

Demonstration of Modified Inactive First Component of Complement (C1) Inhibitor in the Plasmas of C1 Inhibitor-deficient Patients

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Abstract

The first component of complement (C1) inhibitor plays a critical role in the regulation of the classical complement pathway and the contact system, and the deficiency of C1 inhibitor protein or function is associated with recurrent angioedema. In this study we evaluated the size of the C1 inhibitor antigens present in the plasmas of C1 inhibitor-deficient patients. We found that the C1 inhibitor in the plasmas existed in three forms: high molecular weight forms in complex with proteases, native 110-kD C1 inhibitor, and a modified inactive 94-kD form. The proportion of the total C1 inhibitor in the 94-kD form was 28% in nine hereditary angioedema patients, 92% in five acquired C1 inhibitor-deficiency patients, and 1.2% in five normal controls. In vitro activation of normal plasma with kaolin, but not heat-aggregated gamma-globulin generated 94-kD C1 inhibitor from 110-kD C1 inhibitor. Neither kaolin activation nor heat-aggregated gamma-globulin activation generated 94-kD C1 inhibitor in Hageman factor-deficient plasma. These results suggest that 94-kD C1 inhibitor is generated in vitro by activation of the contact system. The in vivo mechanism of 94-kD C1 inhibitor generation in C1 inhibitor-deficient patients is not known.

Introduction

Three distinct syndromes of C1 inhibitor deficiency occur in humans and are associated with complement activation and recurrent angioedema: hereditary angioedema (HAE)¹ (1) and variant HAE (2) are inherited in an autosomal dominant manner; and acquired C1 inhibitor deficiency (3–5) usually occurs in conjunction with a lymphoproliferative disease or paraproteinemia. The pathogenesis of edema formation in the C1 inhibitor-deficiency syndromes is not completely understood. Increased vascular permeability capable of causing edema formation has been ascribed to complement activation with release of a C2 kinin (6, 7) and/or contact system activation with release of bradykinin (8–12). The relative degrees of complement activation and contact system activation in C1 inhibitor-deficient patients

have not been compared, primarily because of the difficulty of assessing contact system activation in plasma.

C1 inhibitor is a heavily glycosylated single-chain polypeptide with a reported molecular weight of ~108 kD (13–19). It is a multifunctional serine protease inhibitor that is normally present in high concentrations in human plasma (174–240 mg/liter). C1 inhibitor is the only known plasma inhibitor of C1r and C1s (20, 21), the activated proteases of the first component of complement (C1). C1 inhibitor is also the major plasma inhibitor of activated Hageman factor (22–27), the first protease in the contact system. It is also one of the major inhibitors of plasma kallikrein (8, 23, 28–32), the contact system protease that cleaves kininogen and releases bradykinin. Although C1 inhibitor can inhibit coagulation factor XIa (24) and plasmin (23), the inhibition rates for these reactions are slow, suggesting that these inhibitions are not significant in vivo (33). Thus the primary physiologic roles of C1 inhibitor appear to be regulation of activation of the classical complement and contact systems. However, the functional activity of C1 inhibitor in the plasmas of C1 inhibitor-deficient patients is often even less than that predicted by its plasma concentration as determined by single radial immunodiffusion (34). The basis of this discrepancy is not entirely clear.

When C1 inhibitor regulates activated C1, it generates stable complexes of C1 inhibitor with C1r and C1s (35). C1 inhibitor forms sodium dodecyl sulfate (SDS)-stable complexes with C1r, C1s, kallikrein, activated Hageman factor, and plasmin (14, 15, 20, 26). Harpel and Cooper (15) showed that plasmin and trypsin interact in vitro with C1 inhibitor to yield smaller C1 inhibitor fragments, at least one of which retained functional activity. Subsequently, other investigators have demonstrated in vitro modification or proteolytic cleavage of C1 inhibitor by C1s, kallikrein, plasmin, elastase, and other serine proteases (14, 36–41).

In this paper we show that plasma from most if not all patients with C1 inhibitor deficiencies contains a nonfunctional smaller ($M_r = 94$ kD) form of C1 inhibitor in addition to the normal, fully functional 110-kD and protease-complexed high molecular weight forms of C1 inhibitor. The 94-kD form of C1 inhibitor is generated from normal 110-kD C1 inhibitor by kaolin activation of normal plasma in vitro.

Methods

Patients and plasma samples. Blood from five normal, nine HAE, two variant HAE, and five acquired C1 inhibitor-deficiency donors was collected into plastic syringes and mixed with sodium citrate at a final concentration of 0.38% (wt/vol). Unless it was to be activated in vitro, the blood was treated with a cocktail of protease inhibitors as follows (final concentrations): aprotinin 333 IU/ml, benzamidine 0.1 mg/ml, 1,10 phenanthroline 0.01 mM, and phenylmethylsulfonyl fluoride 1 mM. Plasma was obtained by centrifugation and was immediately aliquoted and stored at -70°C . All processing was performed in plastic tubes. The

This is Publication No. 4093BCR from the Research Institute of Scripps Clinic.

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Received for publication 9 September 1985 and in revised form 7 April 1986.

1. *Abbreviations used in this paper:* HAE, hereditary angioedema; PAGE, polyacrylamide gel electrophoresis.

J. Clin. Invest.

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0021-9738/86/08/0567/09 \$1.00

Volume 78, August 1986, 567–575

plasma was thawed immediately before analysis. Prekallikrein-deficient plasma was a gift from Dr. Charles Cochrane (La Jolla, CA). Hageman factor-deficient plasma was generously donated by Mrs. Gunda Hiatt. The acquired C1 inhibitor-deficiency patients had the following associated disorders: chronic lymphocytic leukemia (one patient), paraproteinemia (three patients), and undefined (one patient).

Antibodies. Biotinylated horse anti-mouse IgG was purchased from Vector Laboratories (Burlingame, CA). Goat anti-mouse kappa chain was purchased from Southern Biotechnology Associates (Birmingham, AL). Goat anti-human prekallikrein that recognizes both prekallikrein and kallikrein was a gift from Dr. Charles Cochrane. Anti-human C1 inhibitor and anti-human C1s antisera were prepared by immunizing goats with purified proteins. The antisera were monospecific by Ouchterlony and immunoelectrophoresis analysis. The production of the murine monoclonal anti-C1 inhibitor antibody has been previously described (42).

