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Research Article

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# Molecular Basis for the Deficiency of Complement 1 Inhibitor in Type I Hereditary Angioneurotic Edema

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## Abstract

**Hereditary angioneurotic edema (HANE) results from deficiency of complement 1 inhibitor (C1 INH). In type I HANE, C1 INH is present in serum at levels 5–30% of normals. Using cultured monocytes and biosynthetic labeling of proteins, C1 INH was detected in supernatants of cells from HANE patients at levels 20% of those detected in normals. The intracellular reduction of C1 INH in patients' monocytes approached 50%. The study of C1 INH messenger RNA (mRNA) by Northern blot analysis indicated that in HANE patients' monocytes a message of normal size is present at about half the concentration of that from normal cells. One of the patients analyzed showed the presence of a genetically inherited abnormal mRNA (1.9 kb) in addition to the normal mRNA (2.1 kb). Southern blot analysis of DNA from peripheral blood leukocytes did not show any difference in quantity or in sizes of endonuclease restriction fragments between patients and normals. The defect(s), therefore, in type I HANE is pretranslational, but is not due to a deletion or to a major chromosomal rearrangement.**

## Introduction

Hereditary angioneurotic edema (HANE)[1] results from the deficiency of complement 1 esterase inhibitor (C1 INH) (1, 2) and is the most common human disease related to a genetic defect of a complement component. The disease is inherited as an autosomal dominant trait and it is clinically characterized by recurrent attacks of angioedema, which can be life threatening when the larynx is involved (3). Two genetic types of HANE have been described: one, comprising 85% of patients, is characterized by decreased levels of an apparently normal C1 INH protein (Type I); the other by normal or elevated antigenic levels of C1 INH because of the presence of a dysfunctional mutant protein together with low levels of the normal protein (type II) (4, 5). The mutant proteins have been shown to be electrophoretically and functionally heterogeneous (4, 6). Functional levels of C1 INH in serum of type I HANE patients range from 5 to 30% of normal in contrast with the 50% that would be expected in the heterozygous state. From in vivo turnover studies (7) it has been proposed that at half-normal concentration of C1 INH there is activation of C1 and/or other enzyme systems in which this protein acts as an inhibitor. This in turn could lead to consumption of normal C1 INH that falls below 50% of normal (7, 8).

The hepatocyte is the major site of synthesis of C1 INH (9); recent evidence has been presented that this protein is also synthesized and secreted by cultured human peripheral blood monocytes (10). In the present study, also using human monocytes in culture, we examined the molecular basis for the defect in synthesis of C1 INH in type I HANE patients.

## Methods

*Media and reagents.* Hanks' balanced salt solution, Dulbecco's modified Eagles medium lacking methionine, and fetal bovine serum were purchased from Gibco (Grand Island, NY), and medium 199 from M. A. Bioproducts (Walkersville, MD). [$^{35}$S]Methionine (specific radioactivity 1,000 Ci/mmol), [$^{32}$P]Deoxycytidine triphosphate (specific radioactivity 3,000 Ci/mmol), and EN$^3$HANCE solution were obtained from New England Nuclear (Boston, MA). $^{14}$C-methylated protein standards were from Amersham Corp. (Arlington heights, IL). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) reagents were purchased from Bio-Rad Laboratories (Rockville Centre, NY), and IgG Sorb from Enzyme Center (Cambridge, MA). Goat antihuman C1 INH and antihuman C3 were from Atlantic Antibodies (Westbrook, ME), and restriction endonucleases were from New England Biolabs (Beverly, MA). Nick translation kits were obtained from Bethesda Research Laboratories (Gaithersburg, MD).

*Patients.* We studied six patients from four kindred with type I HANE. Synthesis studies were performed on patients from three of the four kindred, and in the protein synthesis studies and the Northern blot analysis one representative of each kindred is presented. With the two kindred in which two members were studied, one individual is presented from each kindred, since the results in each member of the same kindred were virtually identical. In the Southern blot analysis, one member of each kindred is presented, although the other members were also studied, with identical results. All patients had taken no medications, including attenuated androgens (11), for at least 1 wk before the time blood was drawn for monocyte cultures.

