Interaction between inflammation, coagulation and fibrinolysis during infection

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Chapter 8

Inhibition of the tissue factor-factor VIIa pathway does not influence the inflammatory or antibacterial response to abdominal sepsis induced by Escherichia coli in mice

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Abstract

Anticoagulants have gained increasing attention in the treatment of sepsis. Inhibition of the tissue factor (TF)/Factor (F)VIIa pathway has been shown to attenuate the activation of coagulation and to prevent death in a gram-negative bacteremia primate model of sepsis. To determine the role of the TF/FVIIa complex in the host response to peritonitis, mice received an intraperitoneal injection of live *E. coli* with or without concurrent treatment with recombinant Nematode Anticoagulant Protein c2 (rNAPc2), a selective inhibitor of the TF/FVIIa pathway. Peritonitis was associated with an increase in TF expression at tissue level, activation of coagulation, as reflected by elevated levels of thrombin-antithrombin complexes, and by increased fibrin(ogen) deposition in liver and lungs. rNAPc2 strongly attenuated this procoagulant response, but did not influence the inflammatory response (histopathology, leukocyte recruitment to the peritoneal cavity, cytokine and chemokine levels). Moreover, rNAPc2 did not alter bacterial outgrowth locally or dissemination of the infection, and survival was not different in rNAPc2 treated and control mice. These data suggest that TF-FVIIa activity contributes to the coagulation activation during *E. coli* peritonitis, but does not play a role in the inflammatory response or antibacterial host defense.

Introduction

Severe peritonitis and the accompanying systemic inflammatory response syndrome are important causes of death in adult intensive care units [1]. The mortality of patients with abdominal sepsis can be as high as 60%, which contrasts with 25-30% overall mortality rate of sepsis in general [2]. Although different bacteria have been identified as causative organisms in abdominal sepsis, *Escherichia coli* remains one of the most common pathogens in intraperitoneal infections [2-4].

Sepsis is frequently associated with a profound activation of the coagulation system, which can give rise to the clinical syndrome of disseminated intravascular coagulation (DIC), characterized by extensive fibrin depositions in multiple organs and microvascular thrombosis [5, 6]. A pivotal mechanism in the pathogenesis of DIC is the activation of the tissue factor (TF)/factor (F)VIIa dependent pathway of coagulation [5, 6]. Under physiological conditions, TF cannot be detected on the luminal surface of the vascular endothelium [7], and only in very low quantities on circulating blood cells [7-9]. However, during infection and after stimulation with endotoxin or proinflammatory cytokines, TF can be rapidly induced on blood mononuclear cells [9-11] and on endothelial cells [12-14]. Different strategies that inhibited the TF-FVIIa pathway prevented the activation of the coagulation system in experimental endotoxemia and bacteremia in humans and nonhuman primates, including antibodies directed against TF or FVII/VIIa, active site inhibited FVIIa (Dansyl-Glu-Gly-Arg chloromethylketone or DEGR-FVIIa) and TF pathway inhibitor (TFPI) [15-21]. Importantly, in lethal sepsis in baboons induced by direct intravenous administration of high doses of *E. coli*, inhibition of the TF/FVIIa complex not only prevented DIC, but also resulted in an increased survival [16, 18-20].
contrast with interventions that block the coagulation system more downstream, i.e. administration of catalytically inactive FXa (DEGR-FXa) failed to influence lethality of bacteremic baboons, while completely inhibiting the development of DIC [22]. This has led to the hypothesis that inhibition of the TF-FVIIa pathway protects against death not merely by a reduction in the TF-mediated coagulation response, but also through the attenuation of a TF-mediated inflammatory response that appears distinct from TF-mediated coagulation initiation in this experimental setting. Recent studies have further suggested that during sepsis, activation of the coagulation system and induction of inflammatory responses may be linked in a bimodal manner. Indeed, while cytokines are involved in the changes in the coagulation system following infection or endotoxemia [5, 6], a significant body of evidence supports the concept that in turn, activated coagulation factors can provoke a proinflammatory response [5, 23-26]

