Plasma Levels of C1 Inhibitor Complexes and Cleaved C1 Inhibitor in Patients with Hereditary Angioneurotic Edema

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Abstract

C1 inhibitor (C1-Inh) catabolism in plasma of patients with hereditary angioneurotic edema (HANE) was assessed by measuring the complexes formed by C1-Inh with its target proteases (C1s, Factor XIIa, and kallikrein) and a modified (cleaved) inactive form of C1-Inh (iC1-Inh). This study was performed in plasma from 18 healthy subjects and 30 patients with HANE in remission: 20 with low antigen concentration (type I) and 10 (from 5 different kindreds) with dysfunctional protein (type II). Both type-I and type-II patients had increased C1–C1-Inh complexes ($P < 0.0001$), which in type I inversely correlated with the levels of C1-Inh ($P < 0.001$). iC1-Inh was normal in all type-I patients and in type-II patients from three families with increased C1-Inh antigen, whereas iC1-Inh was higher than 20 times the normal values in patients from the remaining two families with C1-Inh antigen in the normal range. None of the subjects had an increase of either Factor XIIa–C1-Inh or kallikrein–C1-Inh complexes. This study shows that the hypercatabolism of C1-Inh in HANE patients at least in part occurs via the formation of complexes with C1 and that genetically determined differences in catabolism of dysfunctional C1-Inh proteins are present in type-II patients. (*J. Clin. Invest.* 1990. 85:1215–1220.) complement • contact system • C1 inhibitor catabolism • C1 inhibitor deficiency • dysfunctional C1 inhibitor

Introduction

Hereditary angioneurotic edema (HANE)[1] is due to the functional deficiency of the inhibitor of the first component of human complement (C1-Inh) (1). This protease inhibitor is involved in the regulation of several proteolytic systems in plasma including the complement system, the contact system of intrinsic coagulation and kinin release, and the fibrinolytic system (2). Two phenotypic variants of HANE are known: type I in which both functional and antigenic C1-Inh in plasma is reduced and type II with functional deficiency but normal or markedly increased antigenic levels due to the presence of a dysmorphic protein (3, 4). Heterogeneity of the dysmorphic proteins has been demonstrated among different type-II HANE families (5). Studies of in vitro cell cultures have shown that monocytes from patients with HANE synthesize ~ 50% of normal C1-Inh compared with cells from normal subjects (6), which is in agreement with the autosomal dominant inheritance of the disorder (7). Independently from the severity of the disease (8), functional C1-Inh in plasma from patients with HANE ranges between 5 and 30% of the normal value instead of the expected 50%. In vivo turnover studies demonstrated that patients with HANE have an increased catabolism of normal C1-Inh along with its reduced synthesis (9). Lachmann and Rosen have suggested that the discrepancy between expected and actual functional C1-Inh levels in HANE is due to the catabolic behavior of C1-Inh: a level of 50% of normal cannot be maintained due to consumption of C1-Inh via the formation of complexes with its target proteases (C1r, C1s, Factor XIIa, and kallikrein) (10). Indirect proof that C1 is activated in both types of HANE during asymptomatic periods comes from the observation that plasma levels of the natural substrates of activated C1, i.e., C4 and C2, are reduced (11, 12). Zuraw and Curd (13) have found, in type I patients, an increase of a modified inactive form of C1-Inh of 94 kD. Since this 94-kD C1-Inh was generated in vitro by contact system activation but not by complement activation, they suggested that contact system activation may be an important mechanism in the pathophysiology of HANE as first proposed by Landerman (14). However, evidence that C1-Inh is indeed consumed by reacting with its target proteases in asymptomatic HANE patients is lacking, as well as is clear-cut proof that the contact system is hyperactivated (13, 15–19).

Despite the description of several highly sensitive assays for C1-Inh complexes in plasma (13, 20–24), only few data are as yet available concerning complex levels in plasma from HANE patients.

