

# Proteolytic Inactivation of Plasma C $\bar{1}$ Inhibitor in Sepsis

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## Abstract

Activation of both the complement system and the contact system of intrinsic coagulation is implicated in the pathophysiology of sepsis. Because C $\bar{1}$  inhibitor (C $\bar{1}$ -Inh) regulates the activation of both cascade systems, we studied the characteristics of plasma C $\bar{1}$ -Inh in 48 patients with severe sepsis on admission to the Intensive Care Unit at the Free University of Amsterdam. The ratio between the level of functional and antigenic C $\bar{1}$ -Inh (functional index) was significantly reduced in the patients with sepsis compared with healthy volunteers ( $P = 0.004$ ). The assessment of modified (cleaved), inactive C $\bar{1}$ -Inh (iC $\bar{1}$ -Inh), and complexed forms of C $\bar{1}$ -Inh (nonfunctional C $\bar{1}$ -Inh species) revealed that the reduced functional index was mainly due to the presence of iC $\bar{1}$ -Inh. On SDS-PAGE, iC $\bar{1}$ -Inh species migrated with a lower apparent molecular weight ( $M_r$ , 98,000, 91,000, and 86,000) than native C $\bar{1}$ -Inh ( $M_r$ , 110,000). Elevated iC $\bar{1}$ -Inh levels ( $\geq 0.13 \mu\text{M}$ ) were found in 81% of all patients, sometimes up to  $1.6 \mu\text{M}$ . Levels of iC $\bar{1}$ -Inh on admission appeared to be of prognostic value: iC $\bar{1}$ -Inh was higher in 27 patients who died than in 21 patients who survived ( $P = 0.003$ ). The mortality in 15 patients with iC $\bar{1}$ -Inh levels up to  $0.2 \mu\text{M}$  was 27%, but in 12 patients with plasma iC $\bar{1}$ -Inh exceeding  $0.44 \mu\text{M}$ , the mortality was 83%. The overall mortality in the patients with sepsis was 56%. We propose that the cleavage of C $\bar{1}$ -Inh in patients with sepsis reflects processes that play a major role in the development of fatal complications during sepsis.

## Introduction

Sepsis is a life-threatening condition caused by the presence of microorganisms or their products in the circulation (1). Cell wall components of Gram-negative bacteria (e.g., endotoxin) and Gram-positive bacteria (e.g. peptidoglycan) can activate both the complement system and the contact system of intrinsic coagulation (2, 3). Considerable evidence has accumulated that excessive activation of these cascade systems, with release of biologically active peptides (anaphylatoxins and bradykinin), plays a role in the pathophysiology of sepsis, notably

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when complicated by shock and/or adult respiratory distress syndrome (4-12).

Activation of the complement system (via the classical pathway) and the contact system is regulated by C $\bar{1}$  inhibitor (C $\bar{1}$ -Inh),<sup>1</sup> a plasma protein that belongs to the superfamily of serine proteinase inhibitors, the serpins (13-17). C $\bar{1}$ -Inh is the only known inhibitor in plasma of activated C1r and C1s, components of the classical pathway of complement (18), and the major inhibitor of activated Factor XII (Hageman factor) and kallikrein of the contact pathway of coagulation (19-22). Inhibition of these so-called target proteinases by functional C $\bar{1}$ -Inh comprises both proteolytic cleavage of a substrate-like peptide bond in the reactive center of the inhibitor and covalent linkage of the proteinase to the inhibitor: formation of bimolecular proteinase-C $\bar{1}$ -Inh complexes (18, 20, 22-26).

Several studies have shown that the formation of high-molecular weight target proteinase-C $\bar{1}$ -Inh complexes is accompanied by the generation of modified (cleaved), inactive C $\bar{1}$ -Inh (iC $\bar{1}$ -Inh) species with a lower apparent molecular weight than native C $\bar{1}$ -Inh (23, 24, 27-29). In addition, a number of endogenous (e.g., neutrophil elastase) and bacterial nontarget proteinases (i.e., proteinases that are not inhibited by C $\bar{1}$ -Inh) generate iC $\bar{1}$ -Inh species by catalytical cleavage of the inhibitor in its reactive center (30, 31). C $\bar{1}$ -Inh antigen in plasma may thus exist in three forms: functional C $\bar{1}$ -Inh, C $\bar{1}$ -Inh complexed to a proteinase, and iC $\bar{1}$ -Inh.

Many investigators have studied the role of C $\bar{1}$ -Inh in the pathophysiology of sepsis (10, 32-37). Plasma levels of functional C $\bar{1}$ -Inh, measured as the capacity of plasma to inactivate kallikrein, and of C $\bar{1}$ -Inh antigen are increased in patients with uncomplicated sepsis (34, 35), whereas reductions in functional C $\bar{1}$ -Inh and normal or increased concentrations of C $\bar{1}$ -Inh antigen are found in patients with sepsis complicated by shock and/or adult respiratory distress syndrome, as well as in patients with typhoid fever (10, 32-37). The discrepancy between plasma levels of functional and antigenic C $\bar{1}$ -Inh in the latter conditions may indicate the presence of complexed and/or iC $\bar{1}$ -Inh. Colman et al. presented evidence that in patients with typhoid fever this discrepancy is due to prekallikrein activation, demonstrating kallikrein-C $\bar{1}$ -Inh complex formation (33). However, the quantification of plasma Factor XIIa-C $\bar{1}$ -Inh and kallikrein-C $\bar{1}$ -Inh complexes in patients with severe sepsis revealed that the levels of these complexes remained within the normal range in the majority of these patients, in particular in those with septic shock, despite marked reductions in Factor XII and prekallikrein antigen (38).

In this investigation, we have therefore focused on the detection of other inactive C $\bar{1}$ -Inh species in the patients with

1. Abbreviations used in this paper: C $\bar{1}$ -Inh, C $\bar{1}$  inhibitor; C $\bar{1}$ -C $\bar{1}$ -Inh, C $\bar{1}$ rC $\bar{1}$ s(C $\bar{1}$ -Inh); DXS, dextran sulfate; FI, functional index; iC $\bar{1}$ -Inh, modified (cleaved) inactive C $\bar{1}$ -Inh; ICU, Intensive Care Unit.

sepsis. We found that C $\bar{I}$ -Inh is proteolytically inactivated in these patients and moreover, that the extent of plasma C $\bar{I}$ -Inh proteolysis on admission is of prognostic value.