Knowledge of the role of the TF/FVIIa complex in host defense against peritonitis is limited to one earlier investigation [27]. In that study rabbits were given an intraperitoneal inoculation of a suspension containing hemoglobin, porcine mucine and viable E. coli. Treatment with recombinant TFPI had beneficial effects on a number of different physiological parameters, including arterial blood pressure and arterial oxygenation, and enhanced survival. The effect of TF inhibition on antibacterial defense mechanisms was not reported [27]. In the present study, we sought to determine the role of the TF-FVIIa complex in the procoagulant, inflammatory and antibacterial host response to E. coli peritonitis using an established mouse model [28], by treating mice with recombinant Nematode Anticoagulant Protein c2 (rNAPc2), a potent and selective small protein inhibitor of the TF-FVIIa pathway [29, 30].

Material and methods

Animals

Male C57BL/6 wild-type mice were purchased from Harlan CPB (Zeist, The Netherlands). All mice were housed (five per cage) in the same temperature-controlled room with alternating 12h light/dark cycles, and were allowed to equilibrate for at least 5 days before the study. Animals were provided regular mice chow (SRM-A; Hope Farms, Woerden, The Netherlands) and water ad libitum. Mice were used at 8-10 weeks of age. The experiments were approved by the Institutional Animal Care and Use Committee of the Academic Medical Center, Amsterdam, The Netherlands.

rNAPc2

rNAPc2 (Corvas International, Inc, San Diego, California, USA) was produced as described previously [30]. In brief, rNAPc2 was manufactured as a secreted protein in the yeast Pichia pastoris. It was purified to >98% purity using a series of chromatographic steps prior to being formulated in a modified phosphate-buffered saline (PBS) solution at pH 7. Endotoxin levels were determined to be <1 EU/mg and
well within the range considered safe for administration to humans. [31, 32]. In a first experiment, in which the effect of rNAPc2 on the activated prothrombin time (PT) and partial thromboplastin time (aPTT) was determined, rNAPc2 was given as a single subcutaneous dose (10 mg/kg) diluted in 100 μl sterile phosphate buffered saline (PBS). In subsequent peritonitis studies, rNAPc2 was given subcutaneously every 6 h starting 1 h prior to infection for a total of maximal 3 injections (until 17 h after infection) at a dose of 10 mg/kg per injection (in 100 μl PBS). Controls received PBS (100 μl) subcutaneous every 6 h in these studies. We chose to administer rNAPc2 subcutaneously since this route of administration has been used in animals and humans previously [31, 32].

**Induction of peritonitis**

Peritonitis was induced as described previously [28]. In brief, *Escherichia coli* (*E. coli*) O18:K1 was cultured in Luria Bertani medium (LB; Difco, Detroit, MI) at 37°C, harvested at mid-log phase, and washed twice with sterile saline before injection to clear the bacteria of medium. Mice were injected intraperitoneally (i.p) with $10^4$ *E. coli* colony-forming units (CFU) in 200 μl sterile isotonic saline in all experiments except for an additional survival study in which $6 \times 10^3$ CFU was given. The inoculum was plated immediately after inoculation on blood agar plates to determine viable counts.

**Collection of samples**

For comparison of bacterial outgrowth and host responses in rNAPc2 treated and control mice, animals were sacrificed at an early time point (6h) and at a time point directly before mortality occurred (20h). At the time of sacrifice, mice were first anaesthetized by inhalation of isoflurane (Abbott Laboratories Ltd., Kent, UK) / O2 (2% / 21). A peritoneal lavage was then performed with 5 ml sterile isotonic saline using an 18-gauge needle, and peritoneal lavage fluid was collected in sterile tubes (Plastipack; Becton-Dickinson, Mountain View, CA). The recovery of peritoneal fluid was > 90% in each experiment and did not differ between groups. After collection of peritoneal fluid, deeper anesthesia was induced by i.p. injection of 0.07 ml/g FFM mixture (Fentanyl (0.315 mg/ml)-Fluanisone (10 mg/ml) (Janssen, Beersen, Belgium), Midazolam (5 mg/ml) (Roche, Mijdrecht, The Netherlands)). Next, blood was drawn out of the vena cava inferior with a sterile syringe, and transferred to tubes containing heparin or citrate.

