Factor VIIa and Antithrombin III Activity During Severe Sepsis and Septic Shock in Neutropenic Patients

By Rolf M. Meisters, Pier Mannuccio Mannucci, Raffaella Coppola, Thomas Keller, Helmut Ostermann, and Jochen Kienast

Septic shock and multiple organ failure may be associated with coagulation activation, disseminated fibrin formation, and consumption of coagulation inhibitors such as antithrombin III. We have evaluated prospectively coagulation measurements in patients with severe chemotherapy-induced neutropenia. This group of patients was chosen because of their high risk of developing severe septic complications, thus allowing serial prospective coagulation testing before and during evolving sepsis or septic shock. Sixty-two patients with febrile infectious events were accrued to the study. Of these, 13 patients progressed to severe sepsis and 13 additional patients to septic shock as defined according to standard diagnostic criteria. At the onset of fever, factor (F) VIIa activity, FVIIa antigen and antithrombin III (AT III) activity decreased from normal baseline levels and were significantly lower in the group of patients who progressed to septic shock compared with those that developed severe sepsis (medians: 0.3 v 1.4 ng/mL, 21 v 86 U/dL and 45% v 95%; P < .001). The decrease of these measurements in septic shock was accompanied by an increase in prothrombin fragment 1+2 (median: 3.6 v 1.4 nmol/L; P = .05), a marker of thrombin generation. These differences were sustained throughout the septic episode (P < .0001). FVIIa and AT III levels of <0.8 ng/mL and <70%, respectively, at onset of fever predicted a lethal outcome with a sensitivity of 100% and 85%, and a specificity of 75% and 85%, respectively. In contrast, FXIIa-α antigen levels were not different between groups at onset of fever but increased modestly during the course of septic shock (P = .001). Thus, septic shock in neutropenic patients is associated with increased thrombin generation. Furthermore, both FVIIa and AT III measurements are sensitive markers of an unfavorable prognosis.

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MATERIALS AND METHODS

Materials. Recombinant TF used for the TFPI assay was a generous gift from Drs T.S. Edgington and W. Ruf (The Scripps Research Institute, La Jolla, CA) and prepared as described.17 Reconstitution of TF into mixed phosphatidylcholine/phosphatidylserine (70:30, wt/wt) vesicles was performed as reported.18 In this preparation, 8 ng/mL of TF corresponded to one arbitrary unit of TF activity as defined elsewhere.19 Human recombinant FVIIa used for the TFPI assay and as standard in the FVIIa activity assay was purchased from Novo Nordisk (Gentofte, Denmark); human recombinant truncated TF used for the F VIIa assay activity assay was kindly donated by Dr Yale Nemerson (Mount Sinai Hospital, New York, NY); human FX and FXa were purchased from Enzyme Research Laboratories (South Bend, IN). The polyclonal rabbit antirecombinant human TFPI antibody was kindly provided by Drs B.J. Warn-Cramer and S.I. Rapaport (UCSD, La Jolla, CA). All proteins were homogeneous as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.20 All other reagents were of the highest quality available.

Patients. Baseline blood samples were obtained from 97 patients before and after completion of intensive cytostatic chemotherapy. Serial blood sampling was performed for the 62 patients who developed fever (T > 38.3°C) with suspected infection during chemotherapy-induced neutropenia. Herein we report data from the 26 patients who developed either severe sepsis or septic shock. Twenty patients had acute myelogenous leukemia (AML), 4 patients had acute lymphoblastic leukemia (ALL), 1 patient suffered a relapse of non-Hodgkin's lymphoma, and 1 patient had testicular cancer with metas-

From the Department of Internal Medicine, the Division of Hematology/Oncology, University of Münster, Münster, Germany; and the Angelo Bianchi Bonomi Hemophilia and Thrombosis Center, IRCCS Maggiore Hospital and University of Milan, Milan, Italy.

