SHOCK, Vol. 27, No. 5, pp. 488–493, 2007

ALTERED LEVELS AND MOLECULAR FORMS OF GRANZYME K IN PLASMA FROM SEPTIC PATIENTS

Marijana Rucevic,* Loren D. Fast,* Gregory D. Jay,† Flor M. Trespalcios,† Andrew Sucov,† Edward Siryaporn,‡ and Yow-Pin Lim*

**Division of Hematology-Oncology/Department of Medicine, †Department of Emergency Medicine, Rhode Island Hospital/Brown Medical School; and ‡ProThera Biologics LLC, East Providence, Rhode Island*

Received 15 Jun 2006; first review completed 31 Jul 2006; accepted in final form 27 Sep 2006

ABSTRACT—Granzyme K (GrK) is a member of a highly conserved group of potent serine proteases specifically found in the secretory granules of cytotoxic T lymphocytes and natural killer cells. Based on the report indicating that inter-alpha inhibitor proteins are the physiological inhibitors of GrK and on previous findings that showed a significant decrease in plasma inter-alpha inhibitor proteins in patients with sepsis, it was our aim to determine whether increased levels of uninhibited GrK would contribute to the development of sepsis. To test this hypothesis, a competitive enzyme-linked immunosorbent assay system was developed; and the levels of GrK were measured in plasma samples obtained from healthy controls and 2 sets of patients with sepsis: patients admitted to the emergency department with a putative diagnosis of sepsis and patients with severe sepsis enrolled in a clinical trial. In addition, the molecular form(s) of GrK present in these samples was analyzed by Western blot. The levels of GrK were significantly increased in emergency department patients compared with healthy controls and significantly decreased in patients with severe sepsis enrolled in a clinical trial compared with healthy controls. GrK was detected as high-molecular-weight protein complexes in healthy controls but as complexes of lower molecular weight in the septic patients. The decrease in complex size correlated with the appearance of a band at 26 kDa similar to the size of free GrK. Our results indicate that plasma levels of GrK could serve as a useful diagnostic marker to stage sepsis, permitting better classification of septic patients and enabling targeting of specific treatments, and may play a functional role in the development of sepsis.

KEYWORDS—Granzyme K, granzymes, sepsis, biomarker, serine protease, inter-alpha inhibitor proteins

INTRODUCTION

Sepsis is a consequence of a dysregulated systemic inflammatory immune response that occurs mainly as a result of microbial infections (1). The mortality associated with sepsis and its conditions remains high despite antimicrobial therapy and intensive care. The heterogeneous nature of sepsis often makes it difficult to diagnose. Misdiagnoses and inappropriate treatments due to the variety of infectious agents, different sources of infection, and individual differences contribute to the increased mortality associated with sepsis (2). Advances in diagnosis of sepsis will require the identification of more specific, clinically useful biomarkers that may allow better characterization or staging of patients. The use of such biomarkers may well have an implication for future treatment of sepsis.

There is increasing evidence that cytotoxic cells such as natural killer (NK) cells and cytotoxic T lymphocytes (CTL) play a role in the development of sepsis (4–6). The mechanism by which cytotoxic cells contribute to the development of sepsis has not been defined. However, several conditions have been described in which the presence of cytotoxic cells leads to high levels of granzymes (Grz), the serine proteases found in the cytotoxic granules, and results in pathology especially in the absence of protease inhibitors for the Grz (7–11). Increases in the levels of granzyme A (GrA) and granzyme B (GrB) have been observed in septic patients and in patients with systemic bacterial infections (8). Our findings have shown that decreases in the plasma levels of inter-alpha inhibitor proteins (IaIp) are observed in septic patients and that the lowest levels of IaIp are associated with the greatest incidence of mortality (12, 13). Based on a report indicating that IaIp is the physiological inhibitor of granzyme K (GrK) (14), we hypothesized that increased GrK levels in the absence of specific inhibitors (e.g., IaIp) would contribute to the development of sepsis.

To quantitatively analyze the levels of GrK in biological samples, we developed a specific polyclonal antibody (pAb) against human GrK. Using this antibody, we established a competitive immunoassay for the measurement of GrK levels in plasma. We also determined and compared the levels of GrK in healthy controls (HC) and 2 groups of septic patients: patients admitted in the emergency department (group ED) with a putative diagnosis of sepsis and patients with severe sepsis enrolled in clinical trials (group CT). The molecular forms of GrK in these plasma samples were defined by Western blot analysis using the GrK specific pAb. These results suggest that measurement of GrK levels could be used as a marker in patients developing sepsis.

MATERIALS AND METHODS

Materials

Synthetic peptides NSQSYYNGDPFITKDC (pGrK) and NDRNHYNFNP-VIGMNS (pGrA) derived from human GrK and GrA were custom synthesized and obtained from Sigma-Genosys (The Woodlands, Tex). Recombinant human GrK

Address reprint requests to Yow-Pin Lim, MD, PhD, Rhode Island Hospital/Brown Medical School, Division of Hematology-Oncology/Department of Medicine, 593 Eddy Street, Providence, RI 02903. E-mail: Yow-Pin_Lim_MD@brown.edu.