**Assays**

The PT and aPTT were measured in plasma anticoagulated with 3.2% sodium citrate (1/10 vol) by one-stage clotting assays using plasma three times diluted in saline. In short, diluted plasma was incubated with thromboplastin PT-fibrinogen or actin FS (Dade Behring, Buckinghamshire, UK) during 5 minutes at 37°C for measurement of PT and aPTT respectively. Next, PT and aPTT measurements were started by addition.
of 20 mM calcium chloride, and time to agglutination was measured using a ACL 7000 analyzer (Instrumentation Laboratory, Lexington, MA). Thrombin-antithrombin complexes (TATc) were determined in citrated plasma as a measurement of thrombin generation. TATc were measured with a mouse-specific, ELISA-based method [33]. Cytokines and chemokines were measured by cytometric bead array (CBA, Pharmingen, San Diego, CA) (tumor necrosis factor-α (TNF), interleukin (IL)-6, IL-10) or by ELISA (R & D Systems, Abingdon, UK; macrophage inflammatory protein (MIP)-2 and cytokine-induced neutrophil chemoattractant (KC)) following the instructions of the manufactures.

**Histological examination**

Directly after sacrificing the mice, samples from the liver and the lung were removed, fixed in 4% formaline, and embedded in paraffin for routine histology. Sections of 4 μm thickness were stained with haematoxylin and eosin. All slides were coded and scored by a pathologist without knowledge of the type of mice and treatment. TF and fibrin stainings were performed on paraffin slides after deparaffinization and rehydration using standard immunohistochemical procedures. For both stainings endogenous peroxidase activity was quenched using 1.5% H₂O₂ in PBS and non-specific binding blocked with 10% normal goat serum. Primary antibodies used were rabbit anti-mouse TF polyclonal Ab (produced in our laboratory) for the detection of TF, and biotinylated goat anti-mouse fibrinogen Ab (Accurate Chemical & Scientific Corporation, Westbury, NY, USA) for the fibrin staining. The generation and characterization of the anti-TF antibody will be described in detail elsewhere (De Waard V. et al, manuscript in preparation). In brief, rabbits were immunized with five immunogenic murine TF peptides corresponding to different amino acid sequences within the extracellular domain of murine TF. For immunohistochemical staining of TF, the immunoglobulin fraction purified from the polyclonal antiserum against peptide 5, corresponding with amino acids 225-240, was used since this bound native TF in a specific manner. TF staining of brain and trachea of mice exposed to endotoxin colocalized with TF mRNA as determined by in situ hybridization and was abolished in the presence of peptide 5 (data not shown). As secondary antibodies biotinylated swine anti-rabbit Ab (DAKO) were used for the TF staining. For both stainings endogenous peroxidase activity was quenched using 1.5% H₂O₂ in PBS, and ABC solution (DAKO) was used as staining enzyme. 0.03% H₂O₂ and 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma) in 0.05 M Tris pH 7.6 was used as substrate. Examination of immunohistochemical stained slides was performed on coded samples. For TF and fibrin, the presence or absence of positive TF cells or fibrin staining in 25 fields at a magnification of 40 x was determined.

**Enumeration of bacteria**

The number of E. coli CFU was determined in peritoneal fluid, blood and liver homogenates. For this, livers were harvested and homogenized at 4°C in five volumes of sterile isotonic saline with a tissue homogenizer (Biospect Products, Bartlesville, OK), which was carefully cleaned and disinfected with 70% ethanol after each
homogenization. Serial 10-fold dilutions in sterile isotonic saline were made from these homogenates, peritoneal lavage fluid and blood, and 50-µl volumes were plated onto sheep-blood agar plates and incubated at 37°C and 5% CO₂. CFU were counted after 16h.