## Methods

**Patients.** 48 consecutive patients who were admitted to the Intensive Care Unit of the Free University of Amsterdam (ICU) with a clinical diagnosis of severe sepsis entered this study. The diagnosis of "clinical sepsis" was based on the presence of at least four of the following criteria: a suspected infectious focus; shaking chills, fever ( $> 38.5^{\circ}\text{C}$ ) or hypothermia ( $< 35.5^{\circ}\text{C}$ ); tachypnea ( $> 20$  breaths/min); tachycardia ( $> 100$  beats/min); leukocytosis ( $> 15,000$  white blood cells per  $\text{mm}^3$ ); or thrombocytopenia ( $\leq 100,000$  platelets per  $\text{mm}^3$ ). The diagnosis was made before the results of bacteriological studies were known. Septic shock was diagnosed when patients fulfilled the criteria for both sepsis and shock, i.e., a fall in systolic blood pressure of  $\geq 50$  mmHg, or a systolic blood pressure of  $\leq 90$  mmHg, in combination with either oliguria ( $< 20$  ml/h), elevated lactate level ( $> 1.6$  mmol/liter), or altered mentation.

A definite diagnosis of sepsis (definite sepsis) and septic shock was made only when blood and/or local cultures grew pathogenic bacteria. Repeated cultures from 11 patients yielded no pathogenic microorganisms. These patients, many of whom were treated with antibiotics before admission, have therefore been referred to as critically ill. Further clinical features of the patients with sepsis and bacteriologic data are described in studies published elsewhere that focused on the contact and complement system in these patients (38, 39). Blood samples that were obtained on admission were used for the present study.

**Patients without sepsis.** Blood samples were obtained from various patients' groups without sepsis as controls. (a) Critically ill patients without sepsis: (i) 12 patients were admitted to the ICU because of severe burns or multiple traumatic injuries. Blood samples were taken from these patients within 24–48 h after they were admitted. (ii) Four patients with advanced metastatic cancer were admitted to the ICU for monitoring and appropriate management of the anticipated hemodynamic complications of therapy with high doses of IL 2 (see reference 40). The therapeutic regimen consisted of a 12-d period: 2 5-d courses with daily IL 2 administration separated by a 2-d, therapy-free interval. The daily dose of IL 2 (up to  $12 \times 10^6$  U/m $^2$ ) was decided on the basis of clinical judgment, i.e., on the toxicity observed. The treatment with IL 2 induced in all four patients a clinical syndrome that resembled septic shock (41; Thijs, L. G., C. E. Hack, R. J. M. Strack van Schijndel, J. H. Nuijens, A. J. M. Eerenberg-Belmer, and J. Wagstaff, manuscript submitted for publication). Blood samples from these patients were obtained on admission, after the first course of IL 2 treatment (day 5), as well as after the second course of IL 2 administration (day 12). (b) Patients with coronary heart disease: blood samples were obtained from 15 patients with stable coronary heart disease who were admitted to the hospital for coronary-artery bypass grafting. For this study, venous samples obtained on admission and arterial samples obtained on the next day, before surgery, were analyzed.

**Blood collection.** Unless stated otherwise, blood from the various patients' groups was obtained through indwelling ulnar artery catheters (Venflon 17 G,  $1.4 \times 45$  mm; Viggo AB, Helsingborg, Sweden), which were continuously flushed with saline (3 ml/h) containing 2 U of heparin (thromboliquine; Organon Teknika B.V., Boxtel, The Netherlands) per ml. The first 2 ml of blood was discarded; the second 4 ml were used for other purposes. The subsequent 5 ml were used for this study. Blood was collected in siliconized tubes (Vacutainer; Becton Dickinson & Co., Plymouth, UK) to which 10 mM EDTA and 0.05% Polybrene (wt/vol) had been added to prevent in vitro activation of the contact and complement system (42, 43). Experiments performed exactly as described elsewhere to establish optimal conditions to sample blood for contact system studies (see reference 42), revealed that this anticoagulant mixture was also adequate to prevent in vitro cleavage of C $\bar{I}$ -Inh. The tubes were centrifuged at room temperature for 10 min at

1,300 g, and the plasma was divided into aliquots and stored in polystyrene tubes at  $-70^{\circ}\text{C}$  until the tests were performed. Levels of iC $\bar{I}$ -Inh did neither increase upon repeated thawing and freezing nor upon storage at  $-70^{\circ}\text{C}$  for at least 1 yr.

Blood samples from 31 healthy volunteers were obtained by clean venipuncture and processed as described above. Plasma from these healthy donors was stored both individually and in aliquots of pooled normal plasma (prepared by mixing equal volumes of plasma from all donors).

**Reagents.** Dextran sulfate (DXS) (500,000 mol wt), CNBr-activated Sepharose 4B and Con A-Sepharose were obtained from Pharmacia Fine Chemicals AB (Uppsala, Sweden), Tween-20 was obtained from J. T. Baker Chemical Co. (Phillipsburg, NJ), and benzamidine hydrochloride hydrate and hexadimethrine bromide (Polybrene) was purchased from Janssen Life Sciences Products (Beerse, Belgium). Soybean-trypsin inhibitor (SBTI) (type I-S) was obtained from Sigma Chemical Co. (St. Louis, MO).