The present study reports on the quantification of C1-Inh complexes in plasma from asymptomatic HANE patients and supports the notion that levels of C1-Inh in these patients are lower than expected due to activation of C1. Moreover, quantification of modified (cleaved) inactive C1-Inh (iC1-Inh) revealed genetically determined differences in the catabolism of dysfunctional proteins in patients with type-II HANE.

Methods

Subjects and blood sampling

30 HANE patients, aged 14–66 yr, were studied. 16 were males and 14 were females. The patients (20 type I and 10 type II) were in remission (attack-free and without treatment for at least 3 mo). 18 healthy donors

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1. *Abbreviations used in this paper:* C1-Inh, inhibitor of the first component of human complement; iC1-Inh, modified (cleaved) inactive C1-Inh; HANE, hereditary angioneurotic edema; RID, radial immunodiffusion.

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(9 males and 9 females ranging in age between 25 and 62 yr) served as controls. Blood samples were obtained by clean venipuncture without stasis and collected in siliconized vacutainer tubes (Becton Dickinson, Plymouth, UK) to which EDTA (10 mM) and Polybrene (0.05%, wt/vol) had been added to prevent in vitro activation of the complement and the contact system (20, 24). Tubes were centrifuged at 1,300 *g*, at room temperature, for 10 min and plasma was aliquoted and stored in polystyrene tubes at −70°C until tests were performed.

Procedure

RIAs for C1-Inh species were performed as described in detail previously (20, 25–27).

C1-Inh complexes. MAb KOK 12, which binds to both complexed C1-Inh and iC1-Inh, but not to native C1-Inh, was coupled to Sepharose beads and incubated with plasma samples. After washing the Sepharose with saline, bound complexes were detected by subsequent incubation with ^{125}I-labeled polyclonal antiprotease antibodies (^{125}I-anti–Factor XII, ^{125}I-antikallikrein, or ^{125}I-anti-C1s). After another washing procedure, Sepharose-bound radioactivity was measured. As standards in the appropriate assay we used serial dilutions of plasma in which a maximal amount of Factor XIIa–C1-Inh (i.e., 0.375 μM) and kallikrein–C1-Inh (i.e., 0.341 μM) complexes was generated by incubation with dextran sulphate and serum in which a maximal amount of C1–C1-Inh (0.360 μM) complexes was generated by incubation with heat-aggregated human IgG (20, 24, 27). Intra- and interassay coefficients of variation were < 10%. Details on this as well as on the preparation and stability of the standards are given elsewhere (20, 27).

C1-Inh antigen and iC1-Inh. In the assay for C1-Inh antigen, a MAb (R II) that binds all forms of C1-Inh (native C1-Inh, complexed C1-Inh, and iC1-Inh) was used. iC1-Inh was measured with a MAb (K II), that binds to iC1-Inh, but not to native and complexed C1-Inh (25). Binding of C1-Inh species to these MAbs was detected using ^{125}I-labeled polyclonal anti–C1-Inh antibodies. Levels of antigenic C1-Inh and iC1-Inh were expressed in micromolar by reference to pooled normal plasma that contained 2.5 μM of antigenic C1-Inh and 0.08 μM of iC1-Inh.

Functional C1-Inh. This assay is based on the principle that only functional C1-Inh will bind to C1s. Plasma samples were incubated with purified C1s coupled to Sepharose. Binding of C1-Inh to the C1s-Sepharose was detected by subsequent incubation with ^{125}I-labeled polyclonal anti–C1-Inh. Values for functional C1-Inh were expressed in micromolar using pooled normal plasma that contained 2.5 μM of functional C1-Inh as standard.

Prekallikrein and Factor XII were measured by specific RIAs that have been described previously (20). Values were expressed in micromolar by comparison with pooled normal plasma that contained 0.57 μM of prekallikrein and 0.50 μM of Factor XII.