In vitro activation of plasma. For some experiments plasma was activated in vitro with either kaolin (aluminum silicate) or heat aggregated gamma-globulin (20 mg/ml in Ca^{++} containing veronal buffer heated at 56°C for 1 h). Kaolin was added to citrated plasma (without inhibitors) at a final concentration of 1 mg kaolin/ml plasma and incubated at 37°C for 60 min with frequent shaking. Equal volumes of citrated plasma (without inhibitors) and heat-aggregated gamma-globulin were mixed and incubated at 37°C for 60 min with frequent shaking.

Immune adsorption. IgG fractions of polyclonal goat antisera to C1s, C1 inhibitor, and prekallikrein were conjugated to cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) at 5 mg of IgG/ml swollen gel. 80% or more of the IgG was coupled in each case. For immune adsorption 40 μl of a 1:1 suspension of the conjugated Sepharose beads in phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-Tween) was added to 15 μl of plasma (with inhibitors), and incubated at room temperature for 1 h with frequent gentle mixing. The Sepharose beads were then washed three times with PBS-Tween to remove the nonadsorbed plasma constituents, and the Sepharose beads were resuspended in 80 μl of sample buffer (0.0625 M Tris, pH 6.8, 3% SDS, 10% glycerol, and bromphenol blue). Incubation of normal plasma with anti-C1 inhibitor-conjugated Sepharose depleted all the C1 inhibitor from the plasma as determined by rocket immunoelectrophoresis (see below). The anti-prekallikrein-conjugated Sepharose immune adsorbed both prekallikrein and kallikrein, and the anti-C1s conjugated Sepharose immune adsorbed both C1s and C1s. The anti-C1s conjugated sepharose also adsorbed C1r in complex with C1s.

Immunoblotting for C1 inhibitor. After immune adsorption, the proteins on the washed Sepharose beads were released into the sample buffer by incubation for 5 min in a boiling water bath. The Sepharose beads were pelleted, and the protein containing supernatants applied to 7.5% polyacrylamide slab gels and electrophoresed according to the method of Laemmli (43). The gels were then equilibrated for 30 min at 4°C in transfer buffer (0.025 M Tris, pH 8.3, 0.192 M glycine HCl, 20% methanol [vol/vol], 0.01% NaN_3), and electrophoresed for 3 h at 200 V constant voltage onto nitrocellulose paper (BA85, Schleicher & Schuell, Keene, NH) using a trans-blot cell with a supercooling coil (Bio-Rad Laboratories, Richmond, CA) (44). The nitrocellulose was then dried and stored overnight in an airtight container at -20°C. The nitrocellulose was then brought to room temperature, blocked with 3% (wt/vol) nonfat powdered milk in PBS-Tween for 1 h at room temperature, and probed with murine monoclonal anti-C1 inhibitor IgG (5 $\mu\text{g}/\text{ml}$ in 3% milk-PBS-Tween) for 90 min at room temperature. The nitrocellulose was washed and the primary antibody detected either by radioimmunoassay or enzyme immunoassay. For radioimmunoassay the nitrocellulose was incubated with 2-4 μg of ^{125}I -labeled goat anti-mouse kappa (1 $\mu\text{Ci}/\mu\text{g}$) for 30 min at room temperature, followed by a final wash and autoradiography of the dried nitrocellulose. The densities of the bands on the autoradiogram were assessed quantitatively by laser densitometry (Zeineh softlaser, Biomed Instruments Inc., Chicago, IL). The percentage of C1 inhibitor in each band was derived by dividing the density of each band by the sum of the densities of all the bands in that lane, and multiplying the

result by 100. For enzyme immunoassay, the nitrocellulose was incubated with biotinylated horse anti-mouse IgG (1:500 dilution in 3% milk-PBS-Tween) for 30 min at room temperature, washed, incubated with streptavidin-horseradish peroxidase conjugate (1:400 dilution in 1% bovine serum albumin (BSA)-PBS, pH 7.5; Amersham Corp., Arlington Heights, IL) for 30 min at room temperature, washed, and incubated with substrate (3-3'-diaminobenzidine 4 HCl, 0.01% H_2O_2 , 0.1 M Tris HCl, pH 7.4) for 3-5 min at room temperature. Because the radioimmunoassay gave a larger range of measurable band intensities than the enzyme immunoassay did, the radioimmunoassay was used in all immunoblots for which quantitative data was obtained.

Rocket immunoelectrophoresis for C1 inhibitor. C1 inhibitor levels in plasma were determined by rocket immunoelectrophoresis (45). A 1.8% solution of heated agar (SeaKem ME agarose, FMC Corp., Rockland, ME) was mixed with an equal volume of the IgG fraction of polyclonal goat anti-C1 inhibitor (1:20 final concentration) at 56°C, poured onto 3 x 5-in glass slides (5 ml/slide), and allowed to gel at room temperature. Plasma samples (8 μl) were added to 3-mm diam wells and electrophoresed at 15 V into the gel for 16 h at 4°C. The slides were photographed, and the concentration of C1 inhibitor determined by comparing the area within the precipitin band of the sample to the area within the precipitin bands of dilutions of plasma with a known concentration of C1 inhibitor.

Plasminogen depletion and activation. Plasminogen was depleted from plasma by passage over a lysine conjugated sepharose column, and recovered by elution with epsilon aminocaproic acid according to the method of Deutsch and Mertz (46). Streptokinase (Calbiochem-Behring Corp., La Jolla, CA) was dissolved in water (25,000 U/ml) and 50 U added to 0.05 ml plasma in 0.2 ml PBS, pH 7.4. To assess plasmin activity the streptokinase treated plasma was incubated for 20 min at 37°C in ^{125}I -fibrin-coated plastic wells (kindly provided by Dr. Eugene Levin, La Jolla, CA) (47), and the cleavage of the ^{125}I -fibrin determined by counting the released radioactivity in a gamma counter (Iso-Data 20/20, Palatine, IL). The streptokinase-treated plasma was incubated for 60 min at 37°C in plastic tubes in order to assess the effect of plasmin on C1 inhibitor. After the incubation, protease inhibitors were added and the C1 inhibitor was immune-adsorbed, separated, transferred, and immunoblotted as described above.

Purification of C1 inhibitor. C1 inhibitor was purified from 400 ml of normal plasma with added inhibitors according to the method of Salvesen et al. (40). Cibacron blue F3GA was obtained from Polysciences, Inc. (Warrington, PA) and coupled to sepharose CL-6B (Pharmacia Fine Chemicals) (48). The purified C1 inhibitor gave a homogeneous single band in SDS-polyacrylamide gel electrophoresis (PAGE).

Data analysis. All numeric data was entered into the Scripps Clinic CLINFO system. Statistical analyses were performed by Student's *t* test.