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Address reprint requests to Jochen Kienast, MD, Department of Internal Medicine, Division of Hematology/Oncology, University of Münster, Albert-Schweitzer-Straße 33, D-48129 Münster, Germany.

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Patients were treated according to standardized protocols with respect to both cytostatic and antimicrobial chemotherapy. Episodes of fever with suspected infection during neutropenia (defined as white blood cell [WBC] count < 1,000/μL) were classified according to the criteria proposed elsewhere. Accordingly, febrile episodes were defined by body temperature above 38.3°C in the presence of suspected infection with or without tachycardia (> 90 beats/min) and tachypnea (> 20 breaths/min). Severe sepsis was diagnosed if in addition to all of the above criteria there was one or more of the following signs indicating inadequate organ perfusion: elevated plasma lactate levels (> 1.8 mmol/L), hypoxemia (P\textsubscript{O\textsubscript{2}}/Fi\textsubscript{O\textsubscript{2}} ratio < 0.280) without other pulmonary or cardiovascular disease as the cause; or oliguria of less than 0.5 mL/kg body weight for at least 1 hour. Septic shock was diagnosed if, in addition, there was a sustained decrease in systolic blood pressure to < 90 mm Hg or a drop of > 40 mm Hg for at least 1 hour despite adequate volume replacement.

**Blood sampling.** For baseline determinations, blood was drawn from fasting patients between 7:00 and 8:00 AM. The first blood sample (A) was taken before initiation of chemotherapy. The second blood sample (B) was taken after completion of cytostatic chemotherapy when the WBC count had decreased to less than 1,000/μL. When fever (T > 38.3°C) occurred in the presence of suspected infection blood was collected within 2 hours of onset and subsequently every 6 hours for the first 24 hours and every 12 hours for the following 48 hours. Venipunctures were performed using 21-G butterfly needles after applying minimal stasis. Nine volumes of blood was added to 1 vol of 0.12 mol/L citrate anticoagulant in polypropylene tubes (Sarstedt, Numbrecht, Germany). Tubes were immediately put onto melting ice and centrifuged at 2,000 g for 20 minutes at 4°C. Platelet-poor plasma was removed; aliquots were snap frozen in liquid nitrogen and stored at −80°C until analyzed.

**FVIIa activity.** FVIIa activity was determined in citrated plasma using a prothrombin time-based assay that employs a truncated form of human recombinant TF and FVII-deficient plasma. Controls showed that cold activation of FVII by the sampling procedure did not occur. Normal range in 28 healthy volunteers was 3.4 ± 1.2 ng/mL (mean ± SD).

**FVII antigen (Ag).** FVII Ag was measured by enzyme-linked immunosorbent assay (ELISA) in citrated plasma as previously reported. Normal range in 28 healthy volunteers was 102.8 ± 28.3 U/dL (mean ± SD).

**FVIIa-α.** Plasma levels of FVIIa-α antigen were determined by sandwich ELISA (Asserachrom XIIa; Diagnostica Stago, Asnieres, France) using citrated plasma as described. This assay is based on a capture monoclonal antibody (MoAb 2/215) that discriminates between FVIIa and the zymogen with a sensitivity greater than 1 part in 50,000. The epitope recognized by MoAb 2/215 is located in the C-terminal fragment of FXII and is dependent on conformational changes associated with the activation of the protein. The second antibody is a polyclonal antibody raised in sheep against human FXII. The ELISA responds to FVIIa-α rather than FVIIa-β because approximately 50% of FVIIa-α added to normal plasma in vitro was recovered compared with only 1.8% of the FVIIa-β added (concentration ranges, 0 to 500 ng/mL). The antibody recognizes FVIIa-α complexed with C1-inhibitor 0.44 times that of the free enzyme, whereas the recognition of FXIIa-β complexed with the inhibitor is one tenth of the free enzyme. Normal range in 28 healthy volunteers was 3.3 ± 1.4 ng/mL (mean ± SD).