Chromogenic assay for functional C1-Inh was performed with the C1-Inh reagent kit of Immuno (Vienna, Austria). This method is based on the measurement of chromogenic groups released from pyroglutamyl–glycyl-arginin-pNA (Kabi Ab, Stockholm) which is specifically split by C1s. Intra- and interassay coefficients of variation are < 10%.

Radial immunodiffusion (RID) for C1-Inh and C4 antigen was performed according to Mancini et al. (28) (NOR-Partigen; Behring, Marburg, FRG).

SDS-PAGE and immunoblotting analysis of C1-Inh. Total C1-Inh (with MAb RII) and iC1-Inh (with MAb KII) were immunoprecipitated from plasma by incubating 25 μl of plasma for 5 h at room temperature with 80 μg of MAb coupled to Sepharose. After a washing procedure, Sepharose-bound protein was eluted into nonreducing SDS sample buffer by incubation for 10 min at 100°C. The Sepharose beads were pelleted and the supernatants ("immunoprecipitates") were subjected to SDS-PAGE (7% wt/vol) slab. After electrophoretic transfer of proteins from the gel onto nitrocellulose sheets, C1-Inh species on the blots were visualized by incubation with MAb RII purified by affinity chromatography on protein A–Sepharose and radiolabeled with ^{125}I by Iodogen method, followed by autoradiography. Details of the procedures were as described previously (29). The apparent molecular mass of proteins was estimated by comparison with the high-molecular weight protein markers of Bio-Rad Laboratories (Richmond, CA).

Statistics

The results were expressed as median and range; the Kruskal Wallis analysis of variance was performed and the significance of the differences between groups was assessed by the nonparametric test of Wilcoxon-Mann-Whitney. A *P*-value of < 0.05 was considered to indicate a statistically significant difference.

Results

Median values and ranges of the measurements in patients and healthy controls are presented in Table I. The results showed a significant correlation between RID and RIA for C1-Inh antigen ($n = 48$, $r = 0.92$, $x/y = 0.96$) and between chromogenic assay and RIA for functional C1-Inh ($n = 48$, $r = 0.98$, $x/y = 0.99$).

Patients with type-I HANE. Plasma levels of antigenic and functional C1-Inh and of C4 antigen were significantly ($P < 0.0001$) reduced as compared with those in healthy volunteers. Prekallikrein antigen was slightly decreased in patients compared with healthy controls, but the difference did not reach statistical significance. C1–C1-Inh complexes in plasma were significantly increased compared with the controls ($P < 0.0001$); levels of these complexes inversely correlated with functional ($r = -0.78$, $P < 0.001$; Fig. 1) and antigenic ($r = 0.66$, $P < 0.001$) C1-Inh. Plasma levels of Factor XIIa–C1-Inh, kallikrein–C1-Inh, and Factor XII in the patients were not different from those in healthy volunteers.

Patients with type-II HANE. As for type-I patients, C1–C1-Inh complexes were significantly increased ($P < 0.0001$) compared with the controls, but neither correlated with functional C1-Inh nor with C1-Inh antigen. A significant increase in plasma levels of iC1-Inh was found ($P < 0.005$). When data were assembled for families (Table II), two groups could clearly be identified: group 1 (family a and b) with C1-Inh antigenic levels (determined by RID) that ranged between 51 and 67% of normal, and group 2 (family c, d, and e) with C1-Inh levels exceeding 100%. The two groups markedly differed in iC1-Inh levels which were normal in group 2 and higher than 20 times the normal value in group 1. Other measurements were similar in both groups. In group 1, levels of antigenic C1-Inh obtained with the RIA appeared slightly higher compared with those obtained by RID.