Functional C $\bar{I}$ -Inh was purified as previously described (42). For determination of the concentration of C $\bar{I}$ -Inh in purified preparations an extinction coefficient of 3.6 at 280 nm was used (44). iC $\bar{I}$ -Inh was isolated from DXS plasma (38, 45; see below) by two-step affinity chromatography on MAb KII-Sepharose (see below) and Con A-Sepharose. iC $\bar{I}$ -Inh was eluted from KII-Sepharose with PBS (10 mM sodium phosphate, 140 mM NaCl) containing 3 M KSCN (pH 7.4), dialyzed against PBS, and applied to Con A-Sepharose. iC $\bar{I}$ -Inh was eluted from Con A-Sepharose with 2% (wt/vol) methyl- $\alpha$ -D-mannopyranoside in 50 mM Tris with 140 mM NaCl (pH 8.0), and dialyzed against PBS. The iC $\bar{I}$ -Inh concentration in purified iC $\bar{I}$ -Inh preparations was assessed with the RIA for antigenic C $\bar{I}$ -Inh (see below) by using purified functional C $\bar{I}$ -Inh as a reference. On nonreduced SDS-PAGE, purified functional C $\bar{I}$ -Inh appeared as a major protein band of  $M_r$  110,000 and a minor band of  $M_r$  98,000, and purified iC $\bar{I}$ -Inh as a major band of  $M_r$  98,000 and a minor band of  $M_r$  86,000.

Specific antibodies against C $\bar{I}$ -Inh, C $\bar{I}$ s, Factor XII, and prekallikrein were affinity purified from the respective rabbit antisera and labeled with  $^{125}\text{I}$  as previously described (38, 43); the resulting specific activities ranged from 0.1 to 0.4 mBq/ $\mu\text{g}$  of protein. Before use in RIAs,  $^{125}\text{I}$  antibodies were diluted (20 kBq, i.e., 50–200 ng of antibody/ml) in PBS containing 10 mM EDTA, and 0.1% Tween-20 (wt/vol), pH 7.4.

**RIA for functional C $\bar{I}$ -Inh.** This assay is based on the principle that functional C $\bar{I}$ -Inh will bind to C $\bar{I}$ s. Purified C $\bar{I}$ s (45) was coupled to CNBr-activated Sepharose 4B (20 mg of C $\bar{I}$ s to 1 g of Sepharose). The Sepharose beads were suspended (250  $\mu\text{g}/\text{ml}$ ) in PBS containing 10 mM EDTA, 0.1% Tween-20 (wt/vol), and Polybrene (0.05%, wt/vol), pH 7.4. This Sepharose suspension (0.3 ml containing 75  $\mu\text{g}$  of Sepharose, i.e., 1.5  $\mu\text{g}$  of C $\bar{I}$ s) was incubated for 5 h at room temperature by head-over-head rotation with samples (50  $\mu\text{l}$ ) to be tested in 2-ml polystyrene tubes. The Sepharose was then washed with saline (five times with 1.5 ml) and incubated for 16 h at room temperature with 50  $\mu\text{l}$  (1 kBq) of  $^{125}\text{I}$ -anti-C $\bar{I}$ -Inh antibodies together with 0.5 ml of PBS-Tween-20 (0.1%, wt/vol). Thereafter, the Sepharose was washed again with saline (four times with 1.5 ml) and bound radioactivity was measured. Levels of functional C $\bar{I}$ -Inh in plasma samples (tested at 1–250 dilutions) were expressed in micromolar, using pooled normal plasma as a standard (serial dilutions in PBS- 10 mM EDTA-Tween-20 [0.1%, wt/vol]-Polybrene [0.05%, wt/vol]; see reference 46 for standard curve). The concentration of functional and antigenic C $\bar{I}$ -Inh in pooled normal plasma was 275  $\mu\text{g}/\text{ml}$  (2.50  $\mu\text{M}$ ; an  $M_r$  of 110,000 for native plasma C $\bar{I}$ -Inh was used) as was determined by comparing levels of functional and antigenic C $\bar{I}$ -Inh in plasma with those of the purified functional C $\bar{I}$ -Inh preparation. The results obtained with the RIA for functional C $\bar{I}$ -Inh very significantly correlated with those obtained with esterolytic (47) and chromogenic (C $\bar{I}$ -Inh reagent kit; Immuno, Vienna, Austria) assays ( $r = 0.90$ ,  $P < 0.001$ ,  $n = 95$ ,  $X/Y = 1.043$ , and  $r = 0.97$ ,  $P < 0.001$ ,  $n = 60$ ,  $X/Y = 1.045$ , respectively).

**RIA for antigenic C $\bar{I}$ -Inh.** MAb RII, which binds equally well to functional C $\bar{I}$ -Inh, complexed C $\bar{I}$ -Inh, and iC $\bar{I}$ -Inh (45), was partially

purified from ascites fluid by 50% ammonium-sulfate precipitation, and coupled to Sepharose (20 mg of protein to 1 g of Sepharose). The Sepharose beads were suspended (2 mg per ml) in PBS containing 10 mM EDTA, 0.1% Tween-20 (wt/vol), 0.05% Polybrene (wt/vol), 10 mM benzamidine, 0.01% SBTI (wt/vol) and 0.02% NaN<sub>3</sub> (wt/vol), pH 7.4. Further procedures of the RIA for antigenic C $\bar{I}$ -Inh (with 0.3 ml of RII-Sepharose suspension) are as described for the RIA of functional C $\bar{I}$ -Inh. Levels of antigenic C $\bar{I}$ -Inh in plasma samples (tested at 1–250 dilutions) were expressed in micromolar by reference to a standard curve of pooled normal plasma (see reference 46) that contained 2.50  $\mu$ M of antigenic C $\bar{I}$ -Inh.

**RIA for iC $\bar{I}$ -Inh.** This assay is similar to that for antigenic C $\bar{I}$ -Inh. However, MAb KII, which specifically binds to iC $\bar{I}$ -Inh (45), was used instead of MAb RII. Levels of iC $\bar{I}$ -Inh in plasma samples (tested at 1–25 dilutions) were expressed in micromolar using pooled normal plasma as a standard (Fig. 1). The iC $\bar{I}$ -Inh concentration in pooled normal plasma was 0.08  $\mu$ M, as was assessed by reference to the purified iC $\bar{I}$ -Inh preparation with the RIA for iC $\bar{I}$ -Inh.