This work was supported by Lifespan Developmental Grant No. 4792.
DOI: 10.1097/01.shk.0000246905.24895.e5

(rGrK) and GrA (rGrA) were purchased from Alexis Biochemicals (San Diego, Calif). All other chemicals were purchased from Sigma (St Louis, Mo) unless otherwise specified.

Cell culture

The human interleukin (IL) 2 dependent NK cell line 92MI was obtained from the American Type Culture Collection (Manassas, Va). The cells were maintained in alpha minimum essential medium (Gibco, Grand Island, NY) supplemented with 2 mmol/L L-glutamine, 0.2 mmol/L inositol, 0.1 mmol/L 2-mercaptoethanol, 0.02 mmol/L folic acid, 5% heat-inactivated fetal calf serum, and horse serum. The cells were incubated in humidified air with 10% CO_2 at 37°C and subcultured twice per week.

Purification of GrK from human NK cell line

Granzyme K was purified from cytotoxic granules of the IL-2 dependent human NK cell line (NK-GrK) as described by Hanna et al. (15).

Generation, characterization, and purification of GrK specific polyclonal antibody

Polyclonal antibody against human GrK (anti-GrK pAb) was generated in rabbits by immunizing with pGrK, corresponding to amino acid residues 189 to 205 of human GrK (Sigma-Genosys). This sequence was identified using the MacVector software (Accelrys Inc, San Diego, Calif) as a sequence unique to human GrK when compared with the sequence of human GrA, GrB, neutrophil elastase, cathepsin G, or proteinase 3. It was also chosen from the sequence with the highest immunogenicity predicted by this software. Preimmune serum (PRIS) was obtained before immunization. Three immunizations were performed at day 0, 14, and 28. Sera were collected and further characterized for its specificity.

To obtain affinity-purified antibodies, rabbit GrK antisera were passed over an immobilized pGrK column. pGrK was immobilized on *N*-hydroxysuccinimide-activated Sepharose according to the manufacturer's instruction (Amersham Bioscience, Piscataway, NJ). The specific antibodies that were bound to the column were eluted with 0.1 mol/L citric acid, pH 2.3, and immediately neutralized to pH 7.4 by adding 1 mol/L NaOH. The concentration of eluted immunoglobulin G (IgG) was determined by using the Micro BCA Protein Assay Kit (Pierce, Rockford, Ill).

Immunoblot analysis of human GrK

Samples containing GrK, rGrK, NK-GrK, or plasma samples were mixed with nonreducing sodium dodecyl sulfate (SDS) sample buffer, heated to 95°C for 15 min, and separated by 12.5% SDS–polyarylamide gel electrophoresis (PAGE) according to Laemmli (16). The immunodetection was performed using anti-GrK pAb followed by incubation with horseradish peroxidase (HRP)–conjugated goat antirabbit IgG (Biosource, Camarillo, Calif). The blots were visualized by using the enhanced chemiluminescence system (Pierce). For the analysis of high-molecular-weight complex forms of GrK in plasma, samples were separated using 4% to 12% NuPAGE Bis-Tris gels according to the manufacturer's instruction (Invitrogen Corporation, Carlsbad, Calif).

Direct binding and competitive peptide inhibition assay

The specificity of the anti-GrK pAb was determined by a direct solid phase binding assay. pGrK and pGrA or rGrK and rGrA were immobilized onto 96-well microplates (Immulon 4, Dynex, Chantilly, Va) in coating buffer (50 mmol/L sodium bicarbonate, pH 9.6). After blocking with 5% nonfat dried milk, anti-GrK pAb or PRIS diluted in phosphate-buffered serum (PBS) was added to the wells. The bound antibodies were detected by using HRP-conjugated goat antirabbit IgG and visualized by the 1-step ABTS substrate (Zymed, San Francisco, Calif). The color change was measured at 405 nm on BioTek microplate reader (Bio-Tek Instruments, Winooski, Vt). For the competitive inhibition assay, anti-GrK pAb was preincubated with various concentrations (from 0.01 to 10 ng/μL) of pGrK or pGrA at 37°C for 45 min before being added to the microplates coated with rGrK.

Granzyme K enzyme-linked immunosorbent assay

A competitive enzyme-linked immunosorbent assay (ELISA) using affinity-purified anti-GrK pAb was performed as follows: pGrK (250 ng per well) was immobilized in coating buffer onto 96-well microplates overnight at 4°C. After coating, residual binding sites were blocked by 3% bovine serum albumin. A serial dilution of pooled human plasma (healthy donors) in PBS was used to generate a standard curve on each plate. The concentration of GrK in this pooled human plasma was defined as 100 arbitrary units per milliliter (U/mL). For the GrK level analysis, 50 μL of diluted plasma samples (1:10 in PBS) or serially diluted standard plasma was added to individual wells in triplicate along with 50 μL of anti-GrK pAb (0.25 mg/mL) and incubated for 1 h at 37°C. Subsequently, HRP-conjugated goat antirabbit IgG was added to the wells. One-step ABTS was used as a substrate and absorbance was measured at 405 nm. Granzyme K concentration in the samples was calculated based on the standard curve of serially diluted human plasma. The specificity of the developed ELISA was further evaluated by using pGrA or rGrK immobilized on the microplates.

