C1 Inactivator Inhibition by Plasmin

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ABSTRACT Plasmin incubated with partially purified C1 inactivator produced a decrease in inhibitory activity which was related to the time of incubation and to the concentration of plasmin. This effect of plasmin was not influenced by the purity of the inhibitor preparations. Soybean trypsin inhibitor and tosyl arginine methyl ester (TAME), substances which block the active enzymic center of plasmin, prevented the plasmin-induced inactivation. Double diffusion analysis of the functionally deficient, plasmin-treated C1 inactivator using a specific antibody, showed a reaction of identity with the untreated inhibitor. Agarose and acrylamide gel immunoelectrophoresis of a plasmin, inhibitor mixture showed the appearance of an additional precipitin band with immunologic reactivity similar to that of the untreated inhibitor. These results demonstrate that plasmin alters both the functional and immunoelectrophoretic properties of C1 inactivator, and that the active proteolytic site of plasmin is necessary for this interaction. Since C1 inactivator has been shown to inhibit several different proteolytic enzymes including C1, kallikrein, PF/Dil, and plasmin, this investigation provides a theoretical relationship between the fibrinolytic, kallikrein, and complement systems which may have pathophysiologic relevance to various human disease states.

INTRODUCTION

Human serum contains an activity which inhibits C1 (the activated first component of complement [1]), plasma kallikrein, and PF/Dil (a plasma enzyme which enhances cutaneous vascular permeability) (2–4). This inhibitory activity is deficient in the sera of patients with hereditary angioneurotic edema (2, 5) and has been identified as a specific heat and acid labile α1-neuraminoglycoprotein designated C1 inactivator (3, 6, 7). Ratnoff, Pensky, Ogston, and Naff (8) have reported that highly purified preparations of C1 inactivator inhibited the C1r subunit of the first component of complement, retarded the generation of PF/Dil, and inhibited the proteolytic activity of plasmin, the human fibrinolytic enzyme.

Lepow, Ratnoff, and Levy (9) found that streptokinase-treated serum is almost entirely lacking in C1 inactivator. Laurell, Lundh, and Malmquist (10) have postulated that the fall in C1 inactivator in streptokinase-treated serum is due to the activation of plasmin and have demonstrated that the addition of plasmin to human serum results in a reduction of functionally active C1 inactivator. In view of the potential importance of C1 inactivator as an inhibitor of several different plasma enzyme systems, the present studies were undertaken to examine the in vitro effect of plasmin on the functional activity and immunologic reactivity of purified C1 inactivator.

METHODS

Substrates and inhibitor. N-acetyl-L-tyrosine ethyl ester (ATEe), N-α-acetyl-L-lysine methyl ester (ALMe), N-α-acetylglucyl-L-lysine methyl ester (AGLMe), and tosyl arginine methyl ester (TAME) were obtained from Dr. H. Plaut. These esters were chromatographically homogeneous and had an elemental analysis consistent with their formulas. Soybean trypsin inhibitor (SBTI) was crystallized three times and lyophilized.

Plasmin. Spontaneously activated human plasmin in 50% glycerol, containing 32.3 Remmert and Cohen caseinolytic units per ml was obtained from The American Red Cross.

C1s. Partially purified C1s was prepared by a method modified from that of Haines and Lepow (12). 1 vol human serum was added to 8 vol acetate buffer, pH 5.5 ionic strength 0.02 at 0°C. After 16 hr the euglobulin was separated by centrifugation and resuspended in one-tenth the original serum.

2 Cyclo Chemical Div., Travenol Laboratories, Inc., Los Angeles, Calif.

3 Worthington Biochemical Corp., Freehold, N. J.

4 Kindly provided by Dr. Alan Johnson.
volume of 0.05 M Tris (hydroxymethylaminomethane)-chloride, pH 8.0 containing 0.02 M sodium chloride (TSB). The mixture was incubated at 37°C for 30 min to activate the C1 proesterase, then dialyzed against triosodium ethylenediaminetetraacetic acid (Na3HEDTA) in TSB at 4°C for 16 hr.

