Augustine Merriweather is appreciated and the manuscript could not have been completed without the expert secretarial assistance of Anne Marr. Nils U. Bang, M.D., of the Lilly Research Laboratories generously provided the rabbit antibody to human fibronectin, and Dr. Oscar Ratnoff, of University Hospitals, Cleveland, Ohio, provided the synthetic ellagic acid. Dr. Jozef Kleniewski prepared the synthetic ellagic acid. Dr. Jozef Kleniewski prepared the synthetic ellagic acid. Dr. Oscar Ratnoff, of University Hospitals, Cleveland, Ohio, provided the synthetic ellagic acid. Dr. Jozef Kleniewski prepared the synthetic ellagic acid. Dr. Jozef Kleniewski prepared the synthetic ellagic acid. Dr. Jozef Kleniewski prepared the synthetic ellagic acid. Dr. Jozef Kleniewski prepared the synthetic ellagic acid. Dr. Jozef Kleniewski prepared the synthetic ellagic acid. Dr. Jozef Kleniewski prepared the synthetic ellagic acid.


22. Kleniewski J: Unpublished observations


Complexes between C1-inhibitor, kallikrein, high molecular weight kininogen, plasma thromboplastin antecedent, and plasmin in normal human plasma and hereditary angioneurotic edema plasmas containing dysmorphic C1-inhibitors: role of cold activation

VH Donaldson and RA Harrison