SDS-PAGE analysis of dysfunctional C1-Inh proteins. Immunoprecipitates of C1-Inh species from plasma of patients with type-II HANE were subjected to SDS-PAGE and immunoblot analysis (Fig. 2). Immunoblots of antigenic C1-Inh (RII-immunoprecipitate) from pooled normal plasma showed a major protein band of M_r 110,000, whereas those from plasma of all patients from group 1 revealed pronounced protein bands of M_r 110,000 and 98,000. Immunoblots of iC1-Inh (KII-immunoprecipitates) from pooled normal plasma revealed only a faint protein band of M_r 98,000. In contrast, pronounced bands of M_r 98,000 were observed on immunoblots of iC1-Inh from patients' plasma samples of group 1. Immunoblot analysis of antigenic C1-Inh from plasma of patients from family c and d (group 2) revealed a major protein band of M_r 110,000 together with a band of M_r 180,000, whereas no protein bands were observed on immunoblots of iC1-Inh from these plasma samples. The results of RIAs that

Table I. Complement and Contact System Parameters in Healthy Subjects and HANE Patients in Basal Condition

	Healthy subjects median (range) $n = 18$	Type-I HANE median (range) $n = 20$	Type-II HANE median (range) $n = 10$
Functional $C\bar{1}$-Inh (chromogenic) %	94 (69–119)	16* (0–37)	15* (4–30)
$C\bar{1}$-Inh antigen (RIA) μM	2.19 (1.30–3.45)	0.49* (0.08–0.73)	2.94** (2.35–6.55)
$iC\bar{1}$-Inh (RIA) μM	0.08 (0.06–0.13)	0.09 (0.03–0.13)	1.04** (0.05–3.77)
C4 antigen (RID) %	99 (67–130)	19* (0–67)	21* (9–55)
$C\bar{1}$–$C\bar{1}$-Inh (RIA) μM	0.007 (0.001–0.011)	0.013* (0.003–0.028)	0.017* (0.008–0.028)
Kallikrein–$C\bar{1}$-Inh (RIA) μM	0.0002 (<0.0002–0.0005)	<0.0002 (<0.0002)	<0.0002 (<0.0002–0.0004)
Factor XIIa–$C\bar{1}$-Inh (RIA) μM	<0.0002	<0.0002	<0.0002
Prekallikrein (RIA) μM	0.50 (0.35–0.627)	0.43 (0.31–0.62)	0.49 (0.34–0.62)
Factor XII (RIA) μM	0.49 (0.18–0.81)	0.48 (0.21–0.70)	0.49 (0.36–0.68)

% = percentage of normal human pooled plasma. Statistical significance vs. normals: *$P < 0.0001$, **$P < 0.005$.

specifically detect dimers of albumin and $C\bar{1}$-Inh indicated that plasma of patients from family c and d contained high levels of these dimers (unpublished observation). The protein band of M_r 180,000 observed on the blots is compatible herewith.

Discussion

In this study we demonstrated elevated plasma levels of $C\bar{1}$–$C\bar{1}$-Inh complexes in asymptomatic patients with an inherited deficiency of $C\bar{1}$-Inh. In patients with type-I HANE the levels of these complexes significantly correlated with antigenic and functional $C\bar{1}$-Inh ($P < 0.001$). These observations suggest that C1 activation is at least in part responsible for the increased catabolism of $C\bar{1}$-Inh found in asymptomatic patients with HANE (9), as proposed by Curd et al. (30), and thus for the apparent discrepancy between expected and actual levels of functional $C\bar{1}$-Inh in this heterozygous disorder (10). A previous study, using an SDS-PAGE and immunoblotting technique, reported increased levels of $C\bar{1}$–$C\bar{1}$-Inh complexes in type-I HANE patients, but no statistical correlation with $C\bar{1}$-Inh levels was performed (13). Our double antibody assay allows a direct quantification of these complexes and provides specificity by the use of a MAb that binds complexed $C\bar{1}$-Inh. In vitro generation of complexes is avoided by drawing blood samples into EDTA and polybrene, a procedure that prevents activation of the contact system and the complement system via the classical pathway (20, 24, 27, 31).