Cell counts and differentials
Leukocyte counts were determined using a coulter counter (Beckman coulter, Fullerton, CA). Subsequently, peritoneal fluid was centrifuged at 1400 x g for 10 min; the supernatant was collected in sterile tubes and stored at −20°C until determination of cytokines. The pellet was diluted with PBS until a final concentration of 10⁵ cells/ml and differential cell counts were done on cytospin preparations stained with a modified Giemsa stain (Diff-Quick; Dade Behring AG, Düdingen, Switzerland) according to the manufacturer’s instructions.

Statistical analysis
Data were analyzed using the SPSS statistical package. Data are expressed as means ± SEM, unless indicated otherwise. Changes in PT and aPTT after a single dose of rNAPc2 were analyzed by one-way analysis of variance followed by Dunnett’s (posthoc) test. Comparisons between groups were conducted using the Mann-Whitney U test. Survival curves were compared by log-rank test. A value of p < 0.05 was considered to represent a statistically significant difference.

Figure 1: Increased expression of tissue factor (TF) in lung after induction of peritonitis. TF immunostaining of lung 20h after i.p. injection with sterile saline (A), or after i.p. injection of 10⁴ CFU E. coli with PBS subcutaneously every 6 hours (B), or after i.p. injection of 10⁴ CFU E. coli with rNAPc2 (10 mg/kg) subcutaneously every 6 hours (C). Subcutaneous injections were started 1h prior to infection and continued until 17h postinfection. Slides show a similar expression of TF by inflammatory cells in PBS and rNAPc2 treated mice with peritonitis. Original magnification x 40. Representative slides are shown from a total of 5 mice per group.

Results
TF expression is upregulated during E.coli peritonitis
To determine whether TF expression is upregulated during peritonitis, mice received an i.p. injection with 200 µl normal saline containing 10⁴ CFU E.coli or 200 µl sterile 104.
saline as a control. As shown in Figure 1, inflammatory cells that infiltrated the lungs in the course of peritonitis expressed TF (Fig 1b). Basal expression of TF was not observed in the lungs of control mice (Fig 1a). As shown in Fig 1c, TF expression was not altered by rNAPc2 treatment (see further).

Peritonitis induces activation of the coagulation system

Next we determined whether our model of peritonitis was associated with activation of coagulation. To obtain insight into the presence and extent of coagulation activation at the site of the infection, we measured the concentrations of TATc in peritoneal lavage fluid obtained before and at 6 or 20h after infection (Figure 2). Induction of peritonitis resulted in local thrombin generation as reflected by an increase in TATc levels in peritoneal lavage fluid (P < 0.05 for both 6 and 20h versus t=0). In addition, peritonitis was accompanied by fibrin(ogen) depositions in liver and lungs (see further).

![Figure 2: Peritonitis induces a local increase in thrombin-antithrombin complexes (TATc). Mean (± SE) concentrations of TATc in peritoneal lavage fluid obtained 6h and 20h after i.p. administration of 10^4 CFU E. coli. n = 8 per time point. * P < 0.05 vs. t=0h; # P < 0.05 vs. t=6h.](image)
Kinetics of the anticoagulant effect of rNAPc2

To establish the anticoagulant properties of rNAPc2 in mice, rNAPc2 was given as a single subcutaneous dose (10 mg/kg), and the PT and aPTT were measured as a marker for the anticoagulant effect of rNAPc2, before, and at different time points thereafter (Figure 3). rNAPc2 prolonged the PT from 30.3 ± 0.5 sec to 62.5 ± 0.8 sec and the aPTT from 48.6 ± 2.2 sec at baseline to 197.9 ± 3.6 sec 0.5h after the injection. The anticoagulant effect of rNAPc2 lasted 4-6h. Based on these experiments, in further studies rNAPc2 was given subcutaneously every 6 h after infection (10 mg/kg in 100 μl PBS), starting 1h prior to infection; controls received PBS (100 μl) subcutaneously every 6h.