**F1+2, TAT, and AT III activity.** F1+2, TAT, and AT III activity levels were determined in citrated plasma using commercially available kits obtained from Behringwerke (Marburg, Germany); F1+2 (Enzygnost F1+2; normal range, 0.44 to 1.11 mmol/L); TAT (Enzygnost TAT Micro; normal range, 1.0 to 4.1 μg/L); AT III (AT III Berichrom; normal range, 75% to 125%). F1+2 and TAT measurements were performed on a microplate reader (EAR 340 AT EASY Reader; SLT-Lab Instruments, Grödig-Salzburg, Austria) and the Berichrom AT III assay on a chromotimer (Behringwerke).

**Assay of TFPI activity.** The two-stage chromogenic substrate assay was based on the ability of a test sample to inhibit FVIIa/TF catalyzed activation of FX in the presence of FXa as described elsewhere. TFPI activity was expressed in percent of the activity of a reference plasma pool; normal range (30 females, 30 males; < 60 years of age): 85% to 127% (mean ± SD). In control experiments, greater than 90% of measured TFPI activity was inhibited by preincubation of plasma with a polycyclonal rabbit-antihuman TFPI antibody.

**Statistical analysis.** For statistical analysis the SPSS program (SPSS Inc, Chicago, IL) was used. Values were tested for normal distribution using the Littlefors test. Because none of the variables showed a normal distribution, the Kruskal-Wallis test was used for analysis of variance. Differences between groups were calculated by the nonparametric Wilcoxon-Mann-Whitney test for unpaired samples. Two-sided P values of < 0.05 were considered significant.

**RESULTS**

From a total of 62 patients accrued to the study, 36 patients developed uncomplicated febrile episodes. Thirteen patients met the criteria of severe sepsis, and septic shock was diagnosed in 13 additional patients. All patients with septic shock died, whereas all patients with severe sepsis or uncomplicated febrile episodes survived. The patient characteristics, including the primary site of infections, are given in Table 1.

The type of PE and PE-like symptoms had occurred in 7 of 13 patients with septic shock (54%) and 6 of 13 patients with severe sepsis (46%). Of the patients who subsequently developed severe sepsis and those who developed septic shock (Figs 1 and 2; Wilcoxon-Mann-Whitney test for unpaired samples). At the onset of febrile episodes progressing to septic shock (time point 0), an immediate, marked decrease in FVIIa activity, FVII Ag, and AT III activity was accompanied by a progressive increase in F1+2.

**Table 1. Clinical Data of Patients With Severe Sepsis and Septic Shock**

<table>
<thead>
<tr>
<th></th>
<th>Severe Sepsis</th>
<th>Septic Shock</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>Age (yr, mean ± SD)</td>
<td>49 ± 18</td>
<td>54 ± 14</td>
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<tr>
<td>AML/ALL/others</td>
<td>11/2/0</td>
<td>9/2/2</td>
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<td>Primary site of infection:</td>
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<td></td>
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<td>Lung</td>
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<tr>
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<td>Central venous catheters</td>
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<td>Gram-positive bacteria</td>
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<td>4</td>
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<tr>
<td>Yeasts</td>
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<td>1</td>
</tr>
<tr>
<td>WBC count (cells/μL) on set of fever</td>
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<td>100*</td>
</tr>
<tr>
<td>Hematocrit (%) on set of fever</td>
<td>26.7*</td>
<td>26.0*</td>
</tr>
</tbody>
</table>

* Median values with interquartile ranges (IQR) given in parentheses.
FACTOR VIIa AND ANTITHROMBIN III DURING SEPSIS

Fig 1. FVIIa activity, FVII Ag, FVIIa/VII Ag ratio, and FXIIa-α levels in patients with severe sepsis (○) and septic shock (●). Data are presented as medians with interquartile ranges (IQRs). On the abscissa, A denotes the first measurement before initiation of cytostatic chemotherapy, B after completion of cytostatic chemotherapy when the WBC had decreased to <1,000/μL. As soon as fever (T > 38.3°C) occurred in the presence of suspected infection blood was collected within 2 hours (time point 0) and subsequently every 6 hours for the first 24 hours and every 12 hours for the following 48 hours. The respective P value denotes the difference between the two groups evaluated by analysis of variance using the Kruskal-Wallis test.

