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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Background. Anticoagulants have gained increasing attention for 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 primate model of sepsis caused by gram-negative bacteria.

Methods. To determine the role of the TF/FVIIa complex in the host response to peritonitis, mice received an intraperitoneal injection of live Escherichia coli with or without concurrent treatment with recombinant nematode anticoagulant protein c2 (rNAPc2), a selective inhibitor of the TF/FVIIa pathway.

Results. Peritonitis was associated with an increase in the expression of TF at the tissue level and activation of coagulation, as reflected by elevated levels of thrombin-antithrombin complexes and by increased fibrin(ogen) deposition in the liver and lungs. rNAPc2 strongly attenuated this procoagulant response but did not influence the inflammatory response (histopathology, leukocyte recruitment to the peritoneal cavity, and cytokine and chemokine levels). Moreover, rNAPc2 did not alter bacterial outgrowth locally or dissemination of the infection, and survival was not different between rNAPc2-treated mice and control mice.

Conclusions. These data suggest that TF/FVIIa activity contributes to the activation of coagulation during E. coli peritonitis but does not play a role in the inflammatory response or antibacterial host defense.
nonhuman primates, including antibodies directed against TF or FVIIa, active site–inhibited FVIIa (Dansyl-Glu-Gly-Arg chloromethylketone [DEGR]–FVIIa), and TF-pathway inhibitor (TFPI) [15–21]. Of importance, in lethal sepsis in baboons, high doses of E. coli, inhibition of the TF/FVIIa complex not only prevented DIC but also resulted in increased survival [16, 18–20]. These findings contrast with those regarding interventions that block the coagulation system farther downstream—that is, 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 by the attenuation of a TF-mediated inflammatory response that, in this experimental setting, appears to be distinct from initiation of TF-mediated coagulation. 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, although cytokines are involved in the changes in the coagulation system after 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 the host defense against peritonitis is limited to 1 earlier investigation [27]. In that study, rabbits were administered an ip 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 inhibition of TF, 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].

MATERIALS AND METHODS

Animals. Male C57BL/6 wild-type mice were purchased from Harlan CPB. All mice were housed (5 mice/cage) in the same temperature-controlled room, with alternating 12-h light/dark cycles, and were allowed to equilibrate for at least 5 days before the study. Mice were provided regular mouse chow (SRM-A; Hope Farms) 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) was produced as described elsewhere [30]. In brief, rNAPc2 was manufactured as a secreted protein in the yeast Pichia pastoris. It was purified to >98% purity, by use of a series of chromatographic steps, before being formulated in a modified PBS solution (pH 7). Endotoxin levels were determined to be <1 EU/mg and well within the range considered to be safe for administration to humans. [31, 32]. In a first experiment, in which the effect of rNAPc2 on the activated prothrombin time (PT) and the partial thromboplastin time (aPTT) was determined, rNAPc2 was administered as a single subcutaneous (sc) dose (10 mg/kg) diluted in 100 μL of sterile PBS. In subsequent peritonitis studies, rNAPc2 was administered sc every 6 h, starting 1 h before infection, for a total of 3 injections maximum (until 17 h after infection), at a dose of 10 mg/kg/ injection (in 100 μL of PBS). In these studies, control mice received PBS (100 μL) sc every 6 h. We chose to administer rNAPc2 sc since this route of administration has been used in mice and humans previously [31, 32].

Induction of peritonitis. Peritonitis was induced as described elsewhere [28]. In brief, E. coli O18:K1 was cultured in Luria Bertani medium (Difco) 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 ip with 105 cfu of E. coli in 200 μL of sterile isotonic saline in all experiments, except for an additional survival study in which 6 × 105 cfu was administered. To determine viable counts, the inoculum was plated on blood agar plates immediately after inoculation.