After centrifugation at 17,000 rpm for 15 min, 20 ml euglobulin solution was applied to a 2.5×30 cm column of DEAE-cellulose (Whatman DE-52 preswollen microgranular exchanger) at 5°C prepared according to the manufacturer's instruction and equilibrated with TSB containing 0.001 M Na3HEDTA. A linearly increasing gradient of salt concentration at constant pH was produced by allowing 1 liter of limit buffer (0.4 M NaCl in 0.05 M Tris-chloride, containing 0.001 M Na3HEDTA) to flow into 1 liter of 0.05 M Tris-chloride containing 0.02 M NaCl and 0.001 M Na3HEDTA with constant magnetic stirring and into the column by gravity flow. 10-ml aliquots were collected at a rate of 60 ml/hr. Conductivity of the samples was measured using an LKB Conductolyzer flow cell 5313 A and a Beckman model RC 16 B2 conductivity bridge. Conductivity was converted to NaCl activator but not by soybean trypsin inhibitor (15).

Measurement of esterase activity. C1s activity was measured as previously described (10) with the use of a pH stat apparatus (TTT IC Tittrator, SBR 2C Tittrigraph, and an SBU 1A microtitration assembly, Radiometer, Copenhagen, Denmark). C1s was measured in a final volume of 2.5 ml at 37°C, in 0.005 M Tris-chloride containing 0.145 M NaCl, 0.02 M NaOH was the titrand. The substrate, ATEe (1) was dissolved in 2-methoxyethanol (methyl cellosolve) at a concentration of 1.0 mole/liter. 0.13 ml of substrate solution was added to the reaction mixture giving a final concentration of 0.05 mole/liter. One unit of C1s activity was defined as that amount which hydrolyzed 1 µmole ATEe/ml of the reaction mixture per hour under the conditions described. The pH stat method for determining C1 activity was shown to yield about twice the number of enzyme units with the same amount of enzyme (6, 10) as that obtained by the Levy and Lepow microformol titration procedure (16). Since Levy and Lepow defined one unit of activity as being that amount which liberates 0.5 µmole of acid in 15 min, the unit as defined in this report contained approximately 25% the activity of their unit. The preparations of C1s used contained 100 U/ml. The substituted methyl esters of lysine and arginine were dissoluted in buffer and added to the titration vessel reaction mixture at a final concentration of 0.015 mole/liter.

C1 inhibitor preparation. C1 inhibitor was prepared according to the method of Pensky, Levy, and Lepow (6) and was carried through the first Dowex chromatography cycle. The precipitate from fresh serum to which 40% solid ammonium sulfate had been added at 0°C was harvested by centrifugation. The supernatant was dialyzed 48 hr. against large volumes of distilled water and recentlyrifuged. The cleared supernatant was stored at −20°C until use as the starting material for chromatography. Dowex AG 2-X10 anion exchange resin, Bio-Rad Laboratories, Richmond, Calif. was prepared as described (6) and packed by gravity in a 1 liter delivery burette. 50 ml of the dialyzed ammonium sulfate freeze supernatant were applied to the column equilibrated with 0.06 M Tris-chloride buffer, pH 7.3. 20-ml fractions were collected at a flow rate of 2 ml per min. After the protein fraction appeared (not adsorbed to the resin), the C1 inhibitor-containing protein peak was eluted by changing the eluting buffer to 0.09 M NaCl dissolved in starting buffer. Fractions containing C1 inhibitor activity were pooled, dialyzed against distilled water, and lyophilized. The inhibitory activity of this preparation was determined to be about 200 times the specific activity of that in the starting serum and contained 10 inhibitor units per ml. C1 inhibitor of approximately 3 times the specific activity of the above preparation was kindly provided by Dr. J. Pensky.

C1 inhibitor measurement. C1 inhibitor activity was measured in the pH stat (10) using a standardized C1s preparation and conditions identical with those described for the assay of the C1s activity. The inhibitory activity was determined by subtracting the micromolar substrate hydrolyzed per milliliter reaction mixture containing C1 inhibitor per hour from that hydrolyzed in the absence of inhibitor. One unit of C1 inhibitor was defined as that amount which inhibited 10 U of the esterase (16). Pooled serum, obtained from 10 normal donors when assayed by this method contained 14.5 inhibitor units per ml.

