Experimental, clinical, and meta-analytical studies of antithrombotic therapies in venous and arterial thrombosis
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COAGULATION INHIBITION IMPAIRS HOST DEFENSE IN A MOUSE MODEL OF PERITONITIS


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Summary

Gram-negative sepsis is associated with acquired deficiencies of anticoagulant proteins, such as protein C, protein S, and antithrombin, and low levels of these proteins predict poor outcome of this disease. Administration of (activated) protein C improves survival in sepsis patients. These observations suggest that the activity of the coagulation system directly influences host defense. In previous experiments we tested this by evaluating host defense in mice that were homozygous for a prothrombotic thrombomodulin mutation (TM^{pro/pro}). These experiments showed little evidence for the hypothesis that an inherited deficiency of an anticoagulant protein interferes with host defense. In the present experiment, we further disturbed the coagulation system in the TM^{pro/pro} mice by pre-treatment with two thrombin inhibitors (hirudin and low molecular weight heparin, LMWH) just before intraperitoneal challenge with Escherichia coli. As controls we used inoculated TM^{pro/pro} mice that did not receive antithrombotics. With one exception, a significant increase of bacterial outgrowth in peritoneal fluid, blood and liver of the anticoagulant treated mice was observed, in parallel with an increase of IL-6 and TNFα levels in peritoneal fluid and blood. Female mice that were treated with LMWH formed the exception. The conclusion from this experiment is that thrombin inhibition in a prothrombotic mouse impairs host defense in a Gram-negative peritonitis, which further underlines a key role for thrombin in the crosstalk between the coagulation and the innate immune system.
Introduction
In humans, gram-negative sepsis is associated with acquired deficiencies of the plasma coagulation inhibitors protein C (PC), protein S (PS), and antithrombin (AT)\(^\text{1,2}\). Supplementation of these coagulation inhibitors results in reduced mortality and morbidity, as shown in recent clinical trials\(^\text{3,4}\). On the basis of these results, it is possible to hypothesize that poor coagulation inhibition leads to impaired host defense and escalation of sepsis. Inversely, administration of coagulation inhibitors that inhibit thrombin action directly or indirectly, such as hirudin or low molecular weight heparin (LMWH), may lead to improved host defense and protection from sepsis.

In a previous study (own unpublished data) using a mouse model with a prothrombotic state (TM\(^{-/-}\))\(^2\), we showed that host defense was not different between mice with a functional deficiency in thrombomodulin (TM\(^{+/+}\)) and normal littermates. However, it was not certain whether the applied model was sensitive enough to provide evidence for the formulated hypothesis. In the present study we inhibited the prothrombotic state in the TM\(^{+/+}\) mice by treatment with hirudin and LMWH and evaluated the effect of this treatment on host defense in a model of peritonitis. Quite surprising and in contrast with our hypothesis, the use of these thrombin inhibitors appears to impair rather than enhance host defense.

Material and methods

Mice.
The generation of mice lacking the capability of thrombomodulin binding to thrombin by a targeted point mutation in the TM gene (TM\(^{+-/-}\)) was described elsewhere\(^3\). These mice have a severely impaired capacity to generate APC but are viable and without overt spontaneous phenotype. Animals were bred and maintained in a 12-h dark and light cycle room in our animal facility department. Animal experiments were approved by the Institution Review Board for Animal Experiments of the Academic Medical Center and performed according to the guidelines of the American Physiological Society and the Dutch Law for Animal Experiments.

Study design.
Male and female mice, aged 9 –12 weeks were used throughout the study. In a preliminary survival experiment, 12 mice were inoculated intraperitoneally (ip) with 6.5x10\(^5\) CFU Escherichia (E.) coli; half of these mice were treated with LMWH (nadroparin, Sanofi Winthorp, Paris, France) 30 minutes before inoculation. LMWH was administered as an intravenous dose of 5 IU anti-Xa-activity (âXa), and a similar dose subcutaneously (sc) The sc injection was repeated 12 hours after inoculation. On the basis of this survival experiment an experiment was devised,
where mice were sacrificed after 24 hours after inoculation with a ~10-fold lower number of bacteria. An additional group of animals was pretreated with 200 μg per mouse of hirudin, a direct thrombin inhibitor provided by Knoll AG, Ludwigshafen, Germany.