Collection of samples. For comparison of bacterial outgrowth and host responses in rNAPc2-treated mice and control mice, animals were killed at an early time point (6 h) and at a time point directly before mortality occurred (20 h). At the time that the mice were killed, mice were first anesthetized by inhalation of isoflurane (Abbott)/O2 (2%/2 L). A peritoneal lavage was then performed with 5 mL of sterile isotonic saline, by use of an 18-gauge needle, and peritoneal lavage fluid was collected in sterile tubes (Plastipack; Becton Dickinson). 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 ip injection of 0.07 mL/g FFM mixture (0.315 mg/mL fentanyl, 10 mg/mL fluanisone [Janssen], and 5 mg/mL midazolam [Roche]). Next, blood was drawn from the vena cava inferior by use of a sterile syringe and was 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 use of 1-stage clotting assays with plasma diluted 3 times in saline. In brief, diluted plasma was incubated with thromboplastin PT-fibrinogen or actin FS (Dade Behring) for 5 min at 37°C, for measurement of PT or aPTT, respectively. Next, PT and aPTT
measurements were started by addition of 20 mmol/L calcium chloride, and time to agglutination was measured by use of an ACL 7000 analyzer (Instrumentation Laboratory). Thrombin-antithrombin complex (TATc) levels were determined in citrated plasma as a measurement of generation of thrombin. TATc levels were measured by use of a mouse-specific, ELISA-based method [33]. Cytokines and chemokines were measured by use of cytometric bead array (Pharmingen; tumor necrosis factor–α, interleukin [IL]–6, and IL-10) or by use of ELISA (R & D Systems; macrophage inflammatory protein–2 and cytokine-induced neutrophil chemoattractant), in accordance with the manufacturers’ instructions.

**Histological examination.** Immediately after we killed the mice, samples from the liver and lungs were removed, fixed in 4% formaline, and embedded in paraffin, for routine histologic examination. Sections of 4 μm thickness were stained with hematoxylin-eosin. All slides were coded and scored by a pathologist who had no knowledge of the type of mouse or the treatment used. TF and fibrin stainings were performed on paraffin slides after deparaffinization and rehydration, by use of standard immunohistochemical procedures. For both stainings, the activity of endogenous peroxidase was quenched by use of 1.5% H2O2 in PBS, and nonspecific binding was blocked by use of 10% normal goat serum. The primary antibodies used were rabbit anti–mouse TF polyclonal antibody (produced in the Laboratory of Experimental Internal Medicine, University of Amsterdam), for the detection of TF; and biotinylated goat anti–mouse fibrinogen antibody (Accurate Chemical & Scientific Corporation), for fibrin staining. The generation and characterization of the anti-TF antibody will be described in detail elsewhere (V. De Waard, J. Timmerman, P. Reitsma, H. ten Cate, unpublished data). In brief, rabbits were immunized with 5 immunogenic mouse TF peptides corresponding to different amino acid sequences within the extracellular domain of mouse TF. For immunohistochemical staining of TF, the immunoglobulin fraction purified from the polyclonal antiserum against peptide 5, corresponding to aa 225–240, was used since this bound native TF in a specific manner. TF staining of the brains and tracheas of mice exposed to endotoxin co-localized with TF mRNA, as determined by in situ hybridization, and was abolished in the presence of peptide 5 (data not shown). Biotinylated swine anti–rabbit antibody (DAKO) was used as a secondary antibody for TF staining. For both stainings, the activity of endogenous peroxidase was quenched by use of 1.5% H2O2 in PBS, and ABC solution (DAKO) was used as a staining enzyme; 0.03% H2O2 and 3,3′-diaminobenzidine tetrahydrochloride (Sigma) in 0.05 mol/L Tris (pH 7.6) was used as substrate. Examination of immunohistochemically 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, was determined.

**Enumeration of bacteria.** The number of *E. coli* colony-forming units was determined in peritoneal fluid, blood, and liver homogenates. For this determination, livers were harvested and homogenized at 4°C in 5 volumes of sterile isotonic saline, by use of a tissue homogenizer (Biospect Products), 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. Colony-forming units were counted after 16 h.