Plasmin and C1 inhibitor incubation experiments. Varying concentrations of plasmin and C1 inhibitor, or inhibitor alone, were incubated at 37°C in 0.005 M Tris-chloride, pH 7.4 containing 0.145 M NaCl. Since the inhibitor is progressively inactivated at acid pH (6) the pH of the incubation mixture was monitored, and no change was found to occur during the interval of incubation. SBTI, a plasmin inhibitor (17, 18), was added to the incubation mixture at a final concentration of 160 µg per ml and the specimen placed in an ice bath until assayed for residual C1 inhibitor. Control experiments with standard caseinolytic assay (19) established that the proteolytic activity of plasmin was completely inhibited after the addition of this concentration of SBTI. At the time of assay a standardized amount of C1s was added to the mixture in the reaction vessel of the pH stat and the reaction started by the addition of ATEe. The inhibitor concentration remaining was determined as has been described. The per cent C1 inhibitor remaining was expressed as the ratio of units of inhibitor found after incubation to the inhibitor activity at time 0 of the experiment. The inhibitory activity at time 0 was the same for incubation mixtures containing a similar concentration of: inhibitor and buffer; inhibitor and plasmin; or inhibitor, plasmin, and SBTI.

Plasmin, C1 inhibitor, and TAME incubation experiments. In experiments concerning the effect of TAME on the plasmin, C1 inhibitor incubation mixture, the pH of the incubation mixture was maintained at 7.4 in the pH stat by the addition of 0.2 M NaOH. The concentration of TAME varied from 0.02 to 0.14 M. The assay for residual C1 inhibitor activity was modified as additional TAME; a substrate for C1 (20, 21) was used in the place of ATEe in a final concentration of 0.05 M. This was necessary as control experiments showed that TAME in the plasmin, inhibitor incubation mixture interfered with the hydrolysis of ATEe after the addition of C1s, thereby yielding falsely elevated values for C1 inhibitor activity.

Immunodiffusion and immunoelectrophoresis. Microimmunodiffusion (22) was performed according to established
methods. Microimmunoelectrophoresis (23) was performed in an LKB-electrophoresis apparatus,7 with 0.8% agarose8 and 0.03 M sodium barbital buffer, pH 8.2. Specific goat antisera to human C1 inactivator was generously provided by Dr. Fred Rosen.

Acrylamide-gel immunoelectrophoresis. Acrylamide-gel slabs were prepared in a vertical electrophoresis apparatus9 according to a method modified from Davis (24). The separation gel consisted of 7.5% cyanogum made in a 1:8 dilution of a stock buffer solution, pH 8.9, containing 48 ml of 1 N HCl, 36.6 g of Tris, and 1.0 ml of TEMED per 100 ml. 132 mg of catalyst, potassium persulfate, was added to the 160 ml of cyanogum solution necessary to fill the apparatus. The chamber buffer contained 0.6 g of Tris and 2.88 g of glycine per liter, pH approximately 8.1. Electrophoresis was performed at 400 volts until the bromphenol blue marker dye had migrated 10 cm. The temperature of the gel varied between 7 and 12°C.

After electrophoresis, the gel slab was cut into vertical strips and either stained for protein with amido black or used for subsequent immunodiffusion. The unstained gel strips were placed in parallel 7 mm apart on an 8 × 10 cm glass plate. The strips were overlaid and the space between them filled with 1.2% agar.10 After diffusion for 16 hr in a moist chamber, filter paper wicks were inserted into the agar, parallel to the gel strips and equidistant from them. The wicks were saturated with C1 inactivator antiserum and immunodiffusion proceeded until precipitin lines appeared. This usually required 48–60 hr.

Thin-layer Sephadex chromatography and immunodiffusion. Descending thin-layer chromatography on Sephadex G-200 superfine gel11 followed by filter paper transfer to agar slides and subsequent immunodiffusion using C1 inactivator antiserum was performed by a technique described by Williamson and Allison (25).

Sephadex G-200 column chromatography. Sephadex G-200,11 particle size 40-120 μ, was poured into a 0.9 × 120 cm glass column. 0.3 ml of C1 inactivator, or inhibitor-plasmin mixture was applied to the column, and 1.0 ml aliquots were collected at a flow of 3.0 ml per hr. The OD400 of the samples was recorded. C1 inactivator was identified by double immunodiffusion.