*Monocyte cultures.* Monocyte monolayers were established by adherence of dextran purified leukocytes (5–10 × $10^6$ cells/ml) on siliconized glass coverslips as previously described (12). For RNA extraction, cell suspensions were adhered to 100-mm siliconized glass petri dishes rather than coverslips.

*Biosynthetic labeling.* Confluent monolayers grown for different time periods in medium 199 containing 10% fetal bovine serum were rinsed and incubated for specific time periods in methionine-free Dulbecco's modified Eagles medium containing [$^{35}$S]methionine (500 $\mu$Ci/ml). At the end of the pulse period, the culture medium was harvested and monolayers were lysed as previously described (13), or in pulse-chase experiments was transferred for varying time periods to medium 199 containing unlabeled methionine. At the end of each chase period, media were harvested and the monolayers lysed. Total protein synthesis was

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1. *Abbreviations used in this paper:* C1 INH, complement 1 esterase inhibitor; HANE, hereditary antioneurotic edema.

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estimated by trichloroacetic acid (TCA) precipitation of aliquots of cell lysates and culture fluids (14). Incorporation of $^{35}S$ into individual immunoprecipitated proteins was determined in gel slices after incubation with 15% hydrogen peroxide for 16 h and addition of Scinti Verse I (Fisher Scientific Co., Springfield, NJ). For each patient a normal control was cultured at the same time; the TCA-estimated total protein synthesis did not vary by > 20% between patient and control.

*Immunoprecipitation.* Immunoprecipitation from cell lysates and cell supernatants was performed by binding of the immune complex to Staphylococcal protein A after overnight incubation with antibody (13). Samples were then analyzed by SDS-PAGE (7.5% polyacryalmide) (15). Radioactive $^{14}C$-labeled standards were included in each gel. After electrophoresis the gel was stained with Coomassie Brilliant Blue, impregnated with EN$^3$HANCE, dried, and exposed to Kodak XAR-5 film at −70°C. Autoradiographs were scanned by soft laser densitometry.

*RNA isolation and blot analysis.* Total cellular RNA was isolated from adherent monocyte monolayers by lysis with guanidinium thiocyanate and centrifugation through cesium chloride density gradients (16). RNA was quantified by absorbance at 260 nm. The yield of total RNA from normal subjects and from patients was the same (5–10 $\mu g/10^6$ cells). Total cellular RNA denatured with formaldehyde and formamide was separated by electrophoresis in formaldehyde-containing agarose gels. Samples were then transferred to nitrocellulose filters and hybridized with a C1 INH cDNA probe $^{32}P$-labeled by nick translation (17).

*DNA isolation and blot analysis.* DNA was prepared from peripheral blood leukocytes as previously described (18). DNA was digested with restriction enzymes and subjected to agarose gel electrophoresis, transferred to nitrocellulose filters, and hybridized with the $^{32}P$-labeled C1 INH cDNA probe (19) (Southern blot analysis). Isolation and characterization of the 950-base pair C1 INH complementary DNA (cDNA) clone (pC1 INH 1) has been described (20). The probe used for both RNA and DNA analysis was the 600-base pair Pst I fragment of pC1 INH 1 isolated as described (20).

## Results

*Synthesis and secretion of C1 INH.* Pulse experiments were performed with peripheral blood monocytes, and cultured for 1 wk from both normal controls and HANE patients. C1 INH appeared in the cell lysates as a single band with a molecular weight of 80,000, which probably represents an underglycosylated form of the protein, since it is the same size as purified C1 INH deglycosylated with trifluoromethanesulfonic acid (21) (Fig. 1). The intracellular C1 INH levels in the three patients were 43% (patient 1), 53% (patient 2), and 35% (patient 3) of normal controls cultured at the same time, as determined by soft laser densitometry of autoradiographs or by quantitation of $^{35}S$-incorporation in gel slices. The sample from patient 1 is shown in Fig. 1, lane *2*, and a normal's sample is shown in Fig. 1, lane *1*. In comparison with that from normal monocytes, intracellular C1 INH from the monocyte lysates from each of the other type I HANE patients appeared virtually identical to the one shown in Fig. 1, lane *2*. When the protein secreted into the supernatants was analyzed, the difference between normals and patients increased. An immunoprecipitable protein with a molecular weight of 105,000, which is identical in size to fully glycosylated C1 INH, is secreted by cultured monocytes from both normal individuals and from patients. However, the quantity of this secreted C1 INH from patients' monocytes is 24% (patient 1; Fig. 1, lane *7*), 18% (patient 2; Fig. 1, lane *5*), and 14% (patient 3; Fig. 1, lane *4*) that of normal individuals, as estimated both by soft laser densitometry and by measurement of specific $^{35}S$-incorporation in gel slices. For comparison, the patients' C1 INH levels, determined immunochemically, were 23, 25, and 29% of normal for patients 1, 2, and 3, respectively.