**RIAs for complexed C $\bar{I}$ -Inh.** The RIAs for Factor XIIa-C $\bar{I}$ -Inh, kallikrein-C $\bar{I}$ -Inh, and C $\bar{I}$ -C $\bar{I}$ -Inh were performed as previously described (38). Serial dilutions of plasma in which a maximal amount of Factor XIIa-C $\bar{I}$ -Inh and kallikrein-C $\bar{I}$ -Inh complexes was generated by incubation with DXS (DXS plasma; see reference 38) and serum, in which a maximal amount of C $\bar{I}$ -C $\bar{I}$ -Inh complexes was generated by incubation with heat-aggregated human IgG (AHG serum; see reference 43) were used as standards in the appropriate C $\bar{I}$ -Inh complex assays. Levels of C $\bar{I}$ -Inh complexes in plasma samples (tested undiluted in the RIAs for Factor XIIa- and kallikrein-C $\bar{I}$ -Inh and tested at 1–25 dilutions in the RIA for C $\bar{I}$ -C $\bar{I}$ -Inh) were expressed in micromolar.

For estimation of the concentration of Factor XIIa-C $\bar{I}$ -Inh (i.e., 0.375  $\mu$ M) in DXS plasma, a plasma concentration of 30  $\mu$ g/ml and an  $M_r$  of 80,000 for Factor XII was used (14). The concentration of kallikrein-C $\bar{I}$ -Inh (i.e., 0.341  $\mu$ M) in DXS plasma was determined by reference to purified  $\beta$ -kallikrein-C $\bar{I}$ -Inh complexes (42, 48). For estimation of the concentration of C $\bar{I}$ -C $\bar{I}$ -Inh (actual composition: C $\bar{I}$ rC $\bar{I}$ s(C $\bar{I}$ -Inh)<sub>2</sub>; see reference 13) in AHG serum (43), the concentration in serum of both C $\bar{I}$ r and C $\bar{I}$ s was taken as 0.360  $\mu$ M (13).

**Reproducibility of RIAs.** Repeated testing of standards on separate occasions revealed that the RIAs were highly reproducible: intra- and interassay coefficients of variation ranged from 5 to 10%.

**SDS-PAGE and immunoblotting analysis of C $\bar{I}$ -Inh from plasma.** Immunoprecipitation of antigenic C $\bar{I}$ -Inh (with MAb RII) and iC $\bar{I}$ -Inh (with MAb KII) from plasma was performed by incubating 25  $\mu$ l of plasma for 5 h at room temperature with 80  $\mu$ g of MAb coupled to Sepharose in the buffers (0.5 ml) used for the RIAs. After a washing procedure, Sepharose-bound antigen was dissociated into 100  $\mu$ l of

nonreducing SDS sample buffer (6.25 mM Tris-HCl, pH 6.8, containing 2% [wt/vol] SDS, 10% [wt/vol] glycerol, and 0.001% [wt/vol] bromophenol blue) by incubation for 10 min at 100°C. The Sepharose beads were pelleted by centrifugation for 1 min at 1,300 g, and the protein-containing supernatants (immunoprecipitates) were subjected to slab SDS-PAGE (6% wt/vol) (49). Proteins from the gel were then electrophoretically transferred onto nitrocellulose sheets (50). C $\bar{I}$ -Inh species on the blots were visualized by subsequent incubation with <sup>125</sup>I-anti-C $\bar{I}$ -Inh antibodies, followed by autoradiography. Details of the procedures were as previously described (51). The apparent molecular weight of proteins was estimated by comparison with the high-molecular weight protein markers of Bio-Rad Laboratories (Richmond, CA).

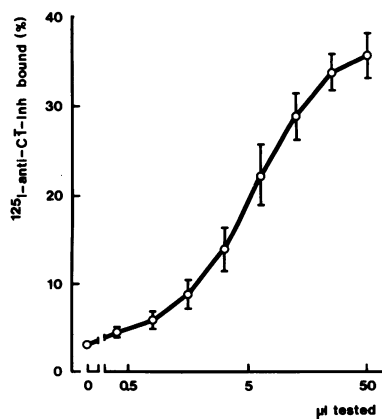
**Statistical analysis.** Statistical calculations were performed on an IBM-compatible personal computer with the use of a standard statistical package (SPSS/PC). When more than two subject groups were analyzed, the Kruskal-Wallis test was used (parameters studied did not appear to be normally distributed) to show significant differences between the various groups. Pairwise comparisons between subject groups were made with the Wilcoxon-Mann-Whitney test. *P* values were corrected for multiple comparisons by the Bonferroni-Holm method. *P* values  $\leq$  0.05 were considered to indicate significant differences.

## Results

**C $\bar{I}$ -Inh species in plasma from patients with sepsis and from healthy volunteers.** Functional C $\bar{I}$ -Inh levels in patients with sepsis were not significantly different from those in healthy controls (Table I), nor when all patients were analyzed together, nor when the data from patients with definite sepsis and from critically ill patients were analyzed separately. Antigenic C $\bar{I}$ -Inh in critically ill patients was higher than in healthy donors (*P* = 0.014). However, the ratio between functional and antigenic C $\bar{I}$ -Inh, i.e., the functional index (FI) was reduced in patients with clinical sepsis (*P* = 0.004), in patients with definite sepsis (*P* = 0.025), as well as in critically ill patients (*P* = 0.002) compared with healthy volunteers (Table I). Differences in functional C $\bar{I}$ -Inh, antigenic C $\bar{I}$ -Inh, and the FI of C $\bar{I}$ -Inh between patients with definite sepsis and critically ill patients were not statistically significant.

To test whether the reduced FI of C $\bar{I}$ -Inh in patients' plasma was caused by the presence of nonfunctional C $\bar{I}$ -Inh, plasma samples were analyzed for iC $\bar{I}$ -Inh and complexed C $\bar{I}$ -Inh. iC $\bar{I}$ -Inh was considerably higher in patients with clinical sepsis than in healthy controls (*P* < 0.0001; Table II), and was increased in 81% of all patients. The levels of iC $\bar{I}$ -Inh in 23 patients with definite sepsis due to infections by Gram-negative bacteria (0.20  $\mu$ M median [range 0.08 to 1.23  $\mu$ M]) were not significantly different from those in 11 patients who suffered from infections by Gram-positive bacteria (0.25  $\mu$ M median [range 0.11 to 1.09  $\mu$ M]). Elevated levels of C $\bar{I}$ -Inh complexes were found in only 21% of all patients (Table II). C $\bar{I}$ -C $\bar{I}$ -Inh and kallikrein-C $\bar{I}$ -Inh complexes in patients with clinical sepsis did not significantly differ from those in healthy donors. However, Factor XIIa-C $\bar{I}$ -Inh complexes in patients with clinical sepsis were higher than in healthy volunteers (*P* = 0.007).

