Correlation between Mortality and the Levels of Inter-Alpha Inhibitors in the Plasma of Patients with Severe Sepsis

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Inter-alpha inhibitor protein (IαIp) is an endogenous serine protease inhibitor in human plasma. Circulating IαIp levels were lower in 51 patients with severe sepsis than in healthy volunteers. Mean levels were 688 ± 295 mg/L in patients with severe sepsis who survived (n = 32), 486 ± 193 mg/L in patients with sepsis who died (n = 19), and 872 ± 234 mg/L in control subjects (n = 25). IαIp levels were lower in patients with shock versus those without (540 ± 246 [n = 33] vs. 746 ± 290 [n = 18] mg/L; P = .0102). IαIp levels were inversely correlated with 28-day mortality rates and Acute Physiology and Chronic Health Evaluation II scores and directly correlated with antithrombin III, protein C, and protein S levels. The administration of IαIp (30 mg/kg body weight intravenously) increased the 50% lethal dose in mice by 100-fold after an intravenous challenge of Escherichia coli. Thus, human IαIp may be a useful predictive marker and potential therapeutic agent in sepsis.

The inter-alpha inhibitor protein (IαIp) family is a group of plasma-associated serine protease inhibitors. Members of this family are composed of heavy and light polypeptide subunits that are covalently linked by a glycosaminoglycan [1]. The light chain, also called bikunin, is responsible for the serine protease inhibitory activity of the molecules. The name “bikunin” reflects the presence of 2 protease-inhibiting domains of the Kunitz type [2].

In normal plasma, bikunin is found mostly in a complex form as inter-alpha inhibitor (IαI), which has a molecular weight of 225 kDa, and pre–alpha inhibitor (PαI), which has molecular weight of 120 kDa [3, 4]. In IαI, bikunin is linked to 2 heavy polypeptide chains, H1 and H2, whereas, in PαI, only a single heavy chain (H3) is linked to bikunin [3]. In these complexed forms, bikunin remains inactive until its release by partial proteolytic degradation, a mechanism that serves as a means to regulate activity [5]. After cleavage from the complex, the activated bikunin is cleared rapidly from plasma by glomerular filtration, a process that is facilitated by its low molecular weight and by receptor-mediated uptake [6]. A growing collection of experimental and clinical data have indicated that members of the IαIp family are involved in many physiological and pathological activities, such as tumor invasion, metastasis [7], and stabilization of the extracellular matrix [8, 9]. Recently, the involvement of IαIp in inflammatory diseases has become an area of intensive investigation.

Sepsis and septic shock are associated with the activation of the innate immunity [10], complement [11], and coagulation systems and the accompanying inhibition of the fibrinolytic system [12]. It is characterized clinically by systemic inflammation, coagulopathy, hypotension, and multiple organ dysfunction. A network of specific proteinases activates clotting, fibrinolytic, and complement factors during severe sepsis. Proteases from endotoxin-activated polymorphonuclear leukocytes can trigger tissue and organ damage [13] and enhance the nonspecific proteolysis of plasma clotting and complement factors [14].

The release of neutrophil proteinases, especially human leukocyte elastase, has been implicated in the pro-
gress of complications in patients with sepsis [15]. Plasma Ip is particularly sensitive to cleavage by neutrophil elastase, and the light chain released from the Ip complex exerts its inhibitory activity on serine proteases [16]. In vitro, Ip has been shown to inhibit several serine proteases that are involved in inflammation, such as elastase, plasmin, and cathepsin G [17]. The results of clinical studies of patients with severe sepsis have revealed significant systemic Ip consumption, together with an extended secretion of elastase [18–20].

In the present article, we describe a quantitative competitive immunoassay for the measurement of Ip (both Ip and Pad) in plasma using a monoclonal antibody (MAb) specific against human bikinin. This assay was used to measure the levels of Ip in 51 septic plasma samples. We also evaluated the potential of the assay to predict the clinical outcome in sepsis, the correlation between Ip levels and the coagulation parameters antithrombin III, protein S, and protein C, and the potential beneficial effect of Ip administration in an experimental mouse model of sepsis.

PATIENTS, MATERIALS, AND METHODS

Patient selection and sample collection. Plasma samples were collected from 51 patients with sepsis who had been randomized to the placebo group of a large, phase 3 multicenter sepsis trial of an interleukin-1 receptor antagonist [21]. Samples were obtained at study entry within 24 h of the onset of severe sepsis. Patients were classified into severe sepsis and/or septic shock groups according to the consensus definitions established at the American College of Chest Physicians/Society of Critical Care Medicine Consensus conference [22]. A clinical evaluation committee reviewed the clinical and laboratory findings in each patient enrolled in the clinical trial. The committee then applied a consistent prespecified and standardized classification system to define the source of infection, organ dysfunction, and probable causative organism(s) for sepsis in each patient. The APACHE II score was also determined for each patient [23]. We used plasma samples from 25 healthy volunteers who had no acute illness as controls.