RESULTS

Effect of plasmin on C1 inactivator activity. In plasmin, C1 inactivator incubation mixtures (Fig. 1) there was a progressive time-dependent fall in C1 inhibitory activity. An inhibitor preparation with three times the specific activity (units per optical density at 280 μm) showed a similar reduction in activity. SBTI added to the mixtures before incubation, prevented this fall as compared to control curves with inhibitor and buffer which showed a similar slight fall in inhibitor activity during the 30 min incubation period. Confirming previous findings, SBTI failed to affect the hydrolysis of ATEE by C1 (1) and plasmin was found to have no ATEE hydrolyzing activity (6, 26).

During a 10 min incubation period, increasing concentrations of plasmin added to a constant concen-

![Figure 1](https://example.com/f1.png)

**Figure 1** Effect of plasmin on C1 inactivator. (The ordinate, %C1 esterase inhibitor, refers to C1 inactivator.) 0.2 C1 inactivator units, prepared as described in the methods (I), or a more highly purified preparation obtained from Dr. J. Pensky (PI), was incubated with 0.8 caseinolytic units plasmin per ml reaction mixture in 0.005 M Tris-chloride pH 7.4, containing 0.145 M NaCl at 37°C. The action of plasmin was stopped by the addition of 160 μg SBTI per ml and residual inhibitor activity was measured by the addition of 3.0 units of C1s and its substrate, ATEE. Inhibition is expressed as a per cent inhibitory activity remaining compared to that in the incubation mixture at time 0. In the control experiment, SBTI was added to the plasmin-inhibitor incubation mixture at time 0.

Inactivation of C1 inactivator caused a concentration-dependent fall in inhibitory activity (Table I).

Effect of TAMe on the C1 inactivator, plasmin incubation. The inclusion of 0.14 M TAMe, a plasmin substrate and competitive inhibitor, effectively prevented the plasmin-induced reduction of C1 inactivator activity (Fig. 2).

The inhibitor was progressively protected from the action of plasmin by increasing concentrations of TAMe added to the incubation mixture (Fig. 3). 0.14 M TAMe prevented the loss of inhibitory activity, whereas a 7-fold reduction in TAMe concentration had only a slight protective effect.

**Table 1**

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<th>Plasmin</th>
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10 Pure Agar Behringwerke Ag., Certified Blood Donor Service, Woodbury, N. Y.
11 Pharmacia Fine Chemicals Inc., Piscataway, N. J.

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Immunodiffusion and immunoelectrophoretic analysis of plasmin, Cl inactivator preparations. Double diffusion experiments (Fig. 4) showed a reaction of identity between Cl inactivator and the plasmin-treated inactivator. Immunoelectrophoresis (Fig. 5) of an inactivator preparation whose activity had been destroyed by plasmin showed two precipitin bands displaying a reaction of partial identity as compared to the one band produced by a Cl inactivator, SBTI, plasmin incubation mixture. One of the bands of the inhibitor, plasmin mixture was sharper than the inhibitor precipitin arc in the preparation protected from plasmin by SBTI and migrated slightly more toward the cathode. A second precipitin band with more anodal electrophoretic mobility than the parent molecule was identified. Plasmin failed to react with the antiserum used.

The heterogeneity of proteins in the first Dowex circle Cl inactivator preparation was apparent after acrylamide gel electrophoresis (Fig. 6). One protein band, with a slow mobility, produced a precipitin arc following immunodiffusion (Fig. 6, bottom) against the Cl inactivator antiserum. Interaction between plasmin and the inhibitor resulted in an altered protein pattern (Fig. 6, top). No band which corresponded in mobility to the parent Cl inactivator could be identified. Two new protein bands were present which produced precipitin arcs. One arc had a slower and the other a faster mobility than the untreated inhibitor. This pattern which suggested immunologic identity was consistent in 10 different electrophoretic runs using various batches of inhibitor. Other alterations occurred in the mobility of several proteins associated with the inhibitor preparation following plasmin treatment. Plasmin, in the concentration used, could not be demonstrated on the acrylamide gel by protein stain. The nature of these changes has not been investigated.

**Figure 2** Effect of TAME on the Cl inactivator, plasmin incubation mixture. (The ordinate, %Cl' esterase inhibitor, refers to Cl inactivator.) Cl inactivator and plasmin, in the concentrations described in Fig. 1, were incubated with buffer, with SBTI, or with 0.14 M TAME. The reaction was stopped at varying time intervals by the addition of SBTI and the remaining inhibitory activity was assayed using 0.05 M TAME as substrate for Cls. Inhibition is expressed as per cent of inhibitory activity remaining as compared to time 0.