Figure 3: rNAPc2 prolongs the PT and aPTT. rNAPc2 (10 mg/kg) was given as a single subcutaneous dose, and the PT and aPTT were measured at the time points indicated. Data are means ± SE of 8 mice per time point. * P < 0.05 vs. t=0h.

Figure 4: rNAPc2 inhibits coagulation activation during peritonitis. Mean ± SE of TAT-c values in peritoneal lavage fluid and plasma. Mice (n=8 per group) were i.p. infected with 10⁴ CFU E. coli at t=0h. rNAPc2 (open bars) was given subcutaneously every 6h after induction of peritonitis at a dose of 10 mg/kg of body weight starting 1h prior to infection until 17h postinfection; controls (filled bars) received PBS subcutaneously every 6h. Mice were sacrificed 20h after infection. *P < 0.05 vs. control mice.
rNAPc2 inhibits coagulation activation during peritonitis

To establish the role of the TF/FVIIa complex in the procoagulant response to peritonitis, we measured TATc levels in plasma and peritoneal lavage fluid obtained 20h after infection from mice treated with rNAPc2 or PBS (Figure 4). Mice treated with rNAPc2 displayed significantly reduced TATc concentrations in both plasma and peritoneal lavage fluid (P < 0.05 versus PBS).

Table I

<table>
<thead>
<tr>
<th></th>
<th>T6h</th>
<th>T20h</th>
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<tr>
<td></td>
<td>Control</td>
<td>rNAPc2</td>
</tr>
<tr>
<td><strong>Total cells</strong>&lt;br&gt;(x10⁶/ml)</td>
<td>2.17 ± 0.42</td>
<td>2.31 ± 1.32</td>
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<tr>
<td><strong>Neutrophils</strong></td>
<td>1.15 ± 0.65</td>
<td>1.17 ± 0.99</td>
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<tr>
<td><strong>Macrophages</strong></td>
<td>0.72 ± 0.23</td>
<td>1.03 ± 0.25</td>
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<tr>
<td><strong>Others</strong></td>
<td>0.30 ± 0.10</td>
<td>0.11 ± 0.07</td>
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</table>

Leukocyte counts in peritoneal lavage fluid Data are mean ± SE (n = 8 mice per group for each time point) at 6 or 20h after i.p. administration of E. coli (10⁴ CFU). rNAPc2 was given subcutaneously every 6h after induction of peritonitis at a dose of 10 mg/kg of body weight starting 1h prior to infection until 17h postinfection; controls received PBS subcutaneously every 6h. Differences between treatment groups were not significant.
Figure 5: Histopathology. Representative H&E staining of liver (A,B) and lung (C,D) and fibrin(ogen) immunostaining of lung (E,F) 20 hours after i.p. infection with 10⁴ CFU E. coli in control mice (A,C,E) and rNAPc2 treated mice (B,D,F). rNAPc2 was given subcutaneously every 6h after induction of peritonitis at a dose of 10 mg/kg of body weight starting 1h prior to infection until 17h postinfection; controls received PBS subcutaneously every 6h. rNAPc2 treated mice displayed less extensive liver necrosis than PBS treated mice. Both groups presented similar interstitial inflammatory infiltrate in the lungs. In PBS-treated mice thrombi were easily found in liver and lung (insert C). Accordingly fibrin(ogen) deposition was also prominent, in contrast to rNAPc2-treated mice in which thrombi could not be found and fibrin(ogen) deposition was minimal. Representative slides of 5 mice per group are shown.
In addition, rNAPc2 abolished the formation of thrombi in liver and lungs which are commonly observed in the course of peritonitis (insert, Figure 5C). Moreover, fibrin(ogen) deposition in vessels and along the pleura (Figure 5E) was also prevented by rNAPc2 treatment (Figure 5F). As expected, rNAPc2 did not alter TF expression in lungs as assessed by immunostaining (Figure 1C). Together these data indicated that rNAPc2 exerted a potent anticoagulant effect during peritonitis.