The results of the measurement of all species of nonfunctional C $\bar{I}$ -Inh, shown in Table II, indicated that the reduced FI of C $\bar{I}$ -Inh was mainly due to the presence of iC $\bar{I}$ -Inh. To assure the reliability of the data obtained with the RIAs for C $\bar{I}$ -Inh complexes with MAb KOK12, which binds to a neodeterminant exposed on both complexed and iC $\bar{I}$ -Inh (38), we tested various amounts of preformed C $\bar{I}$ -Inh complexes mixed



**Figure 1.** Standard curve of pooled normal plasma in the RIA for iC $\bar{I}$ -Inh. 50  $\mu$ l of serial dilutions of plasma were incubated with MAb KII-Sepharose as described in Methods. Bound iC $\bar{I}$ -Inh was detected by subsequent incubation with <sup>125</sup>I-anti-C $\bar{I}$ -Inh antibodies. Results are expressed as percentage binding of the <sup>125</sup>I-anti-C $\bar{I}$ -Inh antibodies added. The volume of plasma tested

(in microliters) is indicated on the abscissa. The figure shows the mean and SD of seven separate dose-response curves, performed over a period of 3 mo.

Table I. Plasma Levels of Functional and Antigenic C $\bar{I}$ -Inh in Patients with Sepsis at the Time of Admission

Clinical group	Functional C $\bar{I}$ -Inh		Antigenic C $\bar{I}$ -Inh		FI	
	Median	Range	Median	Range	Median	Range
	$\mu\text{M}$					
Clinical sepsis (n = 48)	2.58	(1.3–6.73)	2.85	(1.35–6.98)	0.96	(0.49–1.3)*
Definite sepsis (n = 37)	2.48	(1.3–6.73)	2.55	(1.4–6.98)	0.98	(0.66–1.3)‡
Critically ill (n = 11)	3.2	(1.38–3.48)	3.58	(1.35–4.48)§	0.86	(0.49–1.05)
Healthy controls (n = 31)	2.58	(1.68–3.65)	2.38	(1.75–3.35)§	1.06	(0.82–1.21)**

\*  $P = 0.004$ ; ‡  $P = 0.025$ ; §  $P = 0.014$ ; ||  $P = 0.002$  (by the Wilcoxon-Mann-Whitney test).

with 50- $\mu\text{l}$  samples of buffer, normal pooled plasma, and patients' plasma samples containing high levels of iC $\bar{I}$ -Inh, in the RIAs. The results of these experiments revealed a full recovery (98 $\pm$ 5%) of each type of preformed C $\bar{I}$ -Inh complex in plasma compared with that in buffer. Similar experiments, in which the recovery of preformed C $\bar{I}$ -Inh complexes was studied after mixing with 50- $\mu\text{l}$  samples containing increasing amounts of purified iC $\bar{I}$ -Inh, revealed that the amount of MAb KOK12 that was used in the complex RIA, was sufficient for a complete recovery of complexes even when mixed with 50- $\mu\text{l}$  samples containing 1.9  $\mu\text{M}$  of iC $\bar{I}$ -Inh.

*Demonstration of proteolytic degradation of C $\bar{I}$ -Inh in patients' plasma.* Immunoprecipitates of C $\bar{I}$ -Inh species from plasma samples of patients with sepsis were subjected to SDS-PAGE and immunoblotting analysis (Fig. 2). Immunoblots of antigenic C $\bar{I}$ -Inh (RII-immunoprecipitate) from pooled normal plasma showed a major protein band of  $M_r$  110,000, together with faint protein bands of  $M_r$  98,000 and 91,000 (lane 1). Immunoblot analysis of iC $\bar{I}$ -Inh (KII-immunoprecipitate) from pooled normal plasma revealed only a faint protein band of  $M_r$  98,000 (lane 2). In contrast, a pronounced protein band of  $M_r$  98,000, together with additional minor protein bands of  $M_r$  91,000 and 86,000 were observed on immunoblots of iC $\bar{I}$ -Inh from patients' plasma samples that contained an elevated level of iC $\bar{I}$ -Inh as measured by RIA (representative patterns shown in lanes 4 and 6 are from patients M and B, respectively). Immunoblot analysis of antigenic C $\bar{I}$ -Inh from these patients' plasma samples showed a major protein band of  $M_r$  110,000, together with the protein bands of degraded C $\bar{I}$ -Inh (patient M, lane 3; patient B, lane 5). The densities of protein

bands of iC $\bar{I}$ -Inh species on blots were proportional to the amount of iC $\bar{I}$ -Inh in plasma as detected by RIA.

*Relation between C $\bar{I}$ -Inh species in plasma and shock.* Table III shows that functional C $\bar{I}$ -Inh was reduced in patients with septic shock compared with normotensive patients, no matter whether critically ill patients were excluded from the analysis ( $P = 0.017$ ) or not ( $P = 0.041$ ). The reduction of antigenic C $\bar{I}$ -Inh in patients that suffered from septic shock compared with normotensive patients, reached statistical significance only when critically ill patients were included ( $P = 0.046$ ). No significant differences in the FI of C $\bar{I}$ -Inh were noted between patients with septic shock and patients with sepsis without shock. In patients with septic shock iC $\bar{I}$ -Inh was higher compared with normotensive patients, only when critically ill patients were excluded from the analysis ( $P = 0.017$ ).

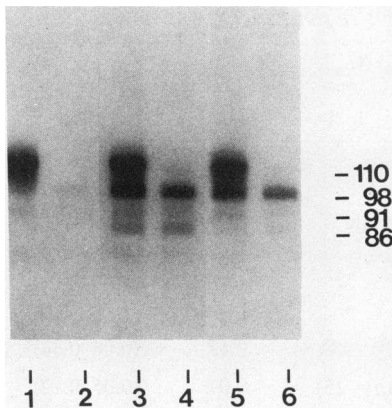
*Relation between inactive C $\bar{I}$ -Inh in plasma and clinical outcome.* Fig. 3 and Table IV show that iC $\bar{I}$ -Inh levels in patients with sepsis who died were significantly higher than in patients who survived, irrespective as to whether critically ill patients were excluded from the analysis ( $P = 0.02$ ) or not ( $P = 0.003$ ). As expected, the FI of C $\bar{I}$ -Inh was reduced in non-survivors compared with survivors, however, this difference reached statistical significance only when data of all patients were included ( $P = 0.05$ ).