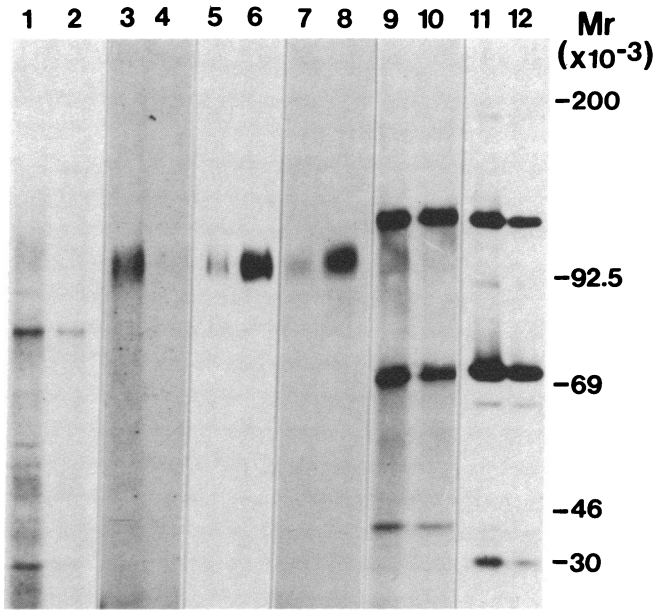



*Figure 1.* Biosynthesis of C1 INH (lanes *1–8*) and C3 (lanes *9–12*) by monocytes from normal individuals (lanes *1, 3, 6, 8, 10,* and *11*) and HANE patients (lanes *2, 4, 5, 7, 9,* and *12*). Cells were in culture for 1 wk or 2 d (lanes *7* and *8*). Lanes *1* and *2* represent immunoprecipitates from cell lysates after a 6-h pulse period. Lanes *3–12* represent immunoprecipitates from cell supernatants pulsed for 24 h. The nonspecific bands seen particularly in cell lysates appear inconstantly and are not blocked by purified C1 INH.

Both intracellular and extracellular C1 INH bands were proven to be specific by their disappearance when antiserum against C1 INH was absorbed with excess purified protein. The specificity of the reduction in C1 INH synthesis in HANE patients was assessed by precipitating C3 from the same samples. Comparable quantities of C3 were detected in normals and patients (Fig. 1, lanes *9–12*). To examine whether C1 INH synthesis and its reduction in HANE patients was also true of monocytes early in culture, cells were pulsed after the first day in culture with the same results as described above (Fig. 1, lanes *7* and *8*). To evaluate the rate of secretion of C1 INH, pulse-chase experiments were performed. With this approach it was possible to show that C1 INH was rapidly secreted from normal monocytes with < 50% detectable intracellularly after 90 min and only trace amounts after 360 min (Fig. 2). Fully glycosylated C1 INH was readily detectable in the extracellular medium by 90 min and continued to accumulate at 360 and 1400 min as it disappeared from the intracellular compartment. Pulse-chase experiments with cultured monocytes from HANE patients confirmed the reductions in both intracellular and secreted C1 INH. However, there were no differences apparent in the kinetics of synthesis or secretion compared with normals. Intracellular C1 INH disappeared and extracellular protein appeared at the same rates as in normal control cultures.