Results

Demonstration of 94-kD C1 inhibitor in plasma. The analysis of C1 inhibitor in plasmas obtained from normal volunteers and C1 inhibitor-deficient patients revealed C1 inhibitor antigen in multiple distinct bands (Fig. 1). The predominant form of C1 inhibitor in normal plasma immunoblotted at an apparent molecular weight of 110 kD. Plasmas obtained from C1 inhibitor-deficient patients also contained significant amounts of a smaller C1 inhibitor that had an apparent molecular weight of 94 kD.

Fig. 1 shows the C1 inhibitor antigens in the plasma of a normal volunteer and a representative patient of each of the three distinct C1 inhibitor-deficiency syndromes. Normal plasma (lane 1) contained 95% of the C1 inhibitor at 110 kD and only 2.5% at 94 kD. HAE plasma (lane 2) contained 16.7% of the C1 inhibitor at 110 kD and 43% at 94 kD. The plasma from a patient with variant HAE (lane 3) contained 51% 110-kD C1 inhibitor and 11% 94-kD C1 inhibitor, while the acquired C1 inhibitor-deficiency plasma (lane 4) contained 100% of the C1 inhibitor at 94 kD.

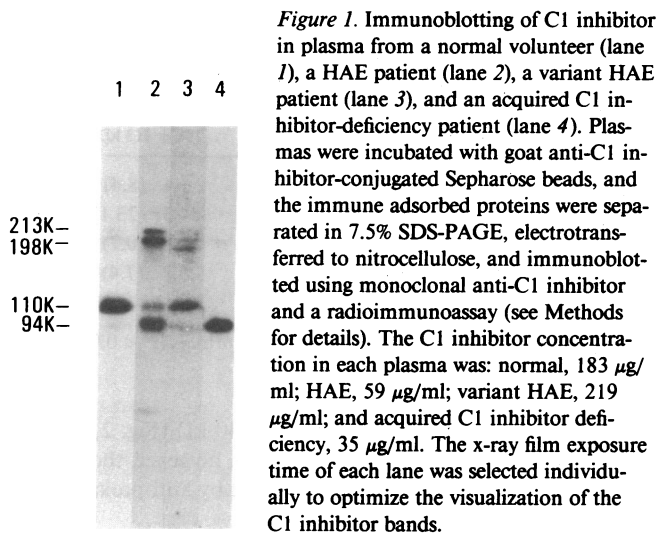


Figure 1. Immunoblotting of C1 inhibitor in plasma from a normal volunteer (lane 1), a HAE patient (lane 2), a variant HAE patient (lane 3), and an acquired C1 inhibitor-deficiency patient (lane 4). Plasmas were incubated with goat anti-C1 inhibitor-conjugated Sepharose beads, and the immune adsorbed proteins were separated in 7.5% SDS-PAGE, electrotransferred to nitrocellulose, and immunoblotted using monoclonal anti-C1 inhibitor and a radioimmunoassay (see Methods for details). The C1 inhibitor concentration in each plasma was: normal, 183 $\mu\text{g}/\text{ml}$; HAE, 59 $\mu\text{g}/\text{ml}$; variant HAE, 219 $\mu\text{g}/\text{ml}$; and acquired C1 inhibitor deficiency, 35 $\mu\text{g}/\text{ml}$. The x-ray film exposure time of each lane was selected individually to optimize the visualization of the C1 inhibitor bands.

Table I shows clinical data, total C1 inhibitor concentration, and percentages of the different forms of C1 inhibitor for the five normal volunteers and 16 patients studied. Some patients were actively swelling or were on androgen therapy at the time that their plasma was obtained, and this is indicated in columns 3 and 4. There was a clear separation without overlap of the total C1 inhibitor concentration, percentage of 94-kD C1 inhibitor, and percentage of 110-kD C1 inhibitor in the HAE and acquired C1 inhibitor-deficiency groups from the normals. Mean total C1 inhibitor levels were significantly reduced in HAE and acquired C1 inhibitor-deficiency patients, and were normal in variant HAE, as expected (Table II). The mean percentage of 110-kD C1 inhibitor was significantly reduced and the mean percentage of 94-kD C1 inhibitor was significantly elevated in the HAE and acquired C1 inhibitor-deficient patients (Table II). The variant HAE group consisted of only two patients, and the distribution of C1 inhibitor in their plasmas was similar to the normal group. The mean percentage of 94-kD C1 inhibitor in an additional 20 normal plasmas was 1.3% with an SD of 0.9.

In addition to the 110-kD and 94-kD bands, high molecular weight C1 inhibitor bands were observed at apparent molecular weights of 198 kD and 213 kD (Fig. 1). The mean percentage of the C1 inhibitor in the high molecular weight bands was 5.4% in five normal volunteers, 34.3% in 9 HAE patients, 2.5% in two variant HAE patients, and 5.2% in five patients with acquired C1 inhibitor deficiency (Table II). The mean percentage of high molecular weight C1 inhibitor in the HAE patients was significantly elevated compared to the normal volunteer group.

The identity of the 198 kD and 213 kD C1 inhibitor bands is demonstrated in Fig. 2. The classical complement pathway of normal plasma was activated *in vitro* with heat-aggregated gamma globulin. Immune adsorption of C1 inhibitor from this activated plasma followed by separation, transfer of the adsorbed protein, and immunoblotting for C1 inhibitor revealed prominent 198-kD and 213-kD C1 inhibitor bands (lane 4). When this complement-activated plasma was incubated with anti-C1s-conjugated Sepharose beads and the immune adsorbed protein was separated, transferred, and immunoblotted for C1 inhibitor, prominent 198-kD and 213-kD bands were again detected (lane 5). No bands were detected when the complement-activated plasma was incubated with anti-prekallikrein-conjugated Sepharose beads and the immune adsorbed protein was separated,

transferred, and immunoblotted for C1 inhibitor (lane 6). Activation of the classical complement pathway with heat-aggregated gamma-globulin was performed in recalcified plasma (see Methods). Under these conditions C1r-C1s-C1 inhibitor complexes (1:1:2) are formed (49), and incubation with anti-C1s-conjugated Sepharose beads will immune adsorb the complex containing both C1r-C1 inhibitor and C1s-C1 inhibitor (50). C1s ($M_r = 87$ kD) and C1r ($M_r = 95$ kD) are predicted to form complexes of M_r 197 and 205 kD, respectively, with C1 inhibitor ($M_r = 110$ kD) (49, 51). The bands seen in lane 5 are consistent with these predictions. The addition of purified C1s to normal plasma generated a prominent 198-kD C1s-C1 inhibitor band on immunoblotting (see Fig. 5 A, lane 2, and Fig. 7 B, lane 1) allowing identification of this band. By deduction, the C1r-C1 inhibitor complex is therefore detected at 213 kD.