The prognostic value of plasma iC $\bar{I}$ -Inh at the time of admission in patients with sepsis is depicted in Fig. 4: the mortality in 15 patients with iC $\bar{I}$ -Inh levels up to 0.20  $\mu\text{M}$  (2.5 times the normal value) was 27%, whereas it was 83% in 12 patients with iC $\bar{I}$ -Inh exceeding 0.44  $\mu\text{M}$  (5.5 times the normal level). The overall mortality in the patients was 56%.

Table II. Plasma Levels of Nonfunctional C $\bar{I}$ -Inh Species in Patients with Sepsis at the Time of Admission and in Healthy Volunteers

	Clinical sepsis (n = 48)			Healthy controls (n = 31)		
	I		II	I		II
	Median	Range		Median	Range	
	$\mu\text{M}$		%	$\mu\text{M}$		%
iC $\bar{I}$ -Inh	0.26	(0.08–1.58)*	81	0.08	(0.04–0.12)*	0
Factor XIIa-C $\bar{I}$ -Inh	<0.0002	(<0.0002–0.0153)‡	21	<0.0002	(<0.0002)‡	0
Kallikrein-C $\bar{I}$ -Inh	0.0002	(<0.0002–0.0476)	21	0.0002	(<0.0002–0.0017)	3
C $\bar{I}$ -C $\bar{I}$ -Inh§	0.013	(0.002–0.036)	21	0.013	(0.006–0.020)	0

II, percent of subjects with increased levels ( $\geq 0.13$   $\mu\text{M}$  for iC $\bar{I}$ -Inh;  $\geq 0.0002$   $\mu\text{M}$  for factor XIIa-C $\bar{I}$ -Inh;  $\geq 0.0010$   $\mu\text{M}$  for kallikrein-C $\bar{I}$ -Inh;  $> 0.020$   $\mu\text{M}$  for C $\bar{I}$ -C $\bar{I}$ -Inh). \*  $P < 0.0001$ ; ‡  $P = 0.007$ . (by the Wilcoxon-Mann-Whitney test). § Actual composition: C $\bar{I}$ rC $\bar{I}$ s(C $\bar{I}$ -Inh) $_2$ ; see reference 13.



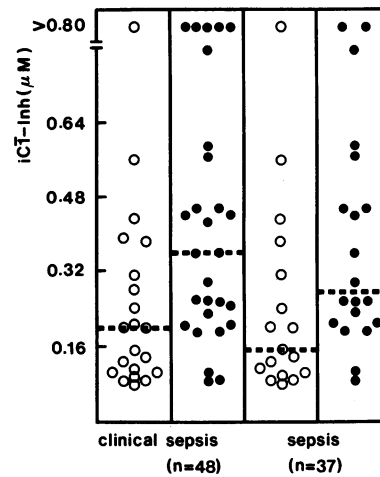
**Figure 2.** SDS-PAGE and immunoblotting analysis of C $\bar{1}$ -Inh from plasma. Antigenic C $\bar{1}$ -Inh and iC $\bar{1}$ -Inh were immunoprecipitated with MAb RII and MAb KII, respectively, and analyzed by SDS-PAGE followed by immunoblotting with  $^{125}$ I-anti-C $\bar{1}$ -Inh, as described in Methods. RII and KII immunoprecipitates from pooled normal plasma (lanes 1 and

2, respectively), from plasma of patient M (lanes 3 and 4, respectively), and from plasma of patient B (lanes 5 and 6, respectively). Patients' plasma M: functional C $\bar{1}$ -Inh, 2.83  $\mu$ M; antigenic C $\bar{1}$ -Inh, 4.5  $\mu$ M; FI, 0.63; iC $\bar{1}$ -Inh, 1.15  $\mu$ M. Patients' plasma B: functional C $\bar{1}$ -Inh, 1.8  $\mu$ M; antigenic C $\bar{1}$ -Inh, 2.08  $\mu$ M; FI, 0.87; iC $\bar{1}$ -Inh, 0.72  $\mu$ M. The right-hand numbers ( $M_r \times 10^{-3}$ ) indicate the apparent molecular weights of protein bands.

**Levels of iC $\bar{1}$ -Inh in plasma from patients without sepsis.** Levels of iC $\bar{1}$ -Inh in the other patients' groups, i.e., patients with severe burns or multiple trauma, patients who were treated with high doses of recombinant IL 2, and patients with stable coronary heart disease, were not increased compared with normal controls (Table V). In addition, no difference in iC $\bar{1}$ -Inh was observed between venous and arterial blood samples (see Table V, patients with coronary heart disease). Thus, the increased proteolysis of C $\bar{1}$ -Inh found in the patients with sepsis, did not as an in vitro artefact result from the collection of blood via heparin-flushed arterial catheters. Similarly, an influence of the collection of blood via indwelling arterial catheters was neither observed on the levels of other C $\bar{1}$ -Inh species, nor on those of other relevant proteins, e.g. Factor XII, prekallikrein, C3a<sub>desarg</sub>, or C4a<sub>desarg</sub> (unpublished observation).

## Discussion

This study demonstrates that in patients with sepsis, a discrepancy exists between plasma levels of functional and antigenic C $\bar{1}$ -Inh that was mainly due to an increase in modified (cleaved) inactive C $\bar{1}$ -Inh (iC $\bar{1}$ -Inh). The extent of plasma C $\bar{1}$ -Inh proteolysis appeared to be related to the clinical outcome.



**Figure 3.** Levels of plasma iC $\bar{1}$ -Inh on admission in survivors (○) and nonsurvivors (●), both with clinical sepsis and definite sepsis. The dashed lines represent the median level in each patients' group.