*Analysis of C1 INH mRNA and DNA.* Monocyte RNA and leukocyte DNA were isolated from patients and controls. Equal quantities of RNA from normal individuals and from patients were subjected to Northern blot analysis. The same size messenger RNA (mRNA), 2.1 kilobase (kb), specific for C1 INH,

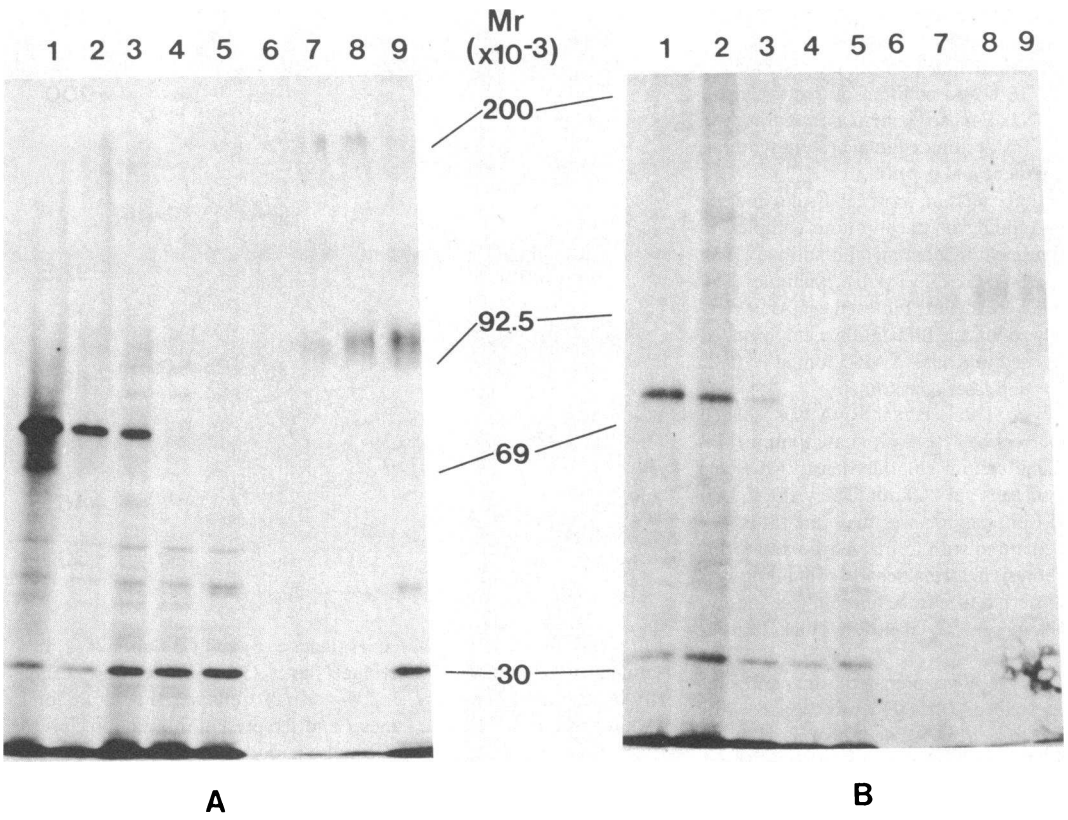


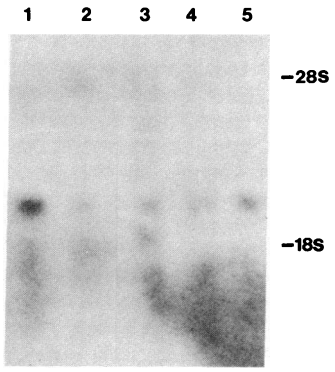

*Figure 2.* Kinetics of synthesis and secretion of C1 INH from (*A*) normal and (*B*) HANE patient monocytes in culture for 1 wk. Cells were pulsed for 1 h and chased at 0, 30, 90, 360, and 1400 min. Lanes *1–5* are cell lysates (0–1400 min), and lanes *6–9* are cell supernatants (30–1400 min).