The contact system in normal plasma was activated *in vitro* with kaolin. Immune adsorption of the C1 inhibitor in the kaolin-activated plasma followed by separation of the adsorbed protein then transfer and immunoblotting for C1 inhibitor revealed a prominent band at 198 kD (lane 7). Incubation of the kaolin-activated plasma with anti-prekallikrein-conjugated sepharose beads followed by separation, transfer, and immunoblotting of the immune adsorbed proteins for C1 inhibitor, again revealed a prominent band at 198 kD (lane 9). Therefore, the 198-kD band from kaolin-activated plasma contained both C1 inhibitor and kallikrein antigens. In addition, kaolin-activated plasma was also incubated with anti-C1 inhibitor-conjugated Sepharose beads and the immune adsorbed proteins separated, transferred, and immunoblotted for kallikrein. In this experiment, a single 198-kD band was demonstrated (Fig. 3, lane 2) showing that

Table I. Concentrations of Different C1 Inhibitor Antigens in Plasmas of Patients with C1 Inhibitor Deficiency and Normal Volunteers

Diagnosis	Patient	Active swelling	Androgen therapy	Total C1 inhibitor $\mu\text{g}/\text{ml}$	Percentage of total C1 inhibitor		
					94 kD	110 kD	198 + 213 kD
Normal	SC	-	-	207	1	79	20
	JC	-	-	183	2	94	4
	BH	-	-	152	0	100	0
	MC	-	-	190	3	94	3
	JAC	-	-	185	0	100	0
HAE	HH	-	-	71	21	54	25
	EC	-	+	66	19	66	15
	PM	+	-	59	28	66	6
	CU	+	-	71	12	28	60
	BU	-	-	38	75	11	14
	SM	+	+	59	36	37	27
	EA	-	-	58	18	37	45
	ST	-	-	62	27	32	41
	KJ	-	-	60	16	8	76
Variant HAE	JB	-	-	219	11	87	2
	RD	-	-	220	0	97	3
AC1D	AH	-	-	75	100	0	0
	LB	+	+	26	75	9	16
	BS	-	+	114	98	2	0
	JL	-	+	81	87	3	10
	JG	-	-	35	100	0	0

Abbreviation: AC1D, acquired C1 inhibitor deficiency.

Table II. Summary of Statistical Significance of the Measurements of the Different C1 Inhibitors in HAE, Variant HAE, and ACID Plasmas

Group	Number	Total C1 inhibitor	Percent of total C1 inhibitor		
			94 kD	110 kD	198 + 213 kD
Normal	5	183 (19.9)*	1.2 (1.3)	93.4 (8.6)	5.4 (8.4)
HAE	9	64 (9.8)‡	28.0‡ (19.1)	36.7‡ (21.2)	34.3‡ (23.1)
Variant HAE	2	220 (0.7)	5.5 (7.8)	92.0 (7.1)	2.5 (0.7)
ACID	5	66 (35.9)‡	92.0‡ (10.9)	2.8‡ (3.7)	5.2 (7.4)

Abbreviation: ACID, acquired C1 inhibitor deficiency. * Mean and standard deviations. ‡ Significantly different from normals ($P < 0.05$).

kallikrein ($M_r = 88$ kD) (52), like C1s, forms a 198-kD complex with C1 inhibitor. The C1s-C1 inhibitor and kallikrein-C1 inhibitor complexes can be differentiated by immune adsorption with either anti-C1s or anti-prekallikrein conjugated sepharose beads as demonstrated in Fig. 2.

Immunoblotting of normal plasma (Fig. 2, lane 1) showed faint C1 inhibitor bands at 198 and 213 kD. These bands were shown to be C1s-C1 inhibitor and C1r-C1 inhibitor complexes by their immune adsorption with anti-C1s conjugated sepharose beads (lane 2). Immunoblotting of the anti-C1 inhibitor-adsorbed protein from kaolin-activated plasma revealed two other C1 in-

hibitor bands of apparent M_r 150 and 140 kD (Fig. 2, lane 7). The identity of these bands is not known, however, the 150-kD C1 inhibitor band was immune adsorbed by anti-prekallikrein-conjugated Sepharose beads (lane 9).

Because the HAE patients had a significantly higher mean percentage of their C1 inhibitor in the high molecular weight complexed form, the identity of the 198-kD band was determined in five HAE patients. Fig. 4 shows an immunoblot from one of these HAE patients. When the HAE plasma was incubated with anti-C1 inhibitor-conjugated Sepharose beads and the immune-adsorbed proteins were separated, transferred, and immunoblotted for C1 inhibitor, bands were seen at 198 and 213 kD (lane 1). These high molecular weight C1 inhibitor bands were detected at the same intensity if the plasma was incubated with anti-C1s-conjugated Sepharose beads prior to immunoblotting (lane 2). However, if the plasma was incubated with anti-prekallikrein-conjugated Sepharose beads prior to immunoblotting, no significant C1 inhibitor band at 198 kD was detectable (lane 3). Kallikrein-C1 inhibitor complex was easily demonstrated at 198 kD in kaolin-activated normal plasma (lane 4) as well as in kaolin-activated HAE plasma (lane 6). Four other HAE plasmas behaved identically.

Kaolin activation of normal plasma generates 94-kD C1 inhibitor. To investigate whether 94-kD C1 inhibitor is generated by complement activation or contact system activation, normal plasma was activated in vitro with heat-aggregated gamma-globulin (a complement activator) or kaolin (a contact system activator). The normal and in vitro activated plasmas were incubated with anti-C1 inhibitor-conjugated Sepharose beads and the immune adsorbed proteins were separated, transferred, and