Until now we did not encounter markedly increased iC $\bar{1}$ -Inh levels in other critically ill patients, i.e., patients without sepsis.

Increased consumption of C $\bar{1}$ -Inh as a consequence of enhanced proteolytic inactivation in combination with increased synthesis due to the acute-phase nature of C $\bar{1}$ -Inh (52) probably accounts for the augmented range in levels of both functional and antigenic C $\bar{1}$ -Inh observed in sepsis. That functional C $\bar{1}$ -Inh in patients with septic shock was lower than in normotensive patients may indicate a relative deficiency of this inhibitor in hypotensive patients and thus a facilitated activation of the contact and complement system, with subsequent release of biologically active peptides. It is tempting to speculate that this process has contributed to the development of hypotension. In agreement with this idea, we observed significantly lower levels of Factor XII (38), and higher C3a<sub>desarg</sub> and C4a<sub>desarg</sub> (39) levels in the patients with septic shock than in normotensive patients. Levels of C3a<sub>desarg</sub> and C4a<sub>desarg</sub> in the patients who died were significantly higher than in those who survived (39). This suggests that complement activation via the classical pathway is involved in the development of fatal complications in sepsis (39). These observations and the high mortality rate (70%) of patients with septic shock (see references 38 and 39) lead us to propose that therapeutic intervention with C $\bar{1}$ -Inh concentrate in patients with septic shock should be seriously considered.

The presence of proteolytically inactivated C $\bar{1}$ -Inh in patients' plasma was visualized by SDS-PAGE and immunoblotting: iC $\bar{1}$ -Inh species had a lower molecular weight (with  $M_r$  98,000, 91,000, and 86,000) than native C $\bar{1}$ -Inh. However,

**Table III.** Relation between C $\bar{1}$ -Inh Species in Patients' Plasma Obtained at the Time of Admission and Shock

Clinical group	Functional C $\bar{1}$ -Inh		Antigenic C $\bar{1}$ -Inh		FI		iC $\bar{1}$ -Inh	
	Median	Range	Median	Range	Median	Range	Median	Range
	$\mu$ M							
<b>Clinical sepsis</b>								
With shock ( $n = 23$ )	2.05	(1.3–4.25)*	2.28	(1.4–4.98)‡	0.95	(0.66–1.3)	0.30	(0.08–1.23)
Without shock ( $n = 25$ )	3.2	(1.38–6.73)*	3.43	(1.35–6.98)‡	0.96	(0.49–1.18)	0.24	(0.08–1.58)
<b>Definite sepsis</b>								
With shock ( $n = 23$ )	2.05	(1.3–4.25)§	2.28	(1.4–4.98)	0.95	(0.66–1.3)	0.30	(0.08–1.23)
Without shock ( $n = 14$ )	3.23	(1.65–6.73)§	2.93	(1.78–6.98)	1.00	(0.71–1.18)	0.16	(0.08–0.56)

\*  $P = 0.041$ ; ‡  $P = 0.046$ ; §  $P = 0.017$ ; ||  $P = 0.017$  (by Wilcoxon-Mann-Whitney test).

**Table IV. Relation between Inactive C $\bar{I}$ -Inh in Plasma from Patients with Sepsis Obtained on Admission and the Clinical Outcome**

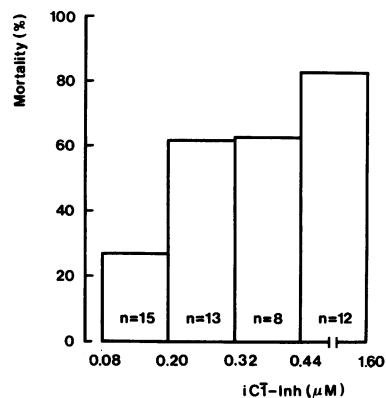
Clinical group	iC $\bar{I}$ -Inh		FI	
	Median	Range	Median	Range
	$\mu\text{M}$			
Clinical sepsis				
Survivors (n = 21)	0.20	(0.08–0.91)*	1.01	(0.71–1.2)‡
Nonsurvivors (n = 27)	0.36	(0.09–1.58)*	0.91	(0.49–1.3)‡
Definite sepsis				
Survivors (n = 17)	0.20	(0.08–0.91)§	1.01	(0.71–1.2)
Nonsurvivors (n = 20)	0.28	(0.09–1.23)§	0.94	(0.66–1.3)
Critically ill				
Survivors (n = 4)	0.25	(0.10–0.39)	0.86	(0.83–1.05)
Nonsurvivors (n = 7)	0.44	(0.24–1.58)	0.80	(0.49–1.02)

\*  $P = 0.003$ ; ‡  $P = 0.05$ ; §  $P = 0.02$ ; ||  $P = 0.038$  (by the Wilcoxon-Mann-Whitney test).

the proteinases that have cleaved the inhibitor cannot be identified from the degradation patterns observed.

Zuraw and Curd (53) have suggested that activation of the contact system of intrinsic coagulation is a major pathway of iC $\bar{I}$ -Inh generation in vivo. This hypothesis is based on several observations. First, substantial amounts of iC $\bar{I}$ -Inh are present in plasma from patients with angioedema due to C $\bar{I}$ -Inh deficiency (53); in these patients activation of the contact system has been shown to occur (54, 55). Second, about one-third of native C $\bar{I}$ -Inh in plasma is cleaved in vitro to iC $\bar{I}$ -Inh upon activation of the contact system by kaolin (53) and DXS (45). Third, analytic gel (SDS-PAGE) studies concerning the interaction of C $\bar{I}$ -Inh with either kallikrein, Factor XIa, or plasmin have demonstrated the formation of stable high-molecular weight complexes and the appearance of iC $\bar{I}$ -Inh species (22, 27–29, 56).

Activation of the contact system of coagulation has been implicated in the pathophysiology of sepsis and septic shock, based on the reduced functional and/or antigenic levels of contact system proteins and inhibitors observed in patients, especially in those with hypotension (10, 32–37). However, the appraisal of these reductions as a measure of consumption is complicated by processes such as hemodilution, impaired syn-



**Figure 4.** Relation between plasma iC $\bar{I}$ -Inh level on admission and mortality. The patients were divided into groups based on their iC $\bar{I}$ -Inh levels on admission (up to 0.20  $\mu\text{M}$ ; 0.21–0.32  $\mu\text{M}$ ; 0.33–0.44  $\mu\text{M}$ , and  $\geq 0.44$   $\mu\text{M}$ ). The mortality of the subgroups is expressed as percentage of the number (n) of patients within each group. The overall mortality (48 patients) was 56%.