Inter-alpha inhibitor protein ELISA

Plasma levels of IaIp were quantitatively measured by sandwich ELISA as described previously by Lim et al. (12).

Clinical samples

The Institutional Review Board for protection of human subjects approved all studies. Blood samples of 41 HC were examined to establish the reference range of GrK levels in plasma. Blood samples from 2 septic patient groups were analyzed. The first group consists of 15 patients presenting to the ED at Rhode Island Hospital (group ED) with hypotension secondary to pneumonia or urosepsis. Each of these patients was identified and treated using the early goal-directed treatment guidelines for sepsis (3). These patients met the following criteria: a systolic blood pressure less than 90 mmHg after 2 L of crystalloid infusion or a serum lactate greater than 4 mmol/L. Monitoring of central venous pressure, which was used in titrating correct amounts of subsequent crystalloid and dobutamine in achieving normotension, was performed in the ED. The acute physiology and chronic health evaluation (APACHE II) score was determined for each patient (17). These patients were subsequently transferred to the medical intensive care unit for further treatment. Collected plasma from patients at the time of admission in the ED was used for the GrK assay. The second septic group consists of 25 severe septic patients enrolled in the phase III multicenter septic clinical trial of an experimental therapeutic agent (group CT). Blood samples were obtained at study entry within 24 h of the onset of severe sepsis (according to the consensus definitions established at the American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference) and were kindly provided by Dr Steven M. Opal, The Memorial Hospital of Rhode Island.

To ensure the comparability and quality of the study, clinical specimens and HC samples were collected under similar conditions. Plasma was separated and obtained from the citrated or EDTA blood collection tubes by centrifugation, transported to the research laboratory, and stored at −80°C until use. The clinical trial specimens were shipped in dry ice to the research laboratory. It has been previously determined that there was no significant difference of GrK levels between plasma obtained at the same time of collection from the same individuals using citrated or EDTA containing blood collection tubes, suggesting that the anticoagulant additives EDTA and citrate do not interfere in the GrK assay.

Statistical analysis

Student *t* test was used to analyze the significance of the plasma GrK levels between the septic groups and HC. All data were presented as mean values ± SD. A *P* value less than 0.05 was considered as statistically significant.

RESULTS

Characterization of anti-GrK pAb

The specificity of anti-GrK pAb was determined in a direct binding ELISA using immobilized peptides, pGrK and pGrA, and with recombinant granzymes, rGrK and rGrA, as shown in Figure 1. Anti-GrK pAb bound specifically to pGrK and rGrK but not to pGrA or rGrA. No binding was observed using PRIS. To verify the specificity of this antibody, a competitive binding assay was performed. pGrK was able to inhibit the binding of anti-GrK pAb to rGrK immobilized on the 96-well microplates in a concentration-dependent manner. A complete inhibition of antibody binding was achieved at 10 ng/μL of pGrK. No significant inhibition was observed with control pGrA (Fig. 2). These results clearly demonstrate that anti-GrK pAb was specifically directed against unique peptide sequence corresponding to human GrK. The anti-GrK pAb was further affinity purified and used in GrK ELISA and Western blot analysis.

Detection of native form of GrK from human NK cell line

It has been known that Grz are expressed by CTL and NK cells and stored in their cytotoxic granules until degranulation (7, 8). To identify the native form of GrK and to demonstrate the ability of anti-GrK pAb to bind GrK in its native form,

GrK was purified from cytotoxic granules obtained from a human IL-2 dependent NK cell line as described by Hanna et al. (15). Human rGrK was included as a positive control. The results of Western blot analysis using affinity-purified anti-GrK pAb showed an identical 26-kDa reactive band in both GrK purified from the human NK cell line and human rGrK (Fig. 3A). PRIS was used as a negative control (Fig. 3B). The results further confirmed the specificity of anti-GrK pAb and demonstrated its ability to bind native form of human GrK.

Development of a competitive ELISA using anti-GrK pAb

To measure the levels of GrK in biological fluids such as plasma, a competitive ELISA using affinity-purified anti-GrK pAb was developed. The immunoassay was based on the ability of GrK in plasma samples to block the binding of anti-GrK pAb to immobilized pGrK. Substitution of pGrK by rGrK showed similar results (data not shown). A standard curve was established by a serial dilution of pooled human plasma (Fig. 4). The detection range of the GrK assay was 1.5 to 100 U/mL. The coefficient of variation between 3 separate experiments was less than 10%.

Granzyme K levels in plasma of septic patients and HC

The developed competitive assay was used to measure the levels of GrK in plasma samples from HC, group ED, and group CT. Analysis of the clinical parameters of these 2 patient groups indicated that the patients were of similar age and APACHE II scores (Table 1). Because of the relatively short length of stay of these patients in the ED, it was not possible to obtain a full assessment of organ dysfunction in this patient group. The results indicated that the levels of GrK were significantly increased in group ED (mean ± SD = 123.4 ± 6.61 U/mL, n = 15) and significantly decreased in group CT (44.9 ± 4.14 U/mL, n = 25) compared with HC (69.14 ± 2.39 U/mL, n = 41) (Fig. 5). The differences between the plasma GrK levels in HC versus group ED and in HC versus group CT were statistically significant ($P < 0.0001$). A statistically significant difference was also found between both septic groups (ED versus CT, $P < 0.0001$).