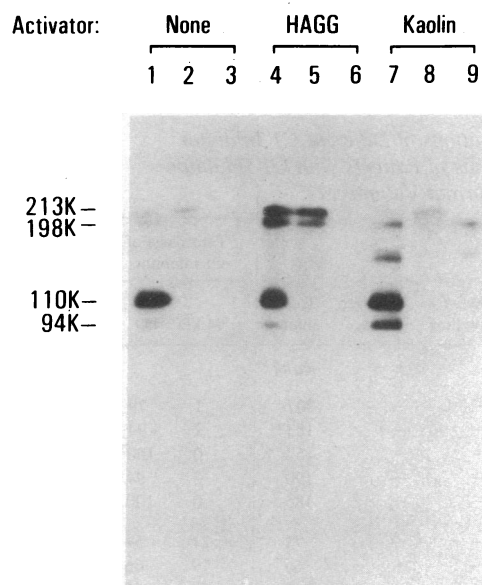


Figure 2. Immunoblotting of C1 inhibitor in normal plasma and in vitro activated normal plasma. Plasma was incubated with polyclonal antibody-conjugated Sepharose beads and the immune adsorbed proteins separated in 7.5% SDS-PAGE, electrotransferred to nitrocellulose, and immunoblotted using monoclonal anti-C1 inhibitor and a radioimmunoassay. The plasma samples and Sepharose-conjugated immunosorbant antibodies were as follows: lane 1, normal plasma and anti-C1 inhibitor; lane 2, normal plasma and anti-C1s; lane 3, normal plasma and anti-prekallikrein; lane 4, heat aggregated gammaglobulin activated plasma and anti-C1 inhibitor; lane 5, heat-aggregated gamma-globulin-activated plasma and anti-C1s; lane 6, heat-aggregated gamma-globulin-activated plasma and anti-prekallikrein; lane 7, kaolin-activated plasma and anti-C1 inhibitor; lane 8, kaolin activated plasma and anti-C1s; lane 9, kaolin-activated plasma and anti-prekallikrein.

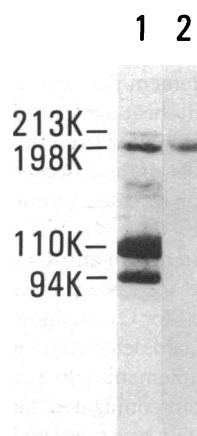


Figure 3. Immunoblotting for C1 inhibitor (lane 1) and (pre)kallikrein (lane 2) in kaolin-activated normal plasma. Kaolin-activated plasma was incubated with goat anti-C1 inhibitor-conjugated Sepharose beads, and the immune adsorbed proteins separated in 7.5% SDS-PAGE, electrotransferred to nitrocellulose, and immunoblotted with either monoclonal anti-C1 inhibitor (lane 1) or affinity purified goat anti-prekallikrein (lane 2) using an enzyme immunoassay.

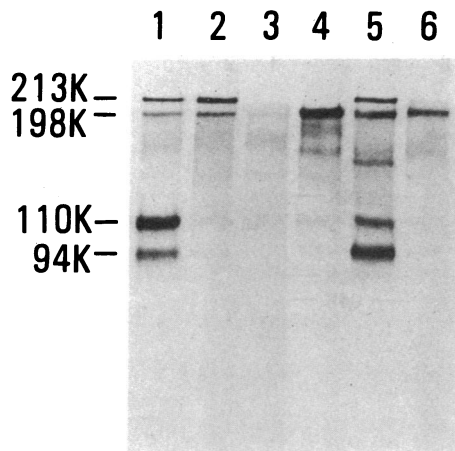


Figure 4. Immunoblotting for C1 inhibitor in HAE plasma (lanes 1–3), kaolin-activated normal plasma (lane 4), and kaolin activated HAE plasma (lanes 5 and 6). Plasmas were incubated with polyclonal antibody-conjugated Sepharose beads and the immune adsorbed proteins separated in 7.5% SDS-PAGE, electrotransferred to nitrocellulose, and immunoblotted using monoclonal anti-C1 inhibitor and an enzyme immunoassay. The plasma samples and Sepharose-conjugated immunosorbent antibodies were as follows: lane 1, HAE plasma and anti-C1 inhibitor; lane 2, HAE plasma and anti-C1s; lane 3, HAE plasma and anti-prekallikrein; lane 4, kaolin-activated normal plasma and anti-prekallikrein; lane 5, kaolin-activated HAE plasma and anti-C1 inhibitor; lane 6, kaolin-activated HAE plasma and anti-prekallikrein.

immunoblotted for C1 inhibitor. The immunoblot of the normal plasma is shown in Fig. 2, lane 1. As expected, 90% of the C1 inhibitor was in the 110-kD form while only 1.7% was in the 94-kD form. The immunoblot of the complement activated plasma is shown in lane 4. Despite considerable complement activation and high molecular weight complex formation, the percentage of 94-kD C1 inhibitor increased to only 3.5%. However, with kaolin activation of plasma the percentage of 94-kD C1 inhibitor increased to 26% (lane 7). Thus, *in vitro* activation of the contact system in normal plasma with kaolin led to a reduction in the level of 110-kD C1 inhibitor and an increase in the level of 94-kD C1 inhibitor.

The ability of C1s to generate 94-kD C1 inhibitor was directly tested by adding purified active C1s (53) to three normal plasma samples (2:1 molar ratio C1s to calculated C1 inhibitor) for 30 min at 37°C prior to immune adsorption and immunoblotting. Fig. 5 A shows an immunoblot from one of the normal plasmas incubated with either C1s (lane 2) or kaolin (lane 3). The mean percentage of 94-kD C1 inhibitor increased from 1% to only 4% after incubation with C1s. By contrast, the same plasma samples were incubated with kaolin to activate the contact system, and the mean percentage of 94-kD C1 inhibitor increased to 30%. When normal plasma was activated with heat-aggregated gamma-globulin or incubated with C1s and then kaolin activated, the percentage of 94-kD C1 inhibitor generated was approximately one half of that generated by kaolin activation alone.

Because kaolin activation of plasma is not specific for the contact system, the roles of the components of the contact system in the generation of 94-kD C1 inhibitor were evaluated directly by kaolin activation of plasmas deficient in Hageman factor, prekallikrein, or high molecular weight kininogen. Fig. 5 B shows an immunoblot of the C1 inhibitor in Hageman factor-deficient

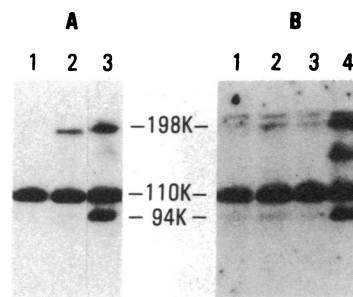


Figure 5. Immunoblotting for C1 inhibitor. Plasmas were incubated with goat anti-C1 inhibitor-conjugated Sepharose beads, and the immune-adsorbed proteins separated in 7.5% SDS-PAGE, electrotransferred to nitrocellulose, and immunoblotted using monoclonal anti-C1 inhibitor and a radioimmunoassay.