**Cell counts and differentials.** Leukocyte counts were determined by use of a Coulter counter (Beckman Coulter). Subsequently, peritoneal fluid was centrifuged at 1400 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, to a final concentration of 107 cells/mL, and differential cell counts were performed on cytopsin preparations stained with a modified Giemsa stain (Diff-Quick; Dade Behring AG), in accordance with the manufacturer’s instructions.

**Statistical analysis.** Data were analyzed by use of the SPSS statistical package. Data are expressed as mean ± SEM, unless indicated otherwise. Changes in PT and aPTT after a single dose of rNAPc2 were analyzed by use of one-way analysis of variance, followed by Dunnett’s (post hoc) test. Comparisons between groups were conducted by use of the Mann-Whitney U test. Survival curves were compared by use of the log-rank test. A value of *P* < .05 was considered to represent a statistically significant difference.

**RESULTS**

**Up-regulation of expression of TF during *E. coli* peritonitis.** To determine whether expression of TF is up-regulated during peritonitis, mice received an ip injection of 200 μL of normal saline containing 106 cfu of *E. coli* or 200 μL of sterile saline as a control. As shown in figure 1, inflammatory cells that infiltrated the lungs during the course of peritonitis expressed TF (figure 1B). Baseline expression of TF was not observed in the lungs of control mice (figure 1A). As shown in figure 1C, expression of TF was not altered by treatment with rNAPc2 (see below).

**Peritonitis-induced 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 activation of coagulation at the site of the infection, we measured the TATc levels in peritoneal lavage fluid obtained before and 6 or 20 h after infection (figure 2). Induction of peritonitis resulted in local generation of thrombin, as reflected by an increase in TATc levels in peritoneal lavage fluid (*P* < .05, for both 6 and 20 h after infection vs.
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Figure 1. Increased expression of tissue factor (TF) in the lungs after induction of peritonitis. TF immunostaining of the lungs at 20 h was performed after intraperitoneal (ip) injection of sterile saline (A), after ip injection of 10⁷ cfu of Escherichia coli with PBS subcutaneously (sc) every 6 h (B), or after ip injection of 10⁴ cfu of E. coli with recombinant nematode anticoagulant protein c2 (rNAPc2; 10 mg/kg) sc every 6 h (C). sc injections were started 1 h before infection and continued until 17 h after infection. Slides show a similar expression of TF by inflammatory cells in PBS- and rNAPc2-treated mice with peritonitis. Original magnification, ×40. Representative slides are shown from a total of 5 mice/group.

Figure 2. Local increase in levels of thrombin-antithrombin complexes (TATc) after induction of peritonitis. Mean ± SE levels of TATc in peritoneal lavage fluid were obtained 6 h and 20 h after intraperitoneal administration of 10⁷ cfu of Escherichia coli. n = 6 mice/time point. *P < .05, vs. time of infection; #P < .05, vs. 6 h after infection.

Kinetics of the anticoagulant effect of rNAPc2. To establish the anticoagulant properties of rNAPc2 in mice, rNAPc2 was administered as a single sc dose (10 mg/kg), and the PT and aPTT were measured as markers for the anticoagulant effect of rNAPc2, before infection and at different time points thereafter (figure 3). rNAPc2 prolonged the PT from 30.3 ± 0.5 to 62.5 ± 0.8 s and the aPTT from 48.6 ± 2.2 to 197.9 ± 3.6 s, from baseline to 0.5 h after infection. The anticoagulant effect of rNAPc2 lasted 4–6 h. On the basis of these experiments, in further studies, rNAPc2 was administered sc every 6 h after infection (10 mg/kg in 100 µL of PBS), starting 1 h before infection; control mice received PBS (100 µL) sc every 6 h.