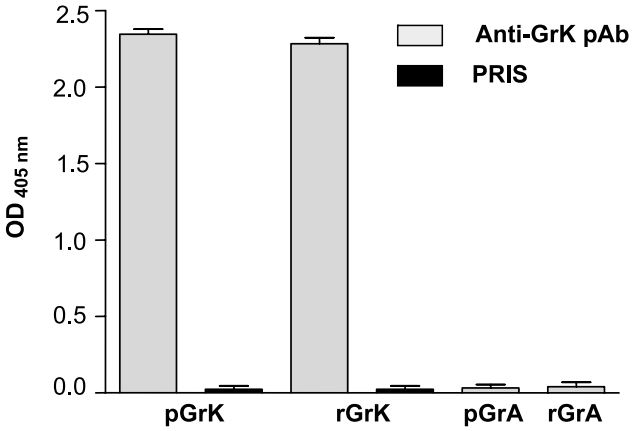

FIG. 1. **Specificity of anti-GrK pAb in ELISA.** Microplates were coated with synthetic peptides (pGrK, pGrA) or human recombinant granzymes (rGrK, rGrA) and incubated with rabbit anti-GrK pAb or PRIS as a negative control. Data represent the average, and error bars represent the SD from 2 separate experiments.

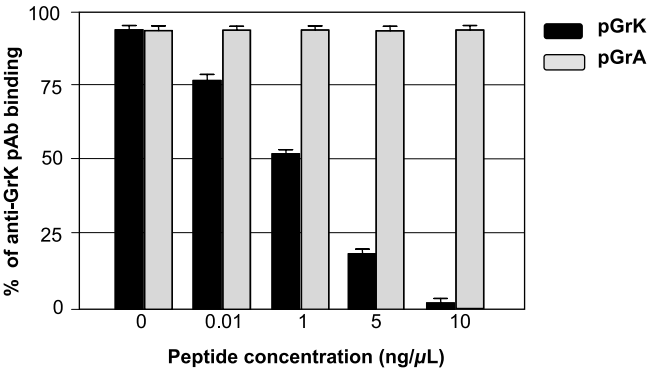

FIG. 2. **The competition of synthetic peptide pGrK for anti-GrK pAb binding.** Various concentrations (from 0.01 to 10 ng/μL) of synthetic peptides pGrK and pGrA were added to anti-GrK pAb and measured for their effect on the binding of the antibody to rGrK. The synthetic peptide pGrK was able to competitively inhibit the binding of anti-GrK pAb to immobilized rGrK in concentration-dependent manner. A complete inhibition of antibody binding was achieved at 10 ng/μL of pGrK, whereas control pGrA had no significant effect. Data represent the average, and error bars represent the SD from 2 separate experiments.

Molecular form of GrK in human plasma

In an effort to determine the molecular form of GrK present in plasma from healthy individuals and septic patients, samples were separated by 4% to 12% or 12.5% SDS-PAGE followed by Western blot analysis using anti-GrK pAb. The 4% to 12% SDS PAGE and subsequent Western blot analysis revealed that GrK circulates in healthy individuals in complex form(s), resistant to SDS and heat, and with major detectable protein bands of >250 and >150 kDa (Fig. 6A). In the plasma of the analyzed septic patients, the >250-kDa protein band was clearly absent. We observed enhanced levels of the 150-kDa band and additional bands corresponding to ~125, 98, and 60 kDa in the plasma of group ED, whereas noticeably less of these GrK reactive bands were detected in the plasma of group CT. Moreover, 12.5% SDS-PAGE and subsequent Western blot analysis showed a lower band of 26 kDa only in plasma of septic patients (groups ED and CT), but not in HC (Fig. 6B). The apparent 26-kDa band was similar in size with the GrK purified from cytotoxic granules of human NK cells, suggesting the presence of systemic free GrK in septic patients.

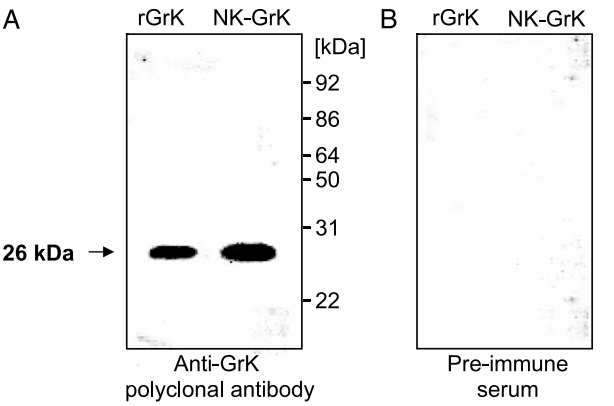

FIG. 3. **The specificity of anti-GrK pAb defined by Western blot analysis with human rGrK and NK-GrK.** An identical 26-kDa reactive band was specifically recognized by anti-GrK pAb in both rGrK and NK-GrK (A). PRIS was used as a negative control (B).