**Figure 4** Double diffusion reaction between Cl inactivator and a Cl inactivator, plasmin mixture. 0.2 U of Cl inactivator was incubated with 0.8 caseinolytic units of plasmin, or with plasmin and 200 μg SBTI, 30 min at 37°C in a final volume of 0.25 ml. The incubation mixtures were analyzed by double diffusion in 0.8% agarose gel. Goat antibody to human Cl inactivator (top well) produced a reaction of identity between the inhibitor (well on left) and the inhibitor whose functional activity was destroyed by plasmin (well on right).

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Elution of the proteins in the acrylamide gel containing the partially purified inhibitor revealed complete localization of Cl inactivator activity to the band which reacted with the antibody.

Sephadex thin-layer chromatography and immunodiffusion. The demonstration of an immunoelectrophoretic split product resulting from the Cl inactivator-plasmin interaction, suggested that the molecular weight of the parent molecule might be altered. Separation by Sephadex G-200, thin-layer, descending chromatography followed by immunodiffusion (Fig. 7) failed to show an alteration in the mobility of the plasmin-treated inhibitor, nor could a lower molecular weight product be identified. Experiments using Sephadex G-200 column chromatography gave similar results.

DISCUSSION

Plasmin is a proteolytic enzyme which hydrolyzes a variety of naturally occurring proteins at neutral pH (27). Plasminogen, the precursor of this enzyme, is found in normal human plasma. While its function in fibrinolytic and thrombolytic phenomena has been the subject of numerous investigations (28, 29), interactions between the fibrinolytic enzyme and other functionally distinct plasma protein systems are only beginning to be explored (30-32). Plasmin has been shown to have a number of effects on the human complement system in vitro. Plasmin inactivates human complement by a process involving two steps (33-35). The enzyme activates Cl from its precursor, the first component of complement (9, 34). Cl then inactivates the hemolytic properties of C2 and C4, the second and fourth components of complement (36). Plasmin has also been demonstrated to convert the C1s subcomponent of Cl into the active enzyme, C1s (15).

Other components of the complement system are altered by plasmin. C3 can be cleaved by plasmin with the generation of a chemotactically active fragment with antigenic reactivity similar to the parent molecule (37). In addition, plasmin destroys the chemotactic factor that is a trimolecular complex consisting of the 5th (C5), 6th (C6), and 7th (C7) components of complement (37).
Lepow, Ratnoff, and Levy (9) found that whereas complement-fixed serum retained most of its Cl inactivator activity, streptokinase-treated serum had negligible inhibitory activity. Laurell, Lundh, and Malmquist (10) reinvestigated the effect of streptokinase on serum complement factors and confirmed the previous findings concerning the reduction in Cl inactivator. They found that the addition of plasmin to serum also resulted in a fall in the functional inhibitory capacity of the treated serum. They postulated that the fall in inhibitor levels in streptokinase-treated serum was due to the activation of plasmin.

Our investigations have supported this thesis by demonstrating that in a plasmin-Cl inactivator mixture, there was a time-dependent reduction in Cl inactivator activity. The possibility that the effect of plasmin on Cl inactivator might be indirect and mediated through another enzyme system associated with the partially purified inhibitor preparations was investigated. The finding that two inhibitor preparations with different specific inhibitory activities had similar reductions in activity following plasmin treatment indicated that plasmin directly interacted with the inhibitor. The active enzymic center of plasmin appeared to be necessary for the destruction of Cl inactivator since two substances which block the active center of plasmin, tosyl arginine methyl ester and soybean trypsin inhibitor (38, 39), prevented inhibitor inactivation.

Alterations were found in the electrophoretic and immuno-electrophoretic patterns of Cl inactivator following plasmin treatment. These changes, consisting of the production of two precipitin arcs with electrophoretic mobilities differing from the parent protein, were prevented by soybean trypsin inhibitor. Since inhibition of the proteolytic center of plasmin blocked both the plasmin-induced reduction of Cl inactivator activity and alteration in the immuno-electrophoretic pattern, it seems reasonable to suggest that the changes observed in the inhibitor are due to proteolytic degradation. Whether the enzymic action of plasmin resulted in an internal restructuring of the inactivator molecule with the production of two species of differing charges with unchanged molecular weights, or in the release of a lower molecular weight degradation product is speculative. It is also possible that the inhibitor may bind to the plasmin molecule in a manner analogous to the action of the α1-macroglobulin inhibitor of plasmin (40).