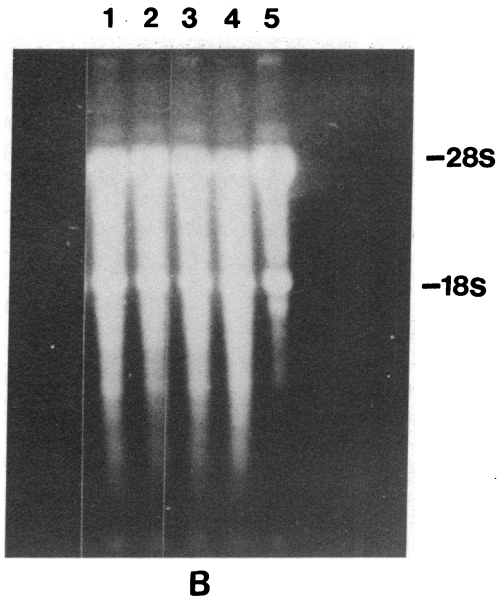
was found in both normals and HANE patients. However, as estimated by soft laser densitometry of the autoradiographs, the patients' C1 INH mRNA levels were reduced to 40% (patient 1; Fig. 3 *A*, lane *2*), 48% (patient 2; Fig. 3 *A*, lane *3*), and 45% (patient 3; Fig. 3 *A*, lane *4*). The same blot, hybridized with a probe for the third component of complement, showed mRNA bands of equal intensity in all the samples (data not shown). In one of the three patients examined (Fig. 3 *A*, lane *3*), a second mRNA band of 1.9 kb, hybridizing with the C1 INH cDNA probe, was detected. This band was of the same intensity as the 2.1-kb band. To avoid the possibility of an artifact and to check whether this abnormality was a genetic characteristic inherited with HANE, different RNA preparations from monocytes from this patient and from two of her siblings were analyzed. The double-band pattern of C1 INH mRNA was present again in the patient and in her brother, who also has HANE (Fig. 3 *C*). The other sibling, who does not have HANE, showed a normal C1 INH mRNA (not shown). Southern blot analysis of the C1 INH gene did not show any difference in the amount of DNA between normals and patients or in the size of fragments produced by digestion with nine different restriction endonucleases (Fig. 4).

## Discussion

In this study we have provided direct evidence that C1 INH deficiency in type I HANE patients is due to a reduction in synthesis of the protein, and that this reduction is related to a pretranslational defect. Monocytes from HANE patients were cultured for various lengths of time and showed a reduction in C1 INH in cell lysates that ranged from 35 to 53% of normal controls. C1 INH mRNA levels were consistent with these findings (see below). However, secreted C1 INH protein in cell supernatants was reduced to 14–24% of normal controls. These values are similar to the serum levels of C1 INH in HANE patients (23–29% of normal in the patients presented here). In most heterozygous deficiencies of plasma proteins, including other complement components, levels are reduced to ~ 50% of normal.

To rule out the possibility that the discrepancy between intracellular and extracellular levels of C1 INH could be due to a co-existent secretory defect, pulse-chase experiments were performed. These experiments demonstrated that the kinetics of secretion were the same in normal and patient monocytes. In each, > 50% of the C1 INH was secreted within 90 min and only trace amounts of the protein were detected intracellularly after 360 min. There was no accumulation of intracellular C1 INH in cultured monocytes from patients. It is therefore unlikely that the reduction in extracellular C1 INH levels compared with intracellular is due to a defect in the kinetics of secretion. The data suggest that C1 INH in patient monocyte cultures is consumed during or after secretion. The data also are compatible with the previous suggestion (7, 8) that protease activation in the presence of half-normal C1 INH concentrations leads to this consumption of C1 INH, which then is decreased further to below 50% of normal. The present study, however, does not define the mechanism by which this happens. Activation of C1 is probably not the main factor, since the phenomenon is also present when cells are analyzed early in culture. At this time, according to previous data (22), active macromolecular C1 is not produced by monocytes. However, it is still possible that other proteases, physiologically inhibited by C1 INH, are acti-



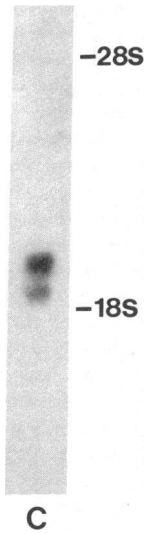




*Figure 3.* Monocyte RNA from normals (lanes *1* and *5*) and HANE patients (lanes *2–4*). *A* is the autoradiograph of the Northern blot; lane *3* shows the patient with two different sizes of C1 INH mRNA. *B* is the ethidium bromide–stained gel from which the Northern blot was generated, showing that comparable amounts of total RNA were loaded in each lane. *C* is the autoradiograph of the Northern blot from the sibling with HANE of the patient shown in *A*, lane *3*. 18S and 28S indicate the positions of 18S and 28S ribosomal RNA.