Activation of coagulation inhibited by rNAPc2 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 20 h after infection from mice treated with rNAPc2 or PBS (figure 4). Mice treated with rNAPc2 displayed significantly reduced TATc levels in both plasma and peritoneal lavage fluid (P < .05, vs. PBS). In addition, rNAPc2 abolished the formation of thrombi in the liver and lungs, which is commonly observed in the course of peritonitis (figure 5C, insert). Moreover, fibrin(ogen) deposition in vessels and along the pleura (figure 5E) was also prevented by treatment with rNAPc2 (figure 5F). As expected, rNAPc2 did not alter expression of TF in the lungs, as assessed by immunostaining (figure 1C). Taken together, these data
Figure 3. Prothrombin time (PT) and partial thromboplastin time (aPTT) prolonged by recombinant nematode anticoagulant protein c2 (rNAPc2). rNAPc2 (10 mg/kg) was administered as a single subcutaneous dose, and the PT and the aPTT were measured at the time points indicated. Data are mean ± SE of 8 mice/time point. *P < .05, vs. time of injection.

Figure 4. Activation of coagulation inhibited by recombinant nematode anticoagulant protein c2 (rNAPc2) during peritonitis. Data are mean ± SE levels of thrombin-antithrombin complexes (TAT-c) in peritoneal lavage fluid and plasma. Mice (6/group) were injected intraperitoneally with 8 × 10⁴ cfu of Escherichia coli at the time of infection. rNAPc2 (white bars) was administered subcutaneously (sc) every 6 h after induction of peritonitis, at a dose of 10 mg/kg of body weight, starting 1 h before infection and continuing until 17 h after infection; control mice (black bars) received PBS sc every 6 h. Mice were killed 20 h after infection. *P < .05, vs. control mice.

Dicate that rNAPc2 exerted a potent anticoagulant effect during peritonitis.

Inflammatory response to peritonitis not influenced by rNAPc2. 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. At the time of histopathological examination, PBS-treated mice displayed foci of liver necrosis associated with formation of thrombi (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 interalveolar septa (figure 5C and 5D, respectively). In addition, rNAPc2 did not influence other inflammatory responses, such as the influx of neutrophils (table 1) or the release of proinflammatory cytokines and chemokines into the peritoneal lavage fluid or the circulation (table 2).

Bacterial outgrowth not influenced by rNAPc2. To obtain insight into the role of activation of TF/FVIIa in early antibacterial defense against peritonitis, we compared the number of E. coli colony-forming units at 6 and 20 h after infection in peritoneal lavage fluid (the site of the infection), blood (to evaluate to what extent the infection became systemic), and the livers of rNAPc2- and PBS-treated mice (figure 6). Both groups had similar bacterial loads at all 3 body sites at each time point. Blood agar plates displayed only the E. coli strain that was administered, excluding dissemination of intestinal organisms.

Survival not influenced by rNAPc2. To investigate the role of TF/FVIIa in the outcome of abdominal sepsis, we performed 2 independent survival studies, 1 using the inoculum that was also administered in the experiments presented above (10⁴ cfu) and 1 using a lower inoculum (6 × 10³ cfu). After infection with the higher bacterial dose, all mice died within 2 days, irrespective of treatment with rNAPc2 (figure 7A). After infection with the lower dose, 4 of 10 control mice and 7 of 10 rNAPc2-treated mice died (P = .2, for the difference between groups; figure 7B).
**DISCUSSION**

The role that the TF/FVIIa pathway plays in activation of the coagulation system during sepsis has been firmly established in experimental models using iv challenges of live bacteria or bacterial products, such as endotoxin [15–21]. In addition, in otherwise lethal bacteremia in baboons that was induced by iv infusion of high doses of live *E. coli*, inhibition of the TF/FVIIa pathway prevented not only 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 have demonstrated here that, in our mouse model of septic peritonitis, expression of TF is increased in the liver and lungs of infected mice, which is associated with activation of coagulation, as reflected by increases in TATc levels in peritoneal lavage fluid and plasma and by fibrin depositions in the liver and lungs. Inhibition of the TF/FVIIa complex by use of rNAPc2 reduced activation of coagulation but did not influence the inflammatory response or antibacterial defense mechanisms. These findings suggest that rNAPc2 functions primarily as an anticoagulant during mouse *E. coli* peritonitis and that, in this setting, TF is likely not involved in the host inflammatory response.