Plasma was obtained from endotoxin-free heparinized blood samples (Chromogenix). Blood samples were placed on ice and then centrifuged at 4°C for 15 min at 1500 g within 30 min. Tubes were then frozen at −70°C and shipped on dry ice to the research laboratory.

Preparation of human Ip. Human plasma–derived Ip was isolated from an industrial-scale clotting factor VIII (FVIII) concentrate (Octapharma Pharmaceuticals), which was found to contain a relatively large amount of Ip. Ip was successfully separated from FVIII/von Willebrand factor by size-exclusion high-performance liquid chromatography (BioSec; Merck). Ip that contained fractions concentrated by diafiltration were freeze-dried in the presence of saline for long-term storage. The purity of Ip was estimated to be ~70%, as determined by SDS-PAGE and a competitive ELISA that used Ip purified by immunoaffinity chromatography on immobilized MAb 69.31 as a standard. The major contaminants in this Ip preparation were FVIII and von Willebrand factor.

Generation of MAbs against Ip. A panel of MAbs against human Ip was generated by the subtractive immunization protocol described by Williams et al. [31]. BALB/c mice were tolerized by intraperitoneal (ip) injection of 100 μg of an Ip-containing solvent/detergent–treated plasma fraction (Octapharma Pharmaceuticals). This was followed by ip injections of cyclophosphamide (Cytoxan; Bristol-Myers Squibb) at a dose of 100 mg/kg body weight 15 min, 24 h, and 48 h after tolerogen exposure. Tolerized mice then were immunized 3 times on days 18, 33, and 48 by ip injection of 100 μg of an Ip-containing solvent/detergent–treated human plasma fraction that had also undergone pasteurization. Three days after the third immunization, spleen cells were harvested and fused with PAI myeloma cells, as described by Lane et al. [32]. After fusion, hybridoma cells were hypoxanthine-aminopterin-thymidine selected and cloned simultaneously in a methylcellulose-based formulation (ClonaCell-HY; Stem Cell Technologies). Individual clones were expanded in 96-well plates, and antibody-secreting hybridomas were screened for reactivity against human Ip in an ELISA or Western blot assay. The positive clone selected for the present study was designated as MAb 69.31.

Determination of Ip levels by competitive ELISA. A competitive ELISA that used murine MAb 69.31 was used to measure Ip levels. Then, 96-well Immunolon-4 plates (Dynex) were coated with purified Ip (300 ng) in 50 mmol/L carbonate buffer (pH 9.6) and incubated overnight at 4°C. A serial dilution of purified Ip in PBS that contained 1% rat serum was used to establish a standard curve. For the quantitative analysis of Ip levels in plasma, 50 μL of plasma samples diluted 1:25 in PBS or serially diluted Ip were added to individual wells of a 96-well plate. After the addition of 50 μL of MAb 69.31 to each well, plates were incubated for 1 h at 37°C and subsequently washed using an automated plate washer (Labsystem). The bound MAb 69.31 was detected by adding horseradish peroxidase–conjugated goat anti–mouse IgG (human absorbed) (Biosource) for 1 h at 37°C. After washing, 100 μL of 1-Step ABTS (Pierce) was added to the wells, and the absorbance at 405 nm was measured on ELISA plate reader (BioTek). Each plasma sample was tested in triplicate, and assays were done and repeated 3 times independently.

Determination of antithrombin III (AT-III), protein C, and protein S levels. Citrated plasma was obtained and stored at −70°C until it was analyzed. AT-III activity was measured by colorimetric methods (Diagnostica Stago). Plasma was incubated with heparin and thrombin in excess. Thrombin was
measured according to its amidolytic activity on the chromogenic substrate. Values between 80% and 120% were considered to be normal. Protein C activity and free protein S were measured by a coagulation-based assay (Compact coagulation analyzer; Asnieres).

**Experimental animals and animal model.** Six-week-old female, specific pathogen–free BALB/c mice (Charles River Laboratories) were used for the mortality study. National Guidelines for Animal Care and Use in Biomedical Research were followed. A standard LD$_{50}$ model with intravenous (iv) injection of both the bacteria and Iolp or saline controls that had been approved by the Institutional Animal Care and Use Committee was used for the studies. *Escherichia coli* O18:K1:H7 (strain Bort, a gift from Dr. A. Cross, University of Maryland, Baltimore) was administered intravenously via tail-vein injection under light CO$_2$ anesthesia, followed in 45–60 min by an iv injection of saline or Iolp at 30 mg/kg body weight. *E. coli* O18:K1:H7 is a highly virulent, serum-resistant isolate with a LD$_{50}$ of 2–100 cfu/mouse, depending on the assay used [33, 34]. The results of preliminary in vitro testing demonstrated that Iolp had no direct inhibitory effects on the growth of this strain of *E. coli* even at concentrations >1000 µg/mL.

Bacteria were grown in culture to the midlog phase in TSB media (BBL) and then were washed in PBS and diluted in PBS to concentrations of 10$^2$–10$^6$ by spectrophotometer measurement. The bacterial concentration was confirmed by direct colony counts of serial 10-fold dilutions of PBS on MacConkey plates (BBL). The mortality rate was monitored for 48 h, and the LD$_{50}$ of the challenge strain of *E. coli* was calculated according to the method of Reed and Muench [24].

**Statistical methods.** Statistical analyses were done using the Statistica software package (version 5.0; Statsoft). The non-parametric Mann-Whitney U test was used for comparisons between the treatment and control groups. Correlation analysis between different variables was done with the Spearman rank correlation test. P < .05 was considered to be significant.

## RESULTS

**Characterization of MAb 69.31.** MAb 69.31 recognized a complex glycoprotein that was composed of several polypeptide chains. Affinity chromatography with immobilized MAb 69.31 isolated polypeptide antigens of ~250 and 125 kDa in size, which were identified by N-terminal amino acid sequence analysis to be Iol and Psl, respectively (data not shown). In Western blot analysis, MAb 69.31 recognized both Iol and Psl (~250 and 125 kDa), as well as the 25-kDa light-chain fragment (bikunin) of Iol and Psl. MAb 69.31 binding was not significantly altered by treatment with hyaluronidase, an enzyme that cleaves the glycoaminoglycan linking the heavy and light chains of Iolp (Iol and Psl) (figure 1). These results suggested that the epitope recognized by MAb 69.31 was located in the light chain common to Iol and Psl.

**Development of a competitive ELISA using MAb 69.31.** To quantitatively measure the levels of Iolp in bodily fluids such as plasma, a competitive ELISA with MAb 69.31 was developed. The immunoassay was based on the ability of Iolp in plasma samples to block the binding of MAb 69.31 to immobilized Iolp. A standard curve, established using a known amount of affinity-purified Iolp (figure 2), indicated that the linear range of the immunoassay was between 0.625 and 80 µg/mL. With diluted plasma samples (1:25), the assay was capable of accurately measuring Iolp levels of 15–2000 mg/L. The inter- and intra-assay precision determined from 5 different plasma samples analyzed independently on 10 consecutive days were 7.1% and 11.9%, respectively.

**Iolp levels are decreased in plasma from patients with severe sepsis.** Plasma levels of Iolp were determined with the competitive ELISA within 24 h of the onset of severe sepsis. The results from the analysis of 51 samples from patients with sepsis showed that the levels of Iolp were significantly decreased, compared with those in plasma obtained from 25 healthy volunteers. When patients were divided into survivors and nonsurvivors on the basis of the clinical outcome after 28 days, the mean level of Iolp in the survivor group was 688 ± 295 mg/L (n = 32), versus 486 ± 193 mg/L (n = 19) in the nonsurvivor group. Plasma levels of Iolp in healthy control

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**Figure 1.** Western blot analysis of monoclonal antibody 69.31, which recognized bikunin that contained inter-alpha inhibitor protein (Iolp; inter-alpha inhibitor, ~250 kDa; pre-alpha inhibitor, 125 kDa; lane 1) and a ~25-kDa bikunin band after hyaluronidase treatment (lanes 2–4, arrow). With increasing amounts of hyaluronidase, bikunin was cleaved and released from the Iolp complexes and detected by MAb 69.31. Lane 5, molecular weight protein standard.

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Figure 2. Curve of the competitive immunoassay with monoclonal antibody 69.31. Each data point was tested in triplicate; mean values were plotted against the inter-alpha inhibitor protein (I\(a\)Ip) concentration. The linear range was 0.625–80 \(\mu\)g/mL (\(y = -0.4569 \ln(x) + 2.1786\), \(R^2 = 0.9967\).

Figure 3. Decreased plasma inter-alpha inhibitor protein (I\(a\)Ip) levels in patients with severe sepsis. The I\(a\)Ip levels differed significantly between the healthy control subjects and patients with severe sepsis (survivors and nonsurvivors, \(P = .0001\)) and between the survivor and nonsurvivor groups with severe sepsis (\(P = .0103\)), respectively). Significant differences were also found between the I\(a\)Ip levels in patients with septic shock (65% of patients) and those without (mean, 540 ± 246 mg/L \([n = 33]\) vs. 746 ± 290 mg/L \([n = 18]\); \(P = .0102\)) (figure 4).

To determine whether there was a correlation between plasma I\(a\)Ip levels and mortality, patients were divided into the following 4 groups, according to the size of the decrease in plasma I\(a\)Ip levels: group 1, plasma levels <300 mg/L; group 2, plasma levels between 300 and 600 mg/L; group 3, plasma levels between 600 and 900 mg/L; and group 4, normal plasma levels ≥900 mg/L. The results revealed that 3 (60%) of 5 patients in group 1, 12 (52%) of 23 in group 2, and 3 (21%) of 14 in group 3 died during the 28-day study. In contrast, only 1 (11%) of 9 patients in group 4, who had normal I\(a\)Ip levels (933 mg/L), died during the same time period (figure 5). The results suggest that plasma I\(a\)Ip levels are inversely correlated with the mortality rate in patients with severe sepsis.

**Plasma levels of I\(a\)Ip correlate with severity of illness in sepsis.** To determine whether there was a correlation between plasma I\(a\)Ip levels and disease severity and/or risk of mortality, patients with sepsis who had been admitted to the intensive care unit were scored using APACHE II, a helpful tool in the prognosis and prediction of the mortality in sepsis. All data were collected within the first 24 h of admission, and the most aberrant values were used for scoring. As shown in figure 6A,
there was a statistically significant ($P = .016$) negative correlation between APACHE II scores and IαIp levels in the plasma of 26 patients with severe sepsis (Spearman correlation coefficient, $r = -0.47$).

**Decrease in plasma IαIp levels in sepsis correlate with consumption of coagulation regulators.** Levels of AT-III, protein C, and protein S in plasma from 26 patients with severe sepsis were measured and evaluated by the Spearman correlation test, to determine whether there was a correlation between changes in the plasma levels of these proteins and the plasma levels of IαIp. The results showed that, in patients with severe sepsis, the decrease of IαIp levels was positively correlated with the decrease in the plasma levels of AT-III ($r = 0.58$; $P = .0021$), protein C ($r = 0.403$; $P = .042$), and protein S ($r = 0.45$; $P = .021$) (figure 6B–6D).

**In vivo IαIp administration improves survival after E. coli infection.** To determine whether the administration of IαIp during sepsis would reduce mortality, a lethality study in mice was done. Mice were inoculated intravenously with *E. coli* O18: K1 (strain Bort) at 5 different doses ($10^2$, $10^3$, $10^4$, $10^5$, and $10^6$ cfu/mouse in groups of 10 mice for each dosage level). One hour after bacterial challenge, mice were injected intraperitoneally with IαIp at a dose of $30$ mg/kg body weight (8 mice from each group) or with an equivalent volume of saline (2 mice from each group).

Lethality was measured as the number of animals that died because of the infectious challenge over the next 48 h. Additionally, IαIp was administered at a dose of $30$ mg/kg body weight in another group of 10 untreated animals, as a control group, to ensure that the IαIp caused no direct toxicity itself. All animals in this control group remained healthy throughout the 48-h study period after the IαIp injection.

For mice treated with saline after inoculation with *E. coli*, the $LD_{50}$ was $\log_{10}$ 1.61. In contrast, the $LD_{50}$ in the IαIp-treated animals was $\log_{10}$ 3.69 ($P < .001$), a difference that indicates that a 100-fold higher doses of *E. coli* was required for lethality in the IαIp-treated animals. These results indicate that a preparation containing human IαIp provides significant benefit in protecting mice from an otherwise lethal challenge with a gram-negative bacteria.

**DISCUSSION**

Bikunin, the light chain of IαIp, has been shown to inhibit a large number of serine proteases, including factor Xa and kallikrein, 2 proteases that are involved in the coagulation pathway. The finding that bikunin inhibits cell-surface plasmin [5] and lipopolysaccharide-induced stimulation of neutrophils [25] indicates that this protein is also a part of the regulatory system that controls acute inflammation.

The physiological role of bikunin is still unknown, but the fact that no persons with a complete absence of bikunin have been ever been reported [5] suggests that bikunin is essential for life. In the present article, the 2 bikunin-containing molecules found in plasma, Iαl and Pαl, (referred to together as IαIp) were both recognized in Western blot and immunoprecipitation assays by MAb 69.31. We also determined that the epitope defined by MAb 69.31 is located in bikunin. In addition, we demonstrated that MAb 69.31 effectively blocks the trypsin inhibitory activity of bikunin, which suggests that the epitope
defined by this MAb is located in the active site of the molecule (authors’ unpublished data).

Using MAb 69.31, we developed a competitive ELISA that can accurately measure the total levels of IαIp (IαI and PαI) in body fluids. This is in contrast to previously described sandwich-type ELISA assays that used 2 different polyclonal antibodies against heavy chains (H1 and H2), and thus could only measure IαI [26], or that relied on polyclonal anti-H3 and antibikunin antibodies that could only detect PαI [27].

We evaluated samples from 51 patients with severe sepsis with our competitive ELISA. Our results demonstrated a significant decrease in plasma IαIp levels (a 20%–90% reduction), compared with plasma levels in healthy individuals. Statistically significant differences were found between the mean plasma IαIp levels of healthy controls, between survivor and nonsurvivor groups of patients with sepsis, and between septic patients with and without shock. Furthermore, the decrease of IαIp levels correlated with the clinical outcome. Although Balduyck et al. [19] also reported that IαI levels were significantly decreased in plasma samples collected on admission from patients with sepsis, their assay only detected IαI and did not predict the clinical outcome of the patients. Taken together, these results suggest that the measurement of total bikunin-containing molecules in the plasma might have greater predictive value in sepsis than measurement of either IαI or PαI alone.

Although total plasma levels of IαIp decrease during sepsis, serum levels of PαI actually increase in patients with inflammatory disease, a finding that is consistent with the resemblance of PαI expression to “positive” hepatic acute-phase proteins. However, the increased synthesis of PαI is counterbalanced by its enhanced catabolism of PαI during inflammatory disease [20, 27]. This is in contrast to IαI, which rapidly decreases during inflammation and sepsis and has been described as a “negative” acute-phase protein. The mechanism responsible for the regulation of both components of IαIp remains to be determined.

It is likely that the increased levels of PαI in early inflammatory states are followed by significant reduction during severe sepsis, when hepatocellular dysfunction causes the inadequate synthesis of many proteins [28]. Systemic proteolysis during sepsis induces degradation of IαIp [29] and leads to the rapid appearance of breakdown products in the urine. It has been reported that PαI is more susceptible than IαI to proteolysis by stimulated neutrophils [27]. Thus, decreased biosynthesis, in combination with increased proteolysis, appears
to be responsible, at least in part, for the reduction in plasma Iα Ip in patients with severe sepsis [20].

Serial measurements in patients with sepsis have consistently demonstrated a prolonged procoagulant state manifested by AT-III and protein C deficiency [30]. In contrast to the results reported by Balduyck et al. [19], a positive correlation between Iα Ip levels and AT-III, protein C, and protein S was found in our study. These findings support the fact that plasma serine protease inhibitors such as Iα Ip and AT-III are consumed during sepsis. A relationship between the Iα Ip levels and the clinical severity of the disease, as indicated in the APACHE II score, was also demonstrated by our results. In addition, we found that the mortality rate for patients with severely decreased levels of Iα Ip was significantly higher than that of patients with more moderate reductions. These findings suggest that plasma Iα Ip levels may be a useful predictor of mortality in sepsis. It is crucial to identify these high-risk patients, so that aggressive therapy can be initiated early, before the disease progresses. The prognostic value of Iα Ip levels might be significantly improved by serial measurements of Iα Ip levels during disease progression. Current studies are investigating this possibility during the full course of sepsis in a cohort of critically ill patients with a wide spectrum of illness.

Although the physiological importance of Iα Ip remains elusive, the fact that the relatively high concentrations of Iα Ip found in normal plasma rapidly diminish during sepsis offers a therapeutic rationale for the replacement of this endogenous serine protease inhibitor during sepsis. The results of current studies with Iα Ip administration in a murine model of gram-negative bacteremia support the therapeutic value of Iα Ip replacement for the treatment of septic shock. This method of therapy is also supported by a recent publication by Yang et al. [35], which showed improved hemodynamics and a significant survival advantage after the administration of Iα Ip in a rat cecal ligation and puncture model for polymicrobial peritonitis. These findings, together with the results presented here, provide a strong justification for further experimental and clinical investigation into the therapeutic utility of Iα Ip and suggest that restoring the balance between plasma proteases and inhibitors may provide an effective strategy for reducing the severe complications of sepsis in humans.

References


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