**Intraperitoneal bacterial challenge model**

Peritonitis was induced by gram-negative E.coli O18:K1:H7, strain Bort, a pathogenic smooth, encapsulated strain presenting the O18 (LPS) serotype and the capsular K1 antigen. After growing in LB medium to mid-log phase, the bacteria were washed and adjusted spectrophotometrically to an optical density (OD) of 1.0 (at 650 nm), corresponding to a bacterial concentration of approximately $2.2 \times 10^8$ colony forming units (CFU) per mL. From this concentration, inoculates of $6.5 \times 10^4$ and $7.5 \times 10^3$ CFU/0.5 mL were prepared by dilution with physiological saline.

**Preparation and analysis of peritoneal lavage fluid and blood**

Blood and peritoneal lavage fluid were collected 24 hours after bacterial challenge for cytokine measurements and bacterial cultures. Blood was drawn from the inferior caval vein. The peritoneal contents were lavaged with 3 mL of PBS and harvested after gentle massage. For cytokine assays plasma was stored at -70°C until assay. Bacterial counts were determined by plating 10-fold dilutions of blood, PF or homogenized liver tissue samples on blood agar and incubating overnight at 37°C.

**Cytokine assays**

Plasmatic levels of circulating early response cytokines TNFα and IL-6 were determined by enzyme-linked immunoabsorbent assay (ELISA, R&D Systems, ITK Diagnostics BV, Uithoorn, the Netherlands) according to the instructions of the manufacturer.

**Coagulation System (TAT, tissue thrombosis)**

Levels of thrombin-anti-thrombin complexes in plasma were assessed using a sandwich ELISA, that was developed in our laboratory (described in Chapter 11). In brief, MaxiSorp plates were coated with purified anti-thrombin antibodies from immunized rabbits, the plates then incubated with plasma and again incubated with purified DIG-conjugated rabbit anti-AT, and finally with HRP-conjugated sheep F(ab)2 anti-DIG fragments (Roche Diagnostics). Quantification was performed using the OPD-method, and OD determined at 490 and 650 nm. The concentration of TAT complexes in serum diluted in buffer of approximately 6000 ng/mL was determined by the human TAT assay (Enzygnost TAT, Dade Behring, Marburg, Germany).
Histo/qgica/preparation and analysis

Immediately before sacrifice, the animals were injected with 400 IU heparin through a tail vein to avoid postmortem thrombus formation. The liver was asserved following peritoneal lavage and blood collection, then carefully placed in a 3.7% formaldehyde solution for at least 1 week, together with the other organs. The lung was perfused with PBS before asservation. The organs were processed for paraffine blocks and sections. Staining was performed with hematoxylin/eosin and fibrin staining. Analysis was performed by two independent blinded investigators. For the liver, spleen and lungs a semiquantitative score was used, allowing findings for the presence of fibrin deposition, thrombosis, necrosis, inflammation, neutrophil influx, and edema to be allocated to 1 to 4 points, depending on the amount or the observed degree. Counts were performed in 5 different microscopical fields at a magnification of 125 x 12.5, and the mean of the different counts was used.

Data analysis

All values are expressed as mean values ± SE. Group comparison was performed using the non-parametric Mann-Whitney test. Survival of different groups was compared with the Kaplan-Meier survival curve.

Results

Survival

After ip inoculation with a dose of 6.5 x 10^4 CFU, 2 of 3 males in the control group became ill and died within 30 to 50 hours, whereas 3 of 3 females in the same group did not show any signs of illness and survived 14 days of observation. In the group pretreated with LMWH all 6 animals died within 30 to 50 hours. This indicates that anticoagulation with LMWH severely compromises the host response to the intraperitoneal bacterial load.

Bacterial outgrowth

Bacterial outgrowth in peritoneum and blood was assessed in a second experiment in which infected and control mice were sacrificed after 24 hours. Because the LMWH treated mice did so poorly in the survival experiment a ~10-fold lower number of bacteria were injected (7.5x10^3 CFU). In addition, a third group of animals was inoculated, that were intravenously pretreated with the direct thrombin inhibitor hirudin ( ) with a dose of 200 i g per mouse. 8 Males and 11 females per group were used in this experiment and sacrificed 24 hours after bacterial challenge for assessment of bacterial outgrowth, cytokine expression and TAT-levels. In order to exclude bleeding complications as the cause for illness and death, two additional control groups received
identical doses of LMWH or hirudin without being inoculated with bacteria. These animals survived the experiment in good health and were not analysed