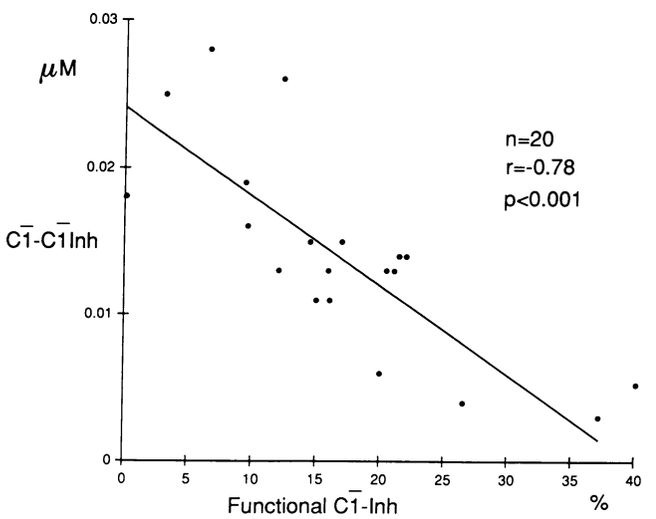

Figure 1. Correlation between plasma levels of functional $C\bar{1}$-Inh, determined by chromogenic assay, and $C\bar{1}$–$C\bar{1}$-Inh complexes in 20 type-I HANE patients.

To our knowledge, no data on the clearance of $C\bar{1}$-Inh complexes in humans have been published. Recently, we estimated an apparent $t_{1/2}$ time of clearance of ~ 50 min, for both Factor XIIa–$C\bar{1}$-Inh and kallikrein–$C\bar{1}$-Inh complexes, from sequential measurements of these complexes in patients with sepsis (20) and we assume that $C\bar{1}$–$C\bar{1}$-Inh complexes also have a similar $t_{1/2}$. Inasmuch as the actual $t_{1/2}$ may be much shorter and may vary among different patients' groups, the contribution of C1 activation to the catabolism of $C\bar{1}$-Inh in HANE patients is difficult to assess. Quastel et al. have shown that the catabolism of $C\bar{1}$-Inh in HANE is increased by ~ 1% of the plasma pool per hour compared with healthy individuals: the fractional catabolic rate was 3.5% of plasma pool per hour in HANE patients, vs. 2.5% in healthy controls (9). Based on the median levels of $C\bar{1}$–$C\bar{1}$-Inh and of $C\bar{1}$-Inh antigen as measured by RIA (Tables I and II), and considering that the actual composition of $C\bar{1}$–$C\bar{1}$-Inh is $C\bar{1}rC\bar{1}s(C\bar{1}\text{-Inh})_2$, the relative amount of $C\bar{1}$-Inh complexed to $C\bar{1}$ was 0.6% in healthy subjects and 5.3% in type-I HANE patients. These data suggest

Table II. Complement Parameters in Type II HANE Patients

Name	Family	Functional $C\bar{1}$-Inh (chromogenic)	$C\bar{1}$-Inh antigen (RID)	$C\bar{1}$-Inh antigen (RIA)	$iC\bar{1}$-Inh (RIA)	$C\bar{1}$–$C\bar{1}$-Inh (RIA)
		%	%	%	μM	μM
Group 1						
A.C.	a	16	51	96	2.23	0.014
M.C.	a	17	67	108	3.77	0.025
P.M.	b	14	56	94	1.89	0.012
I.C.	b	27	63	121	3.01	0.028
L.C.	b	13	52	99	2.25	0.026
median		16	56*	99**	2.25*	0.025
(range)		(13–27)	(51–67)	(94–121)	(1.89–3.77)	(0.012–0.028)
Group 2						
P.P.	c	30	314	262	0.05	0.011
D.C.	d	14	242	226	0.19	0.020
S.T.	d	4	242	125	0.10	0.008
R.B.	e	6	117	114	0.09	0.020
M.R.B.	e	26	194	160	0.07	0.012
median		14	242	160	0.09	0.012
(range)		(4–30)	(117–314)	(114–262)	(0.05–0.19)	(0.008–0.020)