(A) Lane 1, normal plasma; lane 2, 15 μ l of normal plasma incubated with 3 μ g of C1s for 30 min at 37°C; lane 3, kaolin-activated normal plasma (1 mg of kaolin/ml plasma, 30 min, 37°C). (B) Lane 1, Hageman factor-deficient plasma; lane 2, kaolin-activated Hageman factor-deficient plasma; lane 3, normal plasma; lane 4, kaolin-activated normal plasma.

plasma activated with kaolin (lane 2) as compared to kaolin activated normal plasma (lane 4). The percentage of 94-kD C1 inhibitor was <1% after kaolin activation of the Hageman factor-deficient plasma. Table III summarizes the results obtained with other plasmas deficient in contact system factors.

The contribution of plasmin to the generation of 94-kD C1 inhibitor was assessed. Plasminogen was depleted from normal plasma by passage over a lysine-conjugated Sepharose column (46), and the absence of plasminogen confirmed by double immunodiffusion assay using rabbit anti-human plasminogen (Calbiochem Behring Corp.). Kaolin activation of the plasminogen-depleted plasma increased the percentage of 94-kD C1 inhibitor from <1% to 15% (Fig. 6, lanes 3 and 4). Kaolin activation of the non-depleted plasma increased the percentage of 94-kD C1 inhibitor to 20% (lanes 1 and 2). The ability of plasmin to generate 94-kD C1 inhibitor was determined by the addition of streptokinase to plasma. In order to avoid secondary activation of the contact system, Hageman factor-deficient plasma was used. Plasmin activity was monitored by the cleavage of ¹²⁵I-fibrin (Table IV) (47). Streptokinase treatment of Hageman factor-deficient plasma generated <1% 94-kD C1 inhibitor (lane 5). When streptokinase was added to normal plasma, 7% 94-kD C1 inhibitor was generated (lane 6). The addition of streptokinase to a mixture of purified C1 inhibitor and purified plasminogen yielded a 182-kD high molecular weight complex containing C1 inhibitor as well as an 84-kD form of C1 inhibitor (lane 8). These 182- and 84-kD C1 inhibitor bands are clearly distinguishable from the 198- and 213-kD complexes and 94-kD form of C1 inhibitor.

Table III. Plasmas Deficient in Contact System Components Do Not Generate 94-kD C1 Inhibitor after *In Vitro* Activation

Plasma source	Activator:	Percentage of total C1 inhibitor in 94-kD Form			
		None	HAGG	C1s	Kaolin
Normal		<1	<1	<1	31
Hageman factor-deficient		<1	<1	<1	<1
Prekallikrein-deficient		<1	NT*	NT	9
High molecular weight kininogen-deficient		<1	NT	NT	8

Abbreviation: HAGG, heat-aggregated gamma-globulin.

* NT, not tested.

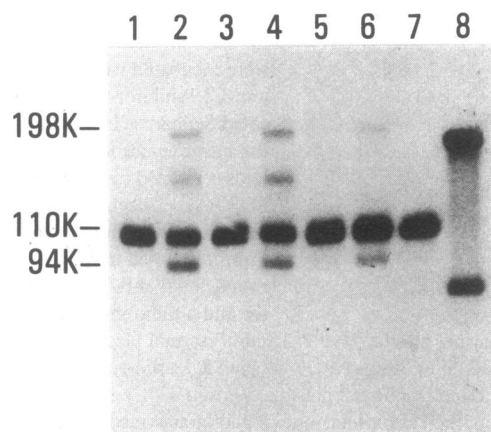


Figure 6. Immunoblotting for C1 inhibitor. Plasmas were incubated with goat anti-C1 inhibitor-conjugated Sepharose beads, and the immune-adsorbed proteins were separated in SDS-PAGE, electrotransferred to nitrocellulose, and immunoblotted using monoclonal anti-C1 inhibitor and a radioimmunoassay. Lane 1, normal plasma; lane 2, kaolin-activated normal plasma; lane 3, plasminogen-depleted plasma; lane 4, kaolin-activated plasminogen-depleted plasma; lane 5, Hageman factor-deficient plasma incubated with streptokinase (50 U in 50 μ l of plasma, 60 min, 37°C); lane 6, normal plasma incubated with streptokinase (same conditions as above); lane 7, purified C1 inhibitor; lane 8, purified C1 inhibitor (10 μ g) plus purified plasminogen (30 μ g) incubated with streptokinase (50 U; 60 min, 37°C).

94-kD C1 inhibitor is functionally inactive. The functional activity of 94-kD C1 inhibitor was assessed by its ability to form stable complexes with C1r, C1s, and kallikrein. Fig. 7 A shows the effects of heat-aggregated gamma-globulin or kaolin activation of plasma from a patient with acquired C1 inhibitor deficiency. The untreated plasma (lane 1) contained virtually 100% 94-kD C1 inhibitor. Activation of the plasma with heat-aggregated gamma-globulin (lane 2) or kaolin (lane 3) did not generate any detectable high molecular weight C1 inhibitor bands. In contrast, untreated normal plasma (lane 4) contained predominantly 110-kD C1 inhibitor, and activation with heat-aggregated gamma-globulin (lane 5) or kaolin (lane 6) resulted in the generation of easily detectable high molecular weight C1 inhibitor bands. Patients with acquired C1 inhibitor deficiency have low plasma concentrations of C1q, C1r, and C1s which could theoretically prevent C1 activation and complex formation with functional C1 inhibitor. Therefore the ability of the 94-kD C1

Table IV. Cleavage of 125 I-Fibrin by Plasmin

Plasmin source	Activator:	Cleavage	
		None	Streptokinase
		<i>cpm released*</i>	
Buffer only		3,278	3,420
Normal plasma		3,339	19,692
HF-deficient plasma		3,556	20,728
Purified C1 inhibitor (10 μ g) + purified plasminogen (30 μ g)		3,623	20,949

Abbreviation: HF, Hageman factor.

* 20-min incubation at 37°C.