rNAPc2 does not influence the inflammatory response to peritonitis

The TF/FVIIa complex has been implicated to play a role in the regulation of inflammatory responses to severe infection by a mechanism that is not strictly linked to its procoagulant properties. To determine the influence of the TF/FVIIa complex on the inflammatory response to E. coli peritonitis several parameters were evaluated. Upon histopathological examination, PBS treated mice displayed foci of liver necrosis associated with thrombi formation (Figure 5A). The extent of liver necrosis was less severe in rNAPc2 treated mice (Figure 5B). In the lungs of both rNAPc2 and PBS treated mice a similar dense inflammatory infiltrate was observed in inter-alveolar septa (Fig 5C and D, respectively). In addition, rNAPc2 did not influence other inflammatory responses, such as the influx of neutrophils (Table I) or the release of proinflammatory cytokines and chemokines into the peritoneal lavage fluid or the circulation (Table II).

Table II

<table>
<thead>
<tr>
<th>Data (pg/ml)</th>
<th>Plasma Control</th>
<th>rNAPc2</th>
<th>Peritoneal Fluid Control</th>
<th>rNAPc2</th>
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<tbody>
<tr>
<td>MIP-2</td>
<td>288 ± 87</td>
<td>223 ± 51</td>
<td></td>
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<tr>
<td>KC</td>
<td>387 ± 183</td>
<td>286 ± 35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>770 ± 40</td>
<td>581 ± 120</td>
<td>851 ± 76</td>
<td>791 ± 88</td>
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<tr>
<td>TNF-α</td>
<td>111 ± 8</td>
<td>102 ± 18</td>
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<td>IL-10</td>
<td>909 ± 195</td>
<td>829 ± 196</td>
<td>684 ± 127</td>
<td>736 ± 173</td>
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</tbody>
</table>

Chemokine and cytokine concentrations Data are mean ± SE (n = 8 mice per group) at 20h after i.p. administration of E. coli (10⁹ CFU). rNAPc2 was given subcutaneously every 6h after induction of peritonitis at a dose of 10 mg/kg of body weight starting 1h prior to infection until 17h postinfection; controls received PBS subcutaneously every 6h. Differences between treatment groups were not significant.
Figure 6: rNAPc2 does not influence bacterial clearance during peritonitis. Bacterial outgrowth (expressed as medians with interquartile ranges of CFU/ml) in peritoneal fluid (left panel), blood (middle panel) and liver (right panel) at 6 and 20h after infection. Mice were i.p. infected with $10^4$ CFU *E. coli* at *t*=0h. rNAPc2 (open bars) was given subcutaneously 1h prior to infection and every 6h thereafter until 17h postinfection; controls (filled bars) received PBS subcutaneously every 6h (n=8 per treatment group at each time point). Differences between treatment groups were not significant.

**Bacterial outgrowth is not influenced by rNAPc2**

To obtain insight into the role of TF/FVIIa activation in early antibacterial defense against peritonitis, we compared the number of *E.coli* CFU at 6 and 20h after infection in peritoneal lavage fluid (the site of the infection), blood (to evaluate to which extent the infection became systemic), and in liver of rNAPc2 and PBS treated mice (Figure 6). Both groups had similar bacterial loads at all three body sites at each time point. Blood agar plates only displayed the *E. coli* strain that was administered, excluding dissemination of intestinal organisms.

**rNAPc2 does not influence survival**

To investigate the role of TF-factor VIIa in the outcome of abdominal sepsis, we performed two independent survival studies, one using the inoculum that was also given in the experiments presented above ($10^4$ CFU) and one with a lower inoculum ($6 \times 10^3$ CFU). After infection with the higher bacterial dose all mice died within 2 days irrespective of rNAPc2 treatment (Figure 7A). After infection with the lower dose, 4/10 control and 7/10 rNAPc2 treated mice died (P = 0.2 for the difference between groups; Figure 7B).
Figure 7: rNAPc2 does not influence survival. Survival after i.p. infection with $10^4$ CFU (A; $n = 15$ per group) or $6 \times 10^3$ CFU (B; $n = 10$ per group) *E. coli* in control treated (closed squares) and rNAPc2 treated mice (open squares). rNAPc2 was given subcutaneously every 6 h after induction of peritonitis at a dose of 10 mg/kg of body weight starting 1 h prior to infection until 17 h postinfection; controls received PBS subcutaneously every 6 h. Differences between treatment groups were not significant.