Group 1 (family a and b) includes patients with elevated concentrations of $iC\bar{1}$-Inh and with $C\bar{1}$-Inh antigen, measured by RID, below 100%. Group 2 (family c, d, and e) includes patients with normal concentrations of $iC\bar{1}$-Inh and with $C\bar{1}$-Inh antigen, measured by RID, above 100% of normal pooled plasma. Patients are designated with their name initials. Percentage values are obtained referring to a curve of serial dilutions of plasma pooled from 30 healthy individuals. Micromolar values are obtained as described in Methods. Median value for $C\bar{1}$-Inh antigen with RID in normals is 98% (range 63–137%), median values for other parameters are reported in Table I. Statistical significance vs. group 2: $*P = 0.0079$; $**P = 0.0159$.

that the increase in $C\bar{1}$-Inh catabolism in type-I HANE is at least in part due to complex formation with $C\bar{1}$.

In this study, we did not find an increase of Factor XIIa–$C\bar{1}$-Inh or kallikrein–$C\bar{1}$-Inh complexes despite the high sensitivity of the assays that can detect 0.05% activation of plasma Factor XII and prekallikrein (20). These results suggest that the extent of contact activation in HANE patients in basal condition is not enough to overcome the clearance of complexes from the circulation. A new approach, such as with recently developed methods (19), may prove useful in defining the role of contact activation envisaged in HANE patients outside acute swellings (13, 14).

Patients with type-II HANE could be divided into two groups based on plasma levels of $C\bar{1}$-Inh antigen and $iC\bar{1}$-Inh. In group 1, antigenic $C\bar{1}$-Inh, as measured by RID, ranges between 51 and 67% of normal and the concentration of $iC\bar{1}$-

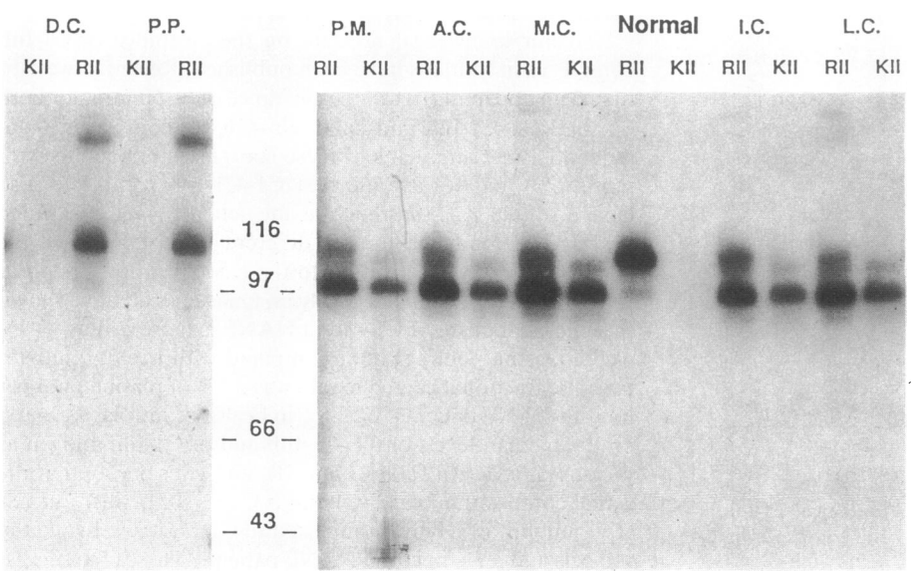

Figure 2. Immunoblotting of $C\bar{1}$-Inh in pooled normal plasma and in patients with type-II HANE, group 1 (*right*) and group 2 (*left*), identified by their initials. Total $C\bar{1}$-Inh antigen and $iC\bar{1}$-Inh were immunoprecipitated from plasma by Sepharose-coupled MAb to total $C\bar{1}$-Inh (RII) and to $iC\bar{1}$-Inh (KII), respectively, and separated on nonreduced 7% SDS-PAGE. After electrotransfer to nitrocellulose and immunoblotting by ^{125}I-labeled MAb RII, bands were visualized by autoradiography.