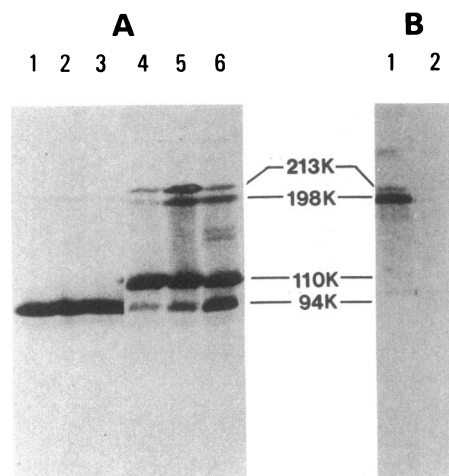


Figure 7. Immunoblotting for C1 inhibitor. (A) Plasmas were incubated with goat anti-C1 inhibitor-conjugated Sepharose beads, and the immune adsorbed proteins were separated in 7.5% SDS-PAGE, electrotransferred to nitrocellulose, and immunoblotted using monoclonal anti-C1 inhibitor and an enzyme immunoassay. Lane 1, acquired C1 inhibitor-deficiency plasma; lane 2, heat-aggregated gamma-globulin-activated acquired C1 inhibitor-deficiency plasma; lane 3, kaolin-activated acquired C1 inhibitor-deficiency plasma; lane 4, normal plasma; lane 5, heat-aggregated gamma-globulin-activated normal plasma; lane 6, kaolin-activated normal plasma. (B) C1s (7.5 μ g) was incubated with 40 μ l of a 1:1 suspension of goat anti-C1s-conjugated Sepharose beads for 1 h at 37°C. The C1s adsorbed sepharose beads were then incubated for 1 h at 37°C with 15 μ l of normal plasma (lane 1) or 15 μ l of acquired C1 inhibitor-deficient plasma (lane 2). The immune adsorbed proteins were separated in 7.5% SDS-PAGE, electrotransferred to nitrocellulose, and immunoblotted using monoclonal anti-C1 inhibitor and an enzyme immunoassay.

inhibitor in acquired C1 inhibitor-deficiency plasma to complex with purified C1s was determined directly. Purified active C1s (53) was immune adsorbed to anti-C1s-conjugated Sepharose beads, and the beads were incubated with either normal or acquired C1 inhibitor deficiency plasma as sources of C1 inhibitor. The adsorbed proteins were separated, transferred and immunoblotted for C1 inhibitor (Fig. 7 B). Functional C1 inhibitor in the plasmas would complex with the adsorbed C1s and be detected as a 198-kD C1s-C1 inhibitor band. As can be seen, C1s-C1 inhibitor complex was formed in normal plasma (lane 1) but not in acquired C1 inhibitor-deficiency plasma (lane 2) indicating that the 94-kD C1 inhibitor cannot complex with C1s. As demonstrated above (Fig. 7 A) 94-kD C1 inhibitor did not form kallikrein-C1 inhibitor complexes after kaolin activation. However, when purified normal C1 inhibitor was added to the acquired C1 inhibitor-deficiency plasma, subsequent kaolin activation did result in the appearance of kallikrein-C1 inhibitor complexes (Fig. 8). Therefore, 94-kD C1 inhibitor cannot form stable complexes with C1r, C1s, or kallikrein, and is inactive or dysfunctional.

Discussion

We demonstrated that C1 inhibitor circulates in plasma in a smaller 94-kD form in addition to its native 110-kD and high molecular weight protease-complexed forms. HAE and acquired C1 inhibitor-deficiency patients had significantly less native 110-

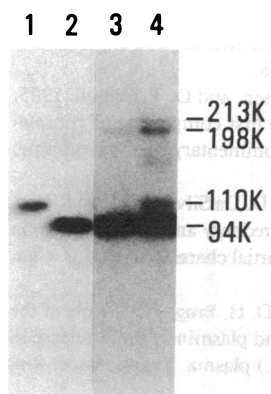


Figure 8. Immunoblotting for C1 inhibitor. Samples were incubated with goat anti-C1 inhibitor-conjugated Sepharose beads, and the immune-adsorbed proteins were separated in 7.5% SDS-PAGE, electrotransferred to nitrocellulose, and immunoblotted using monoclonal anti-C1 inhibitor and a radioimmunoassay. Samples were as follows: lane 1, C1 inhibitor (1 μ g); lane 2, 15 μ l of acquired C1 inhibitor-deficiency plasma; lane 3, 15 μ l kaolin-activated acquired C1 inhibitor-deficiency plasma; lane 4, 15 μ l of kaolin-activated acquired C1 inhibitor-deficiency plasma to which 1 μ g of C1 inhibitor had been added.

kD C1 inhibitor and significantly more 94-kD C1 inhibitor than normal controls. The distribution of C1 inhibitor among 110-kD, 94-kD, and protease-complexed forms segregated into distinct patterns among these groups of patients. Patients with acquired C1 inhibitor deficiency had the highest mean percentage of 94-kD C1 inhibitor (92%), whereas HAE patients had a mean of 28% of their C1 inhibitor in the 94-kD form. Patients with variant HAE had a mean of 5.5% of their C1 inhibitor in the 94-kD form, and the normal volunteers had a mean of 1.2% 94-kD C1 inhibitor.

Activation of the contact system *in vitro* with kaolin generated 94-kD C1 inhibitor and depleted 110-kD C1 inhibitor in normal plasma. However, activation of the classical complement system with heat-aggregated gamma globulin or C1s did not generate 94-kD C1 inhibitor. Plasma deficient in prekallikrein or Hageman factor could not generate significant amounts of 94-kD C1 inhibitor. The 94-kD C1 inhibitor in acquired C1 inhibitor deficiency plasma was not able to form protease-inhibitor complexes with C1r, C1s, or kallikrein. However, the addition of purified 110-kD C1 inhibitor to acquired C1 inhibitor-deficiency plasma restored its ability to form complex.

The structural basis of the generation of 94-kD C1 inhibitor from 110-kD C1 inhibitor has not been elucidated. One possibility involves limited proteolytic cleavage of C1 inhibitor by the protease. There is significant precedent for this mechanism (described below). Because the antibody used to detect C1 inhibitor in our experiments was monoclonal and reacted with the native molecule and the 94-kD form, we would not expect it to also recognize an epitope on a smaller fragment. Therefore, it is possible that such a fragment was generated but not detected. The other possibility is that the C1 inhibitor was conformationally altered by its interaction with protease. Reduction of C1 inhibitor has been shown to cause it to migrate faster on SDS-polyacrylamide gels (14, 37) and has been associated with loss of ordered secondary structures as assessed by circular-dichroism spectra (38). Studies are currently in progress to determine which mechanism is operative.