Discussion

The role of TF/FVIIa pathway in activation of the coagulation system during sepsis has been firmly established in experimental models using intravenous challenges of live bacteria or bacterial products such as endotoxin [15-21]. In addition, in otherwise lethal bacteremia in baboons induced by intravenous infusion of high doses of live *E. coli*, inhibition of the TF/FVIIa pathway not only prevented DIC but also lethality [16, 18-20]. These investigations suggested that blocking the TF/FVIIa pathway might have anti-inflammatory effects that at least in part are unrelated to inhibition of the TF-mediated procoagulant response [5, 6]. In the present study we sought to determine the functional role of the TF/FVIIa complex in the host coagulant, inflammatory and antibacterial response to intra-abdominal sepsis induced by i.p. injection of live *E. coli*. We here demonstrate that in our murine model of septic peritonitis, TF expression is increased in lungs and liver of infected mice, which is associated with activation of coagulation as reflected by rises in TATc levels in peritoneal lavage fluid and plasma, and fibrin depositions in lungs and liver. Inhibition of the TF-FVIIa complex with rNAPc2 reduced coagulation activation, but did not influence the inflammatory response or antibacterial defense mechanisms. These findings suggest that rNAPc2 functions primarily as an anticoagulant during murine *E. coli* peritonitis and that TF is likely not involved in the host inflammatory response in this setting.

In the current investigation rNAPc2 was used to inhibit the TF-FVIIa pathway. This small protein was originally isolated from the hematophagous nematode hookworm *Ancylostoma caninum*, and subsequently produced in recombinant form using the yeast *Pichia pastoris* [30]. rNAPc2 inhibits TF-FVIIa mediated coagulation by a
mechanism of action that differs from that of the physiological inhibitor of TF, TFPI. Indeed, whereas rNAPc2 binds to zymogen factor X (FX) or factor Xa (FXa) prior to the formation of an inhibitory complex with TF-FVIIa [29], TFPI binds only to FXa at its catalytic center, followed by the formation of the quaternary TFPI/FXa – TF/FVIIa complex. The utilization of zymogen FX as an inhibitory scaffold by rNAPc2 obviates the need for forming FXa prior to the inhibition of the TF-FVIIa complex. rNAPc2 also has been demonstrated to inhibit FIX activation by the TF/FVIIa complex [29]. Several previous studies have established the efficacy of rNAPc2 in attenuating coagulation in vivo, i.e. rNAPc2 completely prevented endotoxin-induced coagulation activation in chimpanzees [34], and strongly reduced the incidence of acute deep vein thrombosis in patients undergoing unilateral knee arthroplasty compared to the best current prophylactic regimens [31].

The TF/FVIIa complex has been implicated to play a crucial role in the pathogenesis of sepsis, which goes beyond its role in activation of the coagulation system. Indeed, whereas downstream intervention in the coagulation cascade by treatment with DEGR-FXa (an competitive inhibitor of prothrombinase-mediated thrombin generation) strongly reduced the coagulopathy related to experimental sepsis in baboons, this strategy did not increase survival [22]. However, in the same model of systemic E. coli infection, inhibition of the TF-FVIIa complex by either an anti-TF antibody, TFPI or catalytically inactive FVIIa was associated with both anti-coagulant and anti-inflammatory effects, and an increased survival [16, 18-20]. Other studies have further documented anti-inflammatory effects of TF inhibition, i.e. 1) blocking TF-mediated coagulation in experimental sepsis or acute lung injury models attenuated the inflammatory response in the lung, including neutrophil infiltration, and edema formation [35-37]; 2) recombinant human TF injected intra-articularly induced morphological signs of arthritis and influx of inflammatory cells in synovia [38, 39]; 3) an anti-TF antibody attenuated leukocyte infiltration in a rabbit acute myocardial injury model [40]; 4) treatment with anti-TF antibody or TFPI diminished glomerular inflammation and glomerular fibrin deposition in experimental models of glomerulonephritis [41, 42]; and 5) TFPI protected against experimentally induced spinal cord ischemia [43]. The potential role of TF in inflammatory responses has also been suggested by a number of in vitro studies, which have pointed to TF as a mediator of intracellular signaling, functioning as an intermediate for FVIIa-induced activation of MAP kinases, small GTPases and calcium signaling [44].