In the present 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 was produced in recombinant form by use of the yeast *P. 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) before 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 use of zymogen FX as an inhibitory scaffold, by rNAPc2, obviates the need for forming FXa before the inhibition of the TF/FVIIa complex. rNAPc2 also has been demonstrated to inhibit activation of FIX by the TF/FVIIa complex [29]. Several previous studies have established the efficacy of rNAPc2 in attenuating coagulation in vivo—that is, rNAPc2 completely prevented activation of endotoxin-induced coagulation in chimpanzees [34] and strongly reduced the incidence of acute deep vein thrombosis in patients undergoing unilateral knee arthroplasty, compared with 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 generation of thrombin) 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

### Table 1. Leukocyte counts in peritoneal lavage fluid.

<table>
<thead>
<tr>
<th></th>
<th>Control mice</th>
<th>rNAPc2-treated mice</th>
<th>Control mice</th>
<th>rNAPc2-treated mice</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total cells</strong></td>
<td>2.17 ± 0.42</td>
<td>2.31 ± 1.32</td>
<td>1.37 ± 0.21</td>
<td>1.46 ± 0.91</td>
</tr>
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<td></td>
<td>0.15 ± 0.05</td>
<td>1.17 ± 0.99</td>
<td>0.96 ± 0.12</td>
<td>1.33 ± 0.65</td>
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<tr>
<td><strong>Macrophages</strong></td>
<td>0.72 ± 0.23</td>
<td>1.03 ± 0.25</td>
<td>0.31 ± 0.12</td>
<td>0.12 ± 0.13</td>
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<tr>
<td><strong>Others</strong></td>
<td>0.30 ± 0.10</td>
<td>0.11 ± 0.07</td>
<td>0.10 ± 0.05</td>
<td>0.01 ± 0.09</td>
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**NOTE.** Data are mean ± SE (n = 8 mice/group for each time point) and were obtained at 6 or 20 h after intraperitoneal administration of *Escherichia coli* (10⁴ cfu). Recombinant nematode anticoagulant protein c2 (rNAPc2) was administered subcutaneously (sc) every 6 h after induction of peritonitis, at a dose of 10 mg/kg of body weight, starting 1 h before infection and continuing until 17 h after infection; control mice received PBS sc every 6 h. Differences between treatment groups were not significant.

### Table 2. Chemokine and cytokine concentrations.

<table>
<thead>
<tr>
<th>Chemokine/ cytokine</th>
<th>Plasma</th>
<th>Peritoneal fluid</th>
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<tr>
<td></td>
<td>Control mice</td>
<td>rNAPc2-treated mice</td>
</tr>
<tr>
<td><strong>MIP-2</strong></td>
<td>...</td>
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<tr>
<td><strong>KC</strong></td>
<td>...</td>
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<tr>
<td><strong>IL-6</strong></td>
<td>770 ± 40</td>
<td>581 ± 120</td>
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<tr>
<td><strong>TNF-α</strong></td>
<td>111 ± 8</td>
<td>102 ± 18</td>
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<tr>
<td><strong>IL-10</strong></td>
<td>909 ± 195</td>
<td>828 ± 196</td>
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**NOTE.** Data are mean ± SE (n = 8 mice per group) chemokine/cytokine concentrations (picograms per milliliter) and were obtained at 20 h after intraperitoneal administration of *Escherichia coli* (10⁴ cfu). Recombinant nematode anticoagulant protein c2 (rNAPc2) was administered subcutaneously (sc) every 6 h after induction of peritonitis, at a dose of 10 mg/kg of body weight, starting 1 h before infection and continuing until 17 h after infection; control mice received PBS sc every 6 h. Differences between treatment groups were not significant. IL, interleukin; KC, cytokine-induced neutrophil chemoattractant; MIP-2, macrophage inflammatory protein-2; TNF-α, tumor necrosis factor-α.
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Figure 6. Bacterial clearance not influenced by recombinant nematode anticoagulant protein c2 (rNAPc2) during peritonitis. Bacterial outgrowth (expressed as median with interquartile ranges) in peritoneal fluid (left), blood (middle), and liver (right), at 6 and 20 h after infection, is shown. Mice were injected intraperitoneally with 10^4 cfu of *Escherichia coli* at the time of infection. rNAPc2 (white bars) was administered subcutaneously (sc) 1 h before infection and every 6 h thereafter until 17 h after infection; control mice (filled bars) received PBS sc every 6 h (n = 8 mice/group at each time point). Differences between treatment groups were not significant.