C1 inhibitor shares many structural and functional similarities with the other serine protease-specific plasma inhibitors, and is said to belong to the alpha₁-proteinase inhibitor class (33). The inhibitors of this class are thought to form an acyl-intermediate between a carbonyl carbon of the inhibitor at its reactive site and the gamma oxygen of the protease serine (54). Although this intermediate is usually stable, it can dissociate into active

protease and a modified inhibitor. The dissociation may occur spontaneously or can be experimentally produced by nucleophilic attack (49, 55). The mechanism may be schematically outlined as follows (33, 56): $P + I \rightleftharpoons PI \rightarrow PI^* \rightarrow P + I^*$. In this model P represents the protease, I is the inhibitor, and I^* is the modified inhibitor. Modified forms of α_1 -proteinase inhibitor (57, 58), antithrombin III (55, 59), and α_1 -antichymotrypsin (60) have been generated *in vitro*. Each of the modified inhibitors had a molecular weight from 5,000 to 8,000 D less than the native inhibitor, and each was inactive.

A number of groups of investigators have previously described a smaller form of C1 inhibitor generated by proteases *in vitro* or present in purified preparations of C1 inhibitor (14, 15, 36–41). Harpel and Cooper (15) purified C1 inhibitor from normal plasma and found two forms of C1 inhibitor—a major band at 105 kD and a minor band (15%) at 96 kD. Both of these C1 inhibitor forms were functional. However, incubation of the purified 105-kD C1 inhibitor *in vitro* with plasmin generated two 96-kD C1 inhibitor fragments, one of which was functionally active while the other was inactive. Activated Hageman factor and kallikrein have been reported to be plasminogen activators (61, 62); however, the physiologic relevance of this in plasma has been questioned (63). We have shown that depletion of plasminogen from normal plasma minimally affected the ability of kaolin treatment to generate 94-kD C1 inhibitor. We also demonstrated that plasmin did not generate significant amounts of 94-kD C1 inhibitor in Hageman factor-deficient plasma.

Two forms of C1 inhibitor were also found in C1 inhibitor preparations made from normal plasma by Van der Graaf et al. (14). They demonstrated a major band of 110 kD and a minor inactive band (<10%) of 94 kD. Incubation of the purified C1 inhibitor with either kallikrein or active kallikrein light chain was found to increase the 94-kD C1 inhibitor, decrease the 110-kD C1 inhibitor, and generate kallikrein-C1 inhibitor complexes. During the preparation of this manuscript, De Agostini et al. (39) reported that kallikrein generated a 95-kD form of C1 inhibitor from native 105-kD C1 inhibitor *in vitro*. They described a monoclonal antibody that recognized a neoantigen that is expressed by the kallikrein-C1 inhibitor complex and the 95-kD C1 inhibitor but not the native 105-kD C1 inhibitor.

Other investigators have demonstrated that the *in vitro* formation of C1s-C1 inhibitor complexes generates a small (~5 kD) fragment of C1 inhibitor (37, 38, 40). This fragment has been shown to result from the cleavage of a single arginine-threonine peptide bond at the carboxy end of the C1 inhibitor molecule (38, 40). However, the C1s-modified C1 inhibitor complex remained stable and migrated as a single high molecular weight band on SDS-polyacrylamide gels in two of these three reports (38, 40). One group has reported that the *in vitro* formation of C1s-C1 inhibitor complexes in the presence of heparin (1:1 molar ratio with C1 inhibitor) led to the generation of a modified 95-kD C1 inhibitor on SDS-polyacrylamide gels (37). However the heparin concentration employed in these experiments was at least 10-fold higher than those achieved even during full anticoagulation (64). In addition, the C1s-C1 inhibitor complexes were formed in a tris-HCl buffer, pH 8.0. The pKa of Tris is 8.1, and at this pH Tris has significant nucleophilic reactivity towards carbonyl carbons (65). C1s-C1 inhibitor complexes have previously been shown to be susceptible to dissociation by nucleophilic attack (49). We, as well as other investigators (15, 20, 41, 49), found that neither complement activation with heat-aggregated gamma-globulin nor direct addition of C1s led to

significant generation of 94-kD C1 inhibitor in plasma. Taken together with our other results involving in vitro contact system activation and the use of contact system protease-deficient plasmas, this suggests that contact system activation may be a major pathway of 94-kD C1 inhibitor generation in vivo.

Because the contact system and other proteases may be poorly regulated in C1 inhibitor-deficiency states, it was necessary to exclude in vitro protease activation as the genesis of 94-kD C1 inhibitor generation in our patient samples. We have prepared plasma from C1 inhibitor-deficient patients from blood drawn simultaneously by our usual technique as well as through a large-bore plastic catheter directly into a syringe containing the protease inhibitors. The C1 inhibitor in each of the plasmas was then immune adsorbed and immunoblotted. No differences in C1 inhibitor distribution between the two collection techniques was observed. These results indicate that the 94-kD C1 inhibitor was generated in vivo.

There were consistent differences in the distribution of 94- and 110-kD C1 inhibitor among the C1 inhibitor-deficient groups. The patients with acquired C1 inhibitor deficiency all had very high percentages of their C1 inhibitor in the 94-kD form whereas the common HAE group had elevated but lower percentages of 94-kD C1 inhibitor. The variant HAE group exhibited a lower percentage of 94-kD C1 inhibitor presumably because their dysfunctional C1 inhibitor was unable to bind the relevant protease(s). The total C1 inhibitor concentration did not correlate with the amount of 94-kD C1 inhibitor present in plasma. We suggest that the amount of 94-kD C1 inhibitor present in plasma is dependent on the ability of the C1 inhibitor to bind to the relevant protease(s), and reflects the degree of contact system or other protease activation that has occurred.

This study shows that normal C1 inhibitor undergoes modification during the in vitro kaolin activation of plasma to yield a nonfunctional small C1 inhibitor of 94 kD. The 94-kD C1 inhibitor has been identified and quantified in plasma from normal individuals and C1 inhibitor-deficient patients for the first time. This is also the first in vivo corroboration that inactive modified serine protease inhibitors are formed by the interaction of protease with inhibitor. These studies suggest that contact system activation may be an important mechanism in the pathophysiology of angioedema as first proposed by Landerman et al. (8). This suggestion is consistent with the previous finding of large amounts of active kallikrein in blister fluids of HAE patients (12). C1s may also be an important mediator of angioedema; however, the results presented in this report do not directly address this possibility. Finally, we suggest that quantitation of 94-kD C1 inhibitor may provide a useful probe for evaluating contact system activation in other inflammatory disorders.

Acknowledgments

We thank Drs. Eng Tan and Charles Cochrane for helpful discussions and support, and Ms. Sandra Sugimoto for expert technical assistance.

This work was supported by research grants RR-00833 and AI-10386 from the National Institutes of Health.

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