In the present study we demonstrated the important role of TF/VIIa in activation of coagulation during E. coli induced peritonitis. In accordance, TFPI was previously found to diminish fibrinogen consumption during peritonitis in rabbits, induced by i.p. administration of a suspension containing hemoglobin, porcine mucin and live E. coli [27]. In this latter study, TFPI treatment also reduced lethality, and had beneficial effects on a number of different physiologic parameters, including arterial blood pressure, arterial oxygenation, and lactate levels. In contrast, in our study rNAPc2 did not influence survival. Moreover, rNAPc2 did not impact on inflammatory responses.
including recruitment of leukocytes to the site of infection, local cytokine and chemokine production, plasma cytokine concentrations and lung injury, responses that were not investigated in the TFPI/peritonitis study [27]. Although a firm explanation for these seemingly discrepant results is lacking, differences in the experimental models to induce peritonitis and differences in species may have played a role. Alternatively, the different mechanism of TF-FVIIa inhibition for TFPI and rNAPc2, described above, may have played a role. In this respect it should be noted in vitro studies have suggested that rNAPc2 may facilitate activation of protease-activated receptors 1 and 2 by FXa by stabilizing the ternary TF-FVIIa-FXa complex [45]. Indeed, rNAPc2 inhibits FVIIa but not FXa in this complex, which contrasts with TFPI.

Several issues should be kept in mind when interpreting our data. First, rNAPc2 was administered every 6 hours in our study, whereas a single injection of this protein prolonged the PT for only 4 hours in a statistically significant way. Thus, it is likely that the TF-FVIIa pathway was not completely blocked during the whole observation period. However, from a clinical point of view strong and prolonged elimination of the TF-FVIIa pathway is not desired in patients with severe sepsis. Indeed, recombinant TFPI caused bleeding complications in such patients when given at higher doses [46] and the pivotal phase III trial with recombinant TFPI was done using lower doses [47]. Second, we used a virulent invasive *E. coli* strain that after intraperitoneal injection rapidly enters the circulation. Thus, our model results in early systemic infection, mimicking the condition of severe abdominal sepsis. As a consequence, we cannot generally conclude that rNAPc2 does not influence the outcome of peritonitis. For this, the effect of rNAPc2 should also be investigated in other models of abdominal infection such as induced by cecal ligation and puncture or by local instillation of an infected clot. Third, rNAPc2 was given in the absence of concurrent antibiotic therapy, and therefore, our data do not provide insight into the effects of rNAPc2 in animals treated with antibiotics. Finally, one should realize that our data were obtained in C57BL/6 mice. Hence, we can not exclude that the use of other mouse strains would have yielded different results.

Inhibition of TF activity has been considered as a potential treatment for patients with sepsis [46, 48]. The initial optimism regarding this approach, fueled by the strong protective effects of different anti-TF strategies in lethal bacteremia models, has recently been tempered by the negative phase III trial with recombinant TFPI in patients with severe sepsis [47] Our present data, using a murine model of *E. coli* peritonitis, suggest that TF/VIIa indeed is important for activation of coagulation and the occurrence of fibrin depositions in organs, but that the role of TF-FVIIa complex in the inflammatory and antibacterial response may be limited.
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