Figure 7. Survival not influenced by recombinant nematode anticoagulant protein c2 (rNAPc2). Survival after intraperitoneal injection of 10^4 cfu (A; n = 15/group) or 6 × 10^3 cfu (B; n = 10/group) of *Escherichia coli* in treated control mice (black squares) and rNAPc2-treated mice (white squares) is shown. rNAPc2 was administered subcutaneously (sc) every 6 h after induction of peritonitis, at a dose of 10 mg/kg of body weight, starting 1 h before infection and continuing until 17 h after infection; control mice received PBS sc every 6 h. Differences between treatment groups were not significant.

FVIIa was associated with both anticoagulant and anti-inflammatory effects and an increased survival [16, 18–20]. Other studies have further documented anti-inflammatory effects of inhibition of TF—that is, (1) blocking of 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 an influx of inflammatory cells in synovia [38, 39]; (3) an anti-TF antibody attenuated leukocyte infiltration in a rabbit model of acute myocardial injury [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, func-
tioning as an intermediate for FVIIa-induced activation of MAP kinases, small GTPases, and calcium signaling [44].

In the present study, we have demonstrated the important role of TF/VIIa in activation of coagulation during E. coli-induced peritonitis. In accordance, TFPI was previously found to diminish consumption of fibrinogen during peritonitis in rabbits, by ip administration of a suspension containing hemoglobin, porcine mucin, and live E. coli [27]. In this latter study, treatment with TFPI 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 the present study, rNAPc2 did not influence survival. Moreover, rNAPc2 did not affect inflammatory responses, including recruitment of leukocytes to the site of infection, local production of cytokines and chemokines, 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 and differences in species may have played a role. Alternatively, the different mechanism of inhibition of TF/FVIIa for TFPI and rNAPc2, described above, may have played a role. In this respect, it should be noted that 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, in the present study, rNAPc2 was administered every 6 h, whereas a single injection of this protein prolonged the PT for only 4 h 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 it was administered at higher doses [46], and the pivotal phase 3 trial with recombinant TFPI was performed using lower doses [47]. Second, we used a virulent, invasive E. coli strain that, after ip 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 reason, the effect of rNAPc2 should also be investigated in other models of abdominal infection, such as models that induce peritonitis by cecal ligation and puncture or by local instillation of an infected clot. Third, rNAPc2 was administered in the absence of concurrent antibiotic therapy, and, therefore, our data do not provide insight into the effects of rNAPc2 in mice treated with antibiotics. Finally, one should realize that our data were obtained by use of C57BL/6 mice. Hence, we cannot 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 3 trial of recombinant TFPI in patients with severe sepsis [47]. Our present data, obtained by use of a mouse model of E. coli peritonitis, suggest that TF/VIIa is, indeed, important for the activation of coagulation and the occurrence of fibrin depositions in organs but that the role of the TF/FVIIa complex in the inflammatory and antibacterial response may be limited.

Acknowledgments

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