Fig 2. F1+2, TAT, TFPI, and AT III activity levels in patients with severe sepsis (○) and septic shock (●). Data are presented as medians with IQRs.

and TAT levels (Figs 1 and 2). During the entire study period FVIIa activity, FVII Ag, and AT III activity were significantly lower in the group of patients with septic shock compared to the patients with severe sepsis, whereas F1+2
and TAT remained higher in shock patients (all P values < .0001; Figs 1 and 2). Like FVIIa activity, the FVIIa/FVII Ag ratio was significantly lower in septic shock patients than in patients with severe sepsis (P < .0001, Kruskal-Wallis test; Fig 1). FXIIa-α concentrations increased modestly over time in shock patients, and finally reached significantly higher levels in these patients than in those with severe sepsis (P = .001; Fig 1). TFPI activity did not change over time and was not significantly different between the two groups (Fig 2).

Even at time point 0 (ie, the first blood sample drawn within 2 hours of the onset of fever), FVIIa activity, FVII Ag, and AT III activity were significantly lower in the group of patients who progressed to septic shock compared with the levels found in the group that developed severe sepsis (P values < .001; Fig 3). A similar analysis performed for the other coagulation measurements showed that F1+2 levels tended to be higher in the group of septic shock patients (P = .05; data not shown), whereas FXIIa-α, TAT, and TFPI results for the two groups were not significantly different (P = .51, .09 and .6; data not shown). Furthermore, no significant differences were observed in WBC counts and hematocrit values between both groups at onset of fever (Table 1). The median time that elapsed between detection of fever and the diagnosis of severe sepsis or septic shock was 16 hours (12 to 24 hours) and 12 hours (6 to 24 hours), respectively (interquartile ranges in parentheses). When the peak values of each parameter within 24 hours after onset of fever were used in the comparative analysis, the statistical conclusions were unchanged (data not shown).

The predictive value of each measurement for fatal outcome was evaluated. The results of samples obtained within 2 hours of the onset of fever were used for calculations of sensitivity, specificity, and positive and negative predictive values. Threshold levels were chosen arbitrarily on the basis of the data obtained (Table 2). FVIIa and AT III levels of <.0.8 ng/mL and <70%, respectively, predicted a lethal outcome with a sensitivity of 100% and 85%, and a specificity of 75% and 85%, respectively. Thus, the likelihood that a septic patient with an FVIIa level <0.8 ng/mL or an AT III level <70% ultimately died was 0.81 and 0.85, respectively (positive predictive value). On the other hand, the likelihood that a septic patient with an FVIIa level >0.8 ng/mL or an AT III level >70% survived was 1.0 and 0.85, respectively (negative predictive value). The sensitivity, specificity, and positive and negative predictive values calculated for the remaining coagulation variables showed that FVIIa and AT III levels were the most useful measurements in assessing prognosis in patients with sepsis (Table 2).

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**DISCUSSION**

Under resting conditions in normal individuals coagulation activation is initiated in vivo by the molecular complex of the integral membrane protein TF, functioning as a cofactor and receptor, and the enzyme F VIIa. The importance of the TF pathway for coagulation activation in sepsis is indicated by recent in vivo studies of experimental endo-
toxemia in baboons and chimpanzees and using MoAbs to TF, FVIIa, and FXIIa. However, the mechanisms that initiate coagulation in patients with sepsis are not clear. The clinical situation differs from experimental models in that the causative microorganisms or microbial toxins may vary greatly (eg, gram-negative as well as gram-positive bacteria, fungi) and the septic challenge is less uniform with respect to duration and intensity.