**Table V. Plasma Levels of iC $\bar{I}$ -Inh in Patients without Sepsis**

Clinical group	iC $\bar{I}$ -Inh	
	Median	Range
	$\mu\text{M}$	
Critically ill without sepsis		
Severe burns/multiple traumatic injuries (n = 12)	0.12	(0.04–0.16)
Treatment with IL 2 for malignancy (n = 4)	0.10	(0.08–0.11)*
	0.10	(0.08–0.13)‡
	0.12	(0.06–0.36)§
Stable coronary heart disease (n = 15)	0.07	(0.05–0.12)
	0.07	(0.05–0.12)†

\* Before IL 2 treatment; ‡ after the first course of treatment with IL 2; § after the second course of IL 2 administration; || plasma samples from venous blood; † plasma samples from arterial blood.

thesis, and capillary leakage of protein. So, considering the apparent discrepancy between the pronounced reductions of Factor XII and prekallikrein and the absence of substantial amounts of C $\bar{I}$ -Inh complexes in the patients with sepsis (38), the assessment of the extent to which contact activation is responsible for the increased iC $\bar{I}$ -Inh levels found in patients is still a problem.

The extent to which complement activation via the classical pathway may have contributed to the elevated levels of iC $\bar{I}$ -Inh is even more difficult to assess. Conflicting analytic gel studies have been published concerning the interaction of purified C $\bar{I}$ -Inh and C $\bar{I}$ s. Some studies do show evidence of proteolytic degradation of the inhibitor concomitant to the formation of stable complexes on SDS-PAGE (24, 55, 57), whereas others do not (23, 25, 26, 53). Moreover, studies that have demonstrated proteolysis of C $\bar{I}$ -Inh by C $\bar{I}$ s disagree whether cleavage is enhanced (24) in the presence of heparin or not (57). In experiments using the RIA with Mab KII, we observed the formation of iC $\bar{I}$ -Inh upon interaction of C $\bar{I}$ -Inh with C $\bar{I}$ s, but no enhancement of C $\bar{I}$ s mediated cleavage in the presence of heparin (Nuijens, J. H., et al., unpublished observations). Thus, in our opinion, complement activation via the classical pathway may also have contributed to the elevated iC $\bar{I}$ -Inh levels. Both the modification of C $\bar{I}$ -Inh upon activation of complement via the classical pathway as well as a rapid clearance of C $\bar{I}$ -C $\bar{I}$ -Inh complexes in vivo (see reference 38) may account for the apparent discrepancy between the normal C $\bar{I}$ -C $\bar{I}$ -Inh concentrations and significantly elevated levels of C4<sub>desarg</sub> in the patients with sepsis (39).

A third possible pathway to generate iC $\bar{I}$ -Inh resides in the activation of the fibrinolytic system during sepsis. Harpel and Cooper (22) have shown on SDS-PAGE that iC $\bar{I}$ -Inh species as well as plasmin-C $\bar{I}$ -Inh complexes are formed upon interaction of plasmin with C $\bar{I}$ -Inh. Interestingly, Bing and associates (57) have recently reported that the presence of heparin abrogates the formation of complexes and promotes the enzymatic degradation of C $\bar{I}$ -Inh by plasmin.

The high levels of iC $\bar{I}$ -Inh in sepsis might also reflect the release of lysosomal nontarget proteinases into the circulation. Brower and Harpel (30) have shown proteolytic cleavage and inactivation of purified C $\bar{I}$ -Inh by catalytic amounts of neutrophil elastase. Elevated levels of elastase- $\alpha_1$ -antitrypsin complexes in plasma have been found in both patients with sepsis (58–60) and in healthy persons upon administration of

endotoxin (61). Degranulation of neutrophils, with release of elastase and other lysosomal proteinases into the circulation, can be induced by a variety of agonists, including C5a (62), tumor necrosis factor (63), Factor XIIa (64), kallikrein (65), immune complexes, leukotrienes, and intact bacteria. Although this neutrophil pathway of generating iC $\bar{I}$ -Inh in vivo is probably important, it cannot account for all of the iC $\bar{I}$ -Inh found in our patients: two patients with sepsis complicated by shock and adult respiratory-distress syndrome had high levels of iC $\bar{I}$ -Inh in plasma, despite agranulocytosis due to cytostatic treatment for malignancy that antedated the onset of sepsis. Furthermore, Seitz and co-workers (60) have recently described that the levels of elastase- $\alpha_1$ -antitrypsin in plasma on admission of patients with fatal sepsis are not significantly different from those in patients who survived (60). Thus, other iC $\bar{I}$ -Inh-generating pathways probably account for the difference in levels of iC $\bar{I}$ -Inh between survivors and nonsurvivors at the time of admission.

Catalytical cleavage of C $\bar{I}$ -Inh by bacterial proteinases (e.g., *Pseudomonas aeruginosa* elastase and proteinase; reference 31) provides the fifth possible pathway to account for the elevated iC $\bar{I}$ -Inh observed in sepsis. If this mechanism is operative in sepsis then the balance between C $\bar{I}$ -Inh and its target proteinases is thoroughly disturbed: some bacterial products (e.g., endotoxin) activate the contact and complement system, whereas other products (proteinases) inactivate the main inhibitor of these systems.

In conclusion, we observed high levels of iC $\bar{I}$ -Inh in patients with severe sepsis and demonstrated that the extent of plasma C $\bar{I}$ -Inh proteolysis on admission has prognostic significance. This prognostic value of plasma iC $\bar{I}$ -Inh stresses the necessity to explain the contribution of each of the aforementioned processes in the inactivation of C $\bar{I}$ -Inh and to study the interrelationships of these processes to define their role in the complex pathophysiology of sepsis. A better understanding of the role of these processes in the development of complications during sepsis would allow for rational and specific intervention.

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