Inh is at least 20 times higher than in pooled normal plasma (2,360–4,718%). The measurement of C1̄-Inh antigen by RIA in this group resulted in significantly higher levels than those found by RID. This difference may be ascribed to the presence of high levels of iC1̄-Inh, which is recognized by the monoclonal antibody used in the RIA, but poorly detected by the polyclonal antibody used in RID, as demonstrated by immunoblotting experiments (data not shown). In group 2, C1̄-Inh antigen exceeded 100% (117–314%) and iC1̄-Inh is normal or only slightly increased (66–238%). The immunoblotting experiments revealed that C1̄-Inh in group 1 was, for the major part, degraded into a form with M_r 98,000, whereas no such bands were observed on blots of C1̄-Inh from plasma of patients from group 2. We think that these differences reflect two different molecular mechanisms that may underlie type-II HANE. In group 1 (relatively low antigenic C1̄-Inh and high iC1̄-Inh levels) the mutation in the C1̄-Inh gene probably yields a C1̄-Inh protein which still can form complexes with target proteases such as C1̄s, but these complexes are not stable and are rapidly hydrolyzed into an active protease and iC1̄-Inh. The mutations that underlie this form of HANE thus render C1̄-Inh a true substrate for target proteases. Studies performed with dysfunctional molecules purified from patients with type-II HANE, suggest that some of the dysfunctional molecules indeed behave like substrates for target proteases (5, 32). For example, four of the eight dysfunctional C1̄-Inh molecules studied by Donaldson et al. (32) were cleaved by kallikrein, and did not form stable complexes with this target protease. We have recently prepared a recombinant C1̄-Inh protein with an arginine at position P3. This mutant protein does not form stable complexes with kallikrein, but instead is rapidly degraded by this protease (Eldering, E., C. C. M. Huijbregts, J. H. Nuijens, and C. E. Hack, manuscript in preparation). In group 2, the mutant C1̄-Inh protein probably does not interact with target proteases at all and accounts for higher levels of C1̄-Inh antigen due to a slower catabolism. The type-II group-2 patients in whom we detected high plasma levels of dimers between albumin and C1̄-Inh are very likely to have a substitution of P1 arginine by cysteine (33). The differences in type-II Hane patients of groups 1 and 2, as well as the variability in C1̄-Inh catabolism revealed by in vivo turnover studies (9), may thus reflect different mutations at the reactive center P1 (33–35) or other functionally important residues. In type-II patients, C1̄–C1̄-Inh complexes did not significantly correlate with the levels of C1̄-Inh function. These complexes were higher in group 1 than in group 2 (Table II), although this difference was not statistically significant probably because of the small number of cases. It is possible that part of the complexes measured in group-1 patients is represented by the unstable complexes formed by the dysfunctional protein as proposed above.

Zuraw and Curd (13) studied nine type-I and 2 type-II HANE patients, using a monoclonal antibody that recognizes both native and cleaved C1̄-Inh. In that study, the ratio between inactive and total C1̄-Inh was increased in type-I patients and was normal in type II. Our present data cannot be directly compared because we used a different method (RIA vs. immunoblotting) and a different monoclonal antibody that does not react with the native C1̄-Inh (25). However, our results in type-I patients, as well as those from Zuraw and Curd, demonstrate that relative amount of modified inactive C1̄-Inh is increased compared with normal when referred to total content of C1̄-Inh, which is markedly reduced in these patients. Therefore, the increase in iC1̄-Inh, which results from the interaction of C1̄-Inh with as yet unknown proteases (25), is consistent with the hypercatabolism of C1̄-Inh, but cannot discriminate the catabolic pathway. For type II, it appears that both patients studied by Zuraw and Curd (13) belong to those that we called group 2 and we agree with their suggestion that the amount of catabolic product is dependent on the ability of the mutant C1̄-Inh to interact with proteases.

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