We report a prospective, comparative study of coagulation enzymes (FVIIa, FXIIa-α), markers of thrombin generation (F1+2, TAT), and coagulation inhibitors (AT III, TFPI) in patients with sepsis of variable severity as defined by stringent clinical criteria. The homogeneous group of patients chosen was known to be at high risk of developing septic complications because of severe neutropenia induced by chemotherapy. Although our choice of patients with neutropenia may limit the generalization of our conclusions, it allowed prospective serial measurements in patients with a predictably high incidence of severe septic complications.

We have demonstrated a significantly greater decrease from baseline in FVIIa activity, FVII Ag, and AT III activity in patients who developed septic shock compared to those with severe sepsis. This decline was manifest within the first few hours of the febrile infectious events (Figs 1 through 3). Parallel to the decrease of these measurements, in septic shock there was increased thrombin generation as reflected by a marked and sustained increase from baseline values of the plasma markers F1+2 and TAT (Fig 2).

The mechanism of increased thrombin generation in septic shock cannot be established unequivocally from these findings. Initially we surmised that hypercoagulability might be related to a TF-dependent mechanism. However, the decrease of FVIIa levels is not consistent with this hypothesis because an increase of this enzyme would be expected if the TF pathway was activated. The rapid decrease in FVIIa activity, FVII Ag, and AT III activity could be due to increased turnover, redistribution between plasma and the cell surface (eg, binding of FVIIa/VII to cell-surface associated TF), impaired liver synthesis, or combinations of these mechanisms. Although the short half-life of FVII (approximately 6 to 7 hours) renders impaired liver synthesis a tenable explanation, the much longer half-life of AT III (approximately 48 hours) tends to exclude this mechanism. Similarly, it is unlikely that the decrease in FVIIa, FVII Ag, and AT III is due to inactivation or proteolysis by proteinases like elastase released from activated neutrophils, because little elastase is released during septicemia in patients with severe neutropenia. Moreover, no significant differences were observed in hematocrit values between the two groups at onset of fever (Table 1). Thus, the considerable decrease in FVIIa, FVII Ag, and AT III in septic shock cannot be explained by dilution due to volume replacement. On the whole, the decrease in FVIIa/VII Ag ratios with the parallel increase in F1+2 and TAT levels argues in favor of an increased turnover of FVIIa/VII Ag and AT III rather than reduced synthesis or proteolytic degradation (Figs 1 and 2). The modest increase in FXIIa-α in septic shock suggests that the contribution of the contact system to coagulation activation in septic shock is minor. This is in accordance with the data from animal studies. TFPI consumption does not seem to play a role in septic shock because TFPI activity levels were elevated above the normal range and there was no significant difference between severe sepsis and septic shock (Fig 2). This is in agreement with earlier findings.

Initial FVIIa and AT III activity levels were of similar prognostic value for the prediction of fatal outcome in this clinical setting (Table 2). A comparable prognostic value for AT III has been described in several earlier studies which included patients with septic and nonseptic DIC. Similarly, measurements of FVIIa activity may help the assessment of prognosis in severe sepsis. Because the median time that elapsed between detection of fever and the diagnosis of septic shock was 12 hours (interquartile range, 6 to 24 hours), initial FVIIa and AT III activity measurements may be useful markers for impending deterioration in febrile neutropenic patients. They would allow patients at risk of septic shock to be detected in an early phase of the septic episode when therapeutic interventions might improve outcome.

In summary, in patients with septic shock we observed a rapid decrease in FVIIa/VII Ag that has not been shown previously. It seems to be associated with the severity of the inflammatory response because it occurs in patients with fatal septic shock, but not in patients with reversible severe sepsis. This phenomenon may be related to coagulation activation via the TF pathway because it coincided with the increase in plasma levels of markers of thrombin generation. FVIIa as well as AT III activity measurements may prove to be a valuable aid in determining the severity and the prognosis of the septic inflammatory response in individual patients.

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