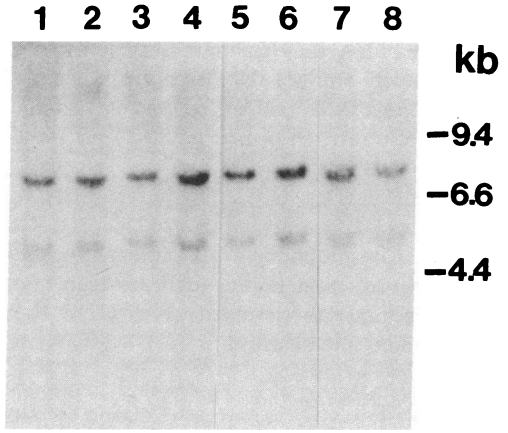



*Figure 4.* Southern blot analysis of leukocyte DNA from normals (lanes *1–4*) and HANE patients (lanes *5–8*). DNA was digested with Bam H1 and hybridized with the C1 INH cDNA probe.

vated in cell supernatants deficient in this protein, resulting in its further reduction.

In previous studies of C1 INH synthesis from normal human monocytes its production was detected after a minimum of 1 wk in culture (10) when the cells became macrophage-like (12), or after stimulation with lymphokines (22). Our data indicate that C1 INH is synthesized from the 1st day in culture (2nd day after drawing blood from the donor). C1 INH mRNA was also detected in freshly isolated mononuclear cells prepared by centrifugation through Ficoll-Hypaque (unpublished observation). This suggests that the production of this protein is a physiologic property of circulating monocytes. This synthesis from monocytes early in culture is also characteristic of alpha-1-antitrypsin, but is not common for complement components (23). C1 INH, as well as alpha-1-antitrypsin, has been demonstrated to belong to the serpin "super family" of protease inhibitors (20). These data may suggest a physiologic role for circulating monocytes in local regulation and prevention of inflammatory phenomena.

The recent isolation of a C1 INH cDNA clone (20) allowed us to investigate the molecular basis for C1 INH deficiency in HANE. Total cellular RNA was extracted from normal human monocytes in culture and, after Northern blot analysis, a C1 INH mRNA of 2.1 kb was detected. This size is consistent with that found for RNA isolated from the human hepatoma-derived cell line HepG2 (20), and with the molecular weight of deglycosylated C1 INH. In agreement with the amount of C1 INH protein in the intracellular compartment, C1 INH mRNA in HANE patients was found to be present at about half the level of normal controls, indicating the existence of a pretranslational defect. An analogous defect has been found in humans in the case of the deficiency of the second component of complement (24), while gene deletion is responsible for deficiency of C4 and 21-hydroxylase (25, 26). The possibility of a major deletion or rearrangement in the C1 INH gene was excluded by the absence of gross differences when genomic DNA from normals and HANE patients was studied by restriction endonuclease digestion and Southern blot analysis.

In two related patients with type I HANE a C1 INH mRNA that was 0.2 kb smaller than the normal mRNA was detected. This band co-existed with the normal message and each made up ~ 50% of the amount of C1 INH mRNA in normals. The

data suggest that this characteristic is genetically determined and is related to the presence of HANE. No abnormal protein could be immunoprecipitated with anti-C1 INH from the cell supernatants or the cell lysates of this patient, suggesting that the abnormal message is not translated or that it is translated into a protein that is rapidly destroyed in the cytoplasm. In conclusion, we suggest that the deficiency of C1 INH in type I HANE is due to a pretranslational defect leading to a reduction in C1 INH synthesis of 50%. Consumption of the protein probably takes place during or after secretion, leading to further reduction. This consumption determines the levels at which C1 INH is detected in cell supernatants and in serum from HANE patients.

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