No change was found in the Sephadex G-200 gel filtration pattern of the Cl inactivator after its functional inactivation by plasmin. The inhibitor was eluted at a position corresponding to the 7S peak of serum. The Cl inactivator has been found to have an apparent sedimentation constant of 4.5S by ultracentrifugation (6). This finding raises the possibility that dimer formation occurred under the conditions of the experiment. An alternative explanation is provided by the anomalous gel filtration behavior of several glycoproteins when compared to carbohydrate-free globular proteins (41–43). These glycoproteins appear to have an expanded structure and therefore have a higher apparent molecular weight as determined by gel filtration than found by other physical methods (41). The Cl inactivator has been identified as a neuraminoglycoprotein (7) containing 42.6% total carbohydrates (44). Changes in the molecular weight of the inhibitor caused by interaction with plasmin might, therefore, have been obscured on gel filtration by its high carbohydrate content.

Rosen, Charache, Pensky, and Donaldson (45) have reported that nine patients from two kindred affected with hereditary angioneurotic edema have normal levels of Cl inactivator determined by immunochemical techniques, but have inhibitor which is nonfunctional. It is unlikely that the defect found in this group of patients was caused by the in vivo action of plasmin, as Rosen et al. identified only one band corresponding to the functionally deficient inhibitor by immuno-electrophoresis. As has been shown in the present study, two bands result from the interaction of Cl inactivator and plasmin.

Ratnoff et al. (8) have reported that Cl inactivator inhibited the hydrolysis of fibrin and casein by plasmin. The present studies have not dealt with this aspect of the plasmin-Cl inactivator interaction. The concentrations of esterase inhibitor in our investigations were lower than those used by Ratnoff et al. in demonstrating a plasmin-inhibiting effect. If, as suggested by our data, Cl inactivator is a plasmin substrate, then the Cl inactivator in appropriate concentrations might function as a plasmin inhibitor through competitive substrate inhibition.

The physiologic significance of the destruction by plasmin of the functional capacity of Cl inactivator, an inhibitor of several plasma proteases has potential pathophysiologic relevance in man. While patients with hereditary angioneurotic edema have a deficiency of Cl inactivator (5) the relationship of this defect to the intermittent attacks of circumscribed edema which occurs in these pateints is not understood. Landerman, Webster, Becker, and Ratcliffe (2) first reported a deficiency of inhibitor to a plasma kallikrein and PF/Dil in hereditary angioneurotic edema serum. Kagen and Becker (3), as well as Ratnoff et al. (8) have demonstrated that Cl inactivator inhibits the permeability effects of kallikrein and PF/Dil. Kinins or kinin-like material has been found in the plasma of patients with Cl inactivator deficiency (2, 46, 47). Cl injected intradermally in man caused an increase in

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vascular permeability (48). Donaldson (49) has shown that activated Hageman factor in vitro accelerates the generation of C1 in the plasma of patients with hereditary angioneurotic edema. She has also demonstrated that plasminogen activation causes generation of C1 in these plasmas (32) and has suggested that plasmin might play a role in the production of C1 in the blood of patients with hereditary angioneurotic edema (32, 50).

Using sensitive assays, several investigators have found that patients with hereditary angioneurotic edema have low but detectable levels of C1 inactivator activity in their plasma (51–53). A fall in the level of circulating inhibitor during an attack of edema has not been reported. The ratios of concentrations of plasmin and C1 inactivator used in the present studies are within the range found in the plasma of patients with hereditary angioneurotic edema.

Transient activation of the fibrinolytic enzyme system at the level of the vascular endothelium, shown to be a source of plasminogen activator (54) might result in a further local reduction of C1, kallikrein inhibitor levels due to the formation of plasmin. In such an inhibitor-deficient setting, the activation of C1 and kinin-forming enzymes might be generated with the resulting production of circumscribed edema. The destruction of the function of C1 inactivator by plasmin provides another example of an interrelationship between the plasma kallikrein, complement, and fibrinolytic enzyme systems.

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