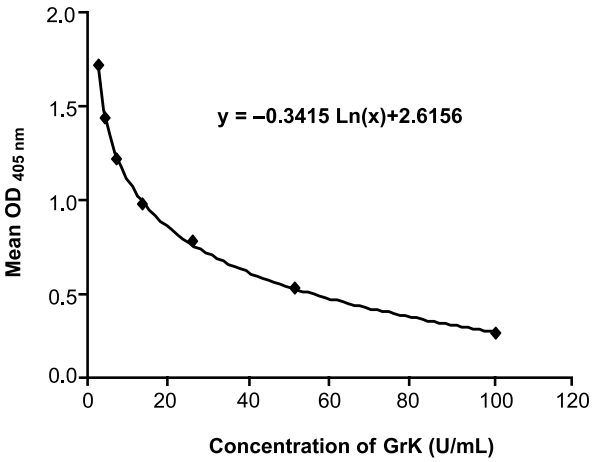

FIG. 4. **A standard curve of GrK assay was established by using serially diluted pooled human plasma with defined amount of GrK.** The concentration of GrK was defined as 100 U/mL in the pooled human plasma. The detection range of the GrK assay was 1.5 to 100 U/mL. The coefficient of variation between 3 separate experiments was less than 10%.

Inter-alpha inhibitor protein levels in plasma of septic patients and HC

Our previous studies have shown significantly decreased plasma IaIp levels of patients with sepsis (12, 13). To determine whether the IaIp levels fluctuated in these septic patient populations, IaIp levels were measured in the same set of samples assayed for GrK levels. The results demonstrated that plasma IaIp levels were significantly decreased in both septic patient groups: group ED (249.6 ± 24.31 mg/L, n = 15) and group CT (176.0 ± 16.15 mg/L, n = 25) compared with HC (597.1 ± 31.69 mg/L, n = 41) (Fig. 7). Statistically significant differences were found between the HC and either septic patient groups ($P < 0.0001$), and these results confirmed our previous findings (12).

DISCUSSION

Sepsis is not a single disease but a complex and heterogeneous process that can progress rapidly, leading to global tissue hypoxia, organ failure, and death. Early detection and intervention were found to be crucial in reducing mortality from sepsis as demonstrated in the early goal-directed therapy study by Rivers et al. (3). However, diagnosis and treatment of sepsis have been hampered by the lack of clinically useful biomarkers for staging patients when they are admitted to the ED. Therefore, discovery of effective biomarkers for sepsis that can identify patients at specific stages when intervention could potentially alter disease progression and ultimately reduce mortality is imperative at the present time.

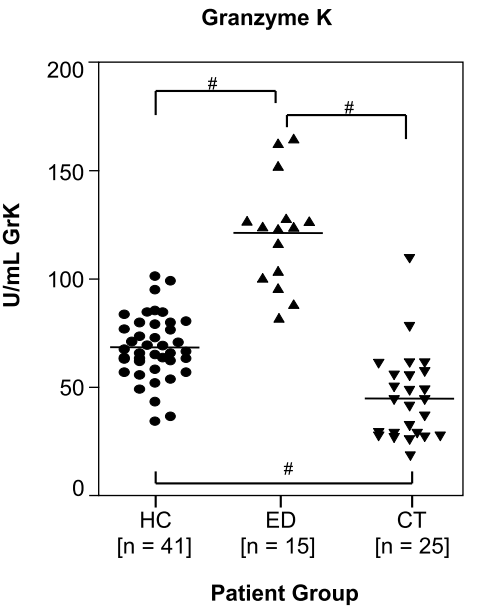

FIG. 5. **The plasma levels of GrK in HC, group ED, and group CT were determined by a novel GrK specific ELISA.** Samples are assayed in triplicate, and the levels are expressed in arbitrary units per milliliter. The differences between the means of septic groups (ED or CT) and HC were statistically significant ($^{\#}P < 0.0001$). Statistically significant difference was also found between both septic groups (ED versus CT, $^{\#}P < 0.0001$).

Low constitutive plasma levels of Grz, serine proteases found in the cytotoxic granules of NK cells and CTL, have been observed (18). Because of the potent activity of Grz, excess protease inhibitors are usually present to inhibit any free Grz, thereby preventing bystander effects (9–11). The absence of these inhibitors has been associated with disease pathology when increased serine protease activity is present (19–21). Based on these findings, it is reasonable to postulate that the decreased plasma levels of protease inhibitors in the presence of increased levels of Grz could result in enhanced inflammatory responses resulting from the cleavage of specific substrates.

Inter-alpha inhibitor proteins are natural serine protease inhibitors found at relatively high concentration in human plasma. These are composed of heavy and light polypeptide subunits that are covalently linked by a chondroitin sulphate chain (22, 23). The light chain (also termed *bikunin* = bi-kunitz inhibitor = inhibitor with 2 Kunitz domains) is responsible for the serine protease inhibitory activity of the molecules. The major forms found in human plasma are inter-alpha inhibitor, which consists of 2 heavy chains (H1 and H2) and a single light chain, and pre-alpha inhibitor, which consists of 1 heavy (H3) and 1 light chain. The relatively high levels of IaIp normally circulating in plasma and the fact

TABLE 1. **The clinical parameters of the 2 patient groups with sepsis**

			Age, years		APACHE II score		# Organ dysfunction
Patient group	n	M/F	Range	Mean ± SD*	Range	Mean ± SD*	Mean ± SD
Group ED	15	11/4	38 to 91	73.2 ± 15.0	11 to 35	20.7 ± 7.1	ND†
Group CT	25	17/8	28 to 85	64.2 ± 17.8	8 to 52	23.6 ± 8.4	2.3 ± 1.1

*No significant difference between the 2 patient groups.
†Not determined during stay in ED.

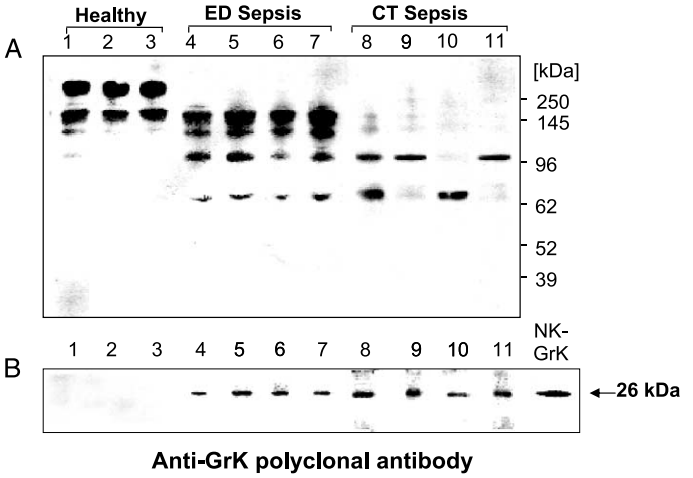

FIG. 6. **Semiquantitative Western blot analysis of components detected by anti-GrK pAb in human plasma.** Plasma samples from HC (lanes 1–3), group ED (lanes 4–7), and group CT (lanes 8–11) were analyzed by SDS-PAGE 4% to 12% gradient gel (A) or 12.5% gel (B) under nonreducing conditions, followed by immunodetection with anti-GrK pAb. NK-GrK: GrK purified from human NK cells cytotoxic granules was used as a control. Results are representative of at least 3 experiments.

that no person with complete absence of IaIp has ever been detected (12) suggest that these proteins have an essential physiological role that remains to be established. Our recent data found a significant decrease in plasma IaIp levels in adult patients and newborns with clinically proven sepsis, and established a correlation between the levels of IaIp and mortality in adult septic patients (12, 13). Inasmuch as IaIp had also been shown to be the physiological inhibitor of GrK (14), the goal of these studies was to investigate the potential role of GrK in pathogenesis of sepsis. Because GrK has not been extensively characterized, as a first step, we generated a highly specific GrK pAb and established a competitive ELISA to quantify the levels of GrK in biological samples. This assay was used to quantitate plasma levels of GrK in 3 groups: HC, group ED patients, and group CT patients. The 2 septic patient groups were found to have similar clinical characteristics as reflected in the APACHE II score (Table 1). Constitutive levels of GrK were observed in HC, whereas analysis of patients admitted to the ED found increased levels of GrK and decreased levels of IaIp, in contrast to the patients enrolled in a clinical trial for severe sepsis who expressed significantly decreased GrK and IaIp levels. One possible explanation for this finding is that the ED patients are at a relatively earlier time point during the disease progression than the CT patients. It seems that increased levels of GrK is a transient event similar to observations made for cytokine levels in response to inflammatory stimuli in human or septic animal models (1, 24–26). Future experiments to address this possible explanation will study the plasma levels of GrK obtained from individual patients at various time points after the admission to the ED.

Although the cells responsible for the production of increased GrK levels need to be defined, the most likely candidates are NK cells. Inflammatory stimuli such as PHA+IL-2 have been shown to induce activation and proliferation of NK cells *in vitro*, resulting in a greater than 20-fold increase in the levels of GrK message (27). *In vivo* injection of LPS into human volunteers induced a 5-fold increase in soluble GrA that peaked at 2 h and an increase in soluble GrB that peaked at 6 h (8). These findings are consistent with an increase in GrK levels early in the inflammatory response.

Whereas the competitive ELISA measures varying levels of GrK in blood, it is clear that the pAb to GrK was detecting GrK in a number of different molecular weight forms. GrK was present in high-molecular-weight protein complexes (>250 and >150 kDa) that were highly specific and stable to SDS and heat treatment. Analysis of the GrK present in samples from ED patients indicated the loss of the >250-kDa complex with a concordant increase in a number of different complexes of lower molecular weight. In addition, a free 26-kDa GrK form not found in HC appeared in these patients. Biochemical analysis of GrK in the plasmas from the CT group indicated further decreases in the size of the GrK complexes and a significant level of the free 26-kDa GrK form. The components of these complexes are currently under investigation. It is possible that serglycin and/or protease inhibitors form GrK complexes as has been shown with GrA (10). Previous studies using a monoclonal antibody to human GrK had also detected high-molecular-weight forms of GrK when the contents of cytotoxic granules were separated by gel filtration (28).

These biochemical findings indicate that anti-GrK pAb is unique in its ability to recognize a specific epitope of GrK in both free form and when complexed. This finding would support the concept that our ELISA can accurately measure the total levels of GrK in biological samples not limited to the free form. Recent studies have used genetic immunization to develop monoclonal antibodies to human GrK, and these antibodies have been used to establish an ELISA assay to measure GrK levels (28). In contrast, in our hands, these monoclonal antibodies failed to react with the GrK complexes

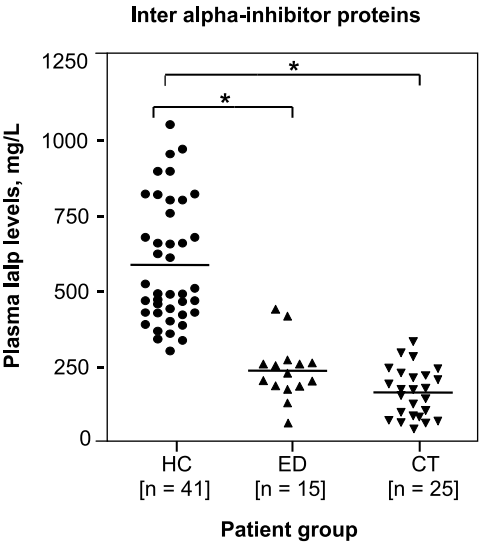

FIG. 7. **The plasma levels of IaIp were determined by a sandwich ELISA using monoclonal antibody 69.26 and pAb against human IaIp.** The lines represent the mean level for each group. Statistically significant decreases of IaIp levels were found in both septic patient groups (groups ED and CT) compared with HC (*$P < 0.0001$).

present in plasma (data not shown). In summary, the use of this pAb revealed that GrK appears in plasma of HC in complexed form. By contrast, free GrK was only found in patients with sepsis. Significantly increased levels of plasma GrK were found in ED patients, suggesting the potential diagnostic and prognostic usefulness of measuring GrK levels for staging sepsis upon admission of the patient to the ED. Because of the temporal changes in GrK levels, additional biomarkers might be needed to complement GrK in assessing patients with a putative diagnosis of sepsis upon their admission to the ED.

The finding of the free from of GrK raises the question of whether the free form is enzymatically active and if this form contributes to inflammatory responses. The potential role of free GrK in inflammatory responses will be assessed most effectively by measuring the consequences of injecting free GrK into murine recipients. This study will address the possible involvement of GrK in the pathogenesis of sepsis and the potential role of GrK as a therapeutic target in sepsis.

Our findings indicate that GrK could become a clinically useful biomarker that may allow better characterization and/or staging of patients with sepsis. The use of such biomarkers may well have an implication for an early intervention therapy that was found the most effective and critically important for improvement of survival in patients with sepsis.

ACKNOWLEDGMENTS

The authors thank Dr Steven M. Opal, The Memorial Hospital of Rhode Island, who kindly provided clinical trial patient samples used in this study.

REFERENCES

1. Carrigan SD, Scott G, Tabrizian M: Toward resolving the challenges of sepsis diagnosis. *Clin Chem* 50:1301–1314, 2004.
2. Marshall JC, Vincent JL, Fink MP, Cook DJ, Rubenfeld G, Foster D, Fisher CJ Jr, Faist E, Reinhart K: Measures, markers, and mediators: toward a staging system for clinical sepsis. A report of the Fifth Toronto Sepsis Roundtable, Toronto, Canada, 2000. *Crit Care Med* 31:1560–1567, 2002.
3. Rivers E, Nguyen B, Havstad S, Ressler J, Muzzin A, Knoblich B, Peterson E, Tomlanovich M: Early goal-directed therapy in the treatment of severe sepsis and septic shock. *N Engl J Med* 345:1368–1377, 2001.
4. Zeerleder S, Hack CE, Caliezi C, van Mierlo G, Eerenberg-Belmer A, Wolbink A, Wuillenmin WA: Activated cytotoxic T cells and NK cells in severe sepsis and septic shock and their role in multiple organ dysfunction. *Clin Immunol* 116:158–165, 2005.
5. van Griensven M, Dalhweid M, Giannoudis PV, Wittwer T, Böttcher F, Breddin M, Pape H: Dehydroepiandrosterone (DHEA) modulates the activity and the expression of lymphocyte subpopulations induced by cecal ligation and puncture. *Shock* 18:445–449, 2002.
6. van Griensven M, Probst C, Muller K, Hoevel P, Pape HC: Leukocyte-endothelial interactions via ICAM-1 are detrimental in polymicrobial sepsis. *Shock* 25:254–259, 2006.
7. Tak PP, Spaeny-Dekking L, Kraan MC, Breedveld FC, Froelich CJ, Hack CE: The levels of soluble granzyme A and B are elevated in plasma and synovial fluid of patients with rheumatoid arthritis (RA). *Clin Exp Immunol* 116:366–370, 1999.
8. Lauw FN, Simpson AJH, Hack CE, Prins JM, Wolbink AM, Chaowagul W, White NJ, Poll TVD: Soluble granzymes are released during human endotoxemia and in patients with severe infection due to gram-negative bacteria. *J Infect Dis* 182:206–213, 2000.
9. Masson D, Tschop J: Inhibition of lymphocyte protease granzyme A by antithrombin III. *Mol Immunol* 25:1283–1289, 1988.
10. Spaeny-Dekking EHE, Kamp A, Froelich C, Hack C: Extracellular granzyme A, complexed to proteoglycan, is protected against inactivation by protease inhibitors. *Blood* 95:1465–1472, 2000.
11. Tsuzuki S, Kokado Y, Satomi S, Yamasaki Y, Hirayasu H, Iwanaga T, Fushki T: Purification and identification of binding protein for pancreatic secretory trypsin inhibitor: a novel role of the inhibitor as an anti–granzyme A. *Biochem J* 372:227–233, 2003.
12. Lim YP, Bendelja K, Opal SM, Siryaporn E, Hixon DC, Palardy JE: Correlation between mortality and the levels of inter-alpha inhibitors in the plasma of patients with severe sepsis. *J Infect Dis* 188:919–926, 2003.
13. Baek WB, Brokat S, Padbury JF, Pinar H, Hixon DC, Lim YP: Inter-α inhibitor proteins in infants and decreased levels in neonatal sepsis. *J Pediatr* 143:11–15, 2003.
14. Wilharm E, Parry MAA, Friebel R, Tschesche H, Matschiner G, Sommerhoff CP, Dieter JE: Generation of catalytically active granzyme K from Escherichia coli inclusion bodies and identification of efficient granzyme K inhibitors in human plasma. *J Biol Chem* 274:27331–27337, 1999.
15. Hanna WL, Zhang X, Turbov J, Winkler U, Hudig D, Froelich CJ: Rapid purification of cationic granule proteases: application to human granzymes. *Protein Expr Purif* 4:398–404, 1993.
16. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685, 1970.
17. Knaus WA, Wagner DP, Draper EA, Zimmerman JE, Bergner M, Bastos PG, Sirio CA, Murphy DJ, Lotring T, Damiano A, et al.: The APACHE III prognostic system. Risk prediction of hospital mortality for critically ill hospitalized adults. *Chest* 100:1619–1636, 1991.
18. Spaeny-Dekking EHA, Hanna WL, Wolbink AM, Wever PC, Kummer AJ, Swaak AJG, Middeldrop JM, Huisman HG, Froelich JC, Hack E: Extracellular granzymes A and B in humans: detection of native species during CTL responses in vitro and in vivo. *J Immunol* 160:3610–3616, 1998.
19. Carrell RW, Lomas DA: Alpha1-antitrypsin deficiency—a model for conformational diseases. *N Engl J Med* 346:45–53, 2002.
20. Tremblay G, Wolbink A, Cormier Y, Hack C: Granzyme activity in the inflamed lung is not controlled by endogenous proteinase inhibitors. *J Immunol* 165:3966–3969, 2000.
21. Lomas DA, Mahadeva R: α1-Antitrypsin polymerization and the serpinopathies: pathobiology and prospects for therapy. *J Clin Invest* 110:1585–1590, 2002.
22. Morelle W, Capon C, Balduyck M, Sautiere P, Kouach M, Michalski C, Fournet B, Mizon J: Chondroitin sulphate covalently cross-links the three polypeptide chains of inter-alpha-trypsin inhibitor. *Eur J Biochem* 221:881–888, 1994.
23. Enghild JJ, Thogersen IB, Cheng F, Fransson LA, Roepstorff P, Rahbek-Nielsen H: Organization of the inter-alpha-inhibitor heavy chains on the chondroitin sulfate originating from Ser(10) of bikunin: posttranslational modification of IalphaI-derived bikunin. *Biochemistry* 38:11804–11813, 1999.
24. Martich G, Danner R, Ceska M, Suffredini A: Detection of interleukin-8 and tumor necrosis factor in normal humans after intravenous endotoxin: the effect of antiinflammatory agents. *J Exp Med* 173:1021–1024, 1991.
25. Maring M, Dunn A, Grail D, Inglese M, Noguchi Y, Richards E, Jungbluth A, Wada H, Moore M, Williamson B, et al.: Characterization of tumor necrosis-factor deficient mice. *Proc Natl Acad Sci U S A* 94:8093–8098, 1997.
26. van der Poll T, Coyle S, Lowry S: Antiinflammatory cytokine responses during clinical sepsis and experimental endotoxemia: sequential measurements of plasma soluble interleukin (IL-1 receptor type II, IL-10, and IL-13. *J Infect Dis* 175:118–122, 1997.
27. Hanna J, Bechtel P, Zhai Y, Youssef F, McLachlan K, Mandelboim O: Novel insights on human NK cells' immunological modalities revealed by gene expression profiling. *J Immunol* 173:6547–6563, 2004.
28. Bade B, Lohrmann J, ten Brinke A, Wolbink AM, Wolbink GJ, ten Berge IJM Jr, Virchow JC, Luttmann W, Hack CE: Detection of soluble human granzyme K in vitro and in vivo. *Eur J Immunol* 35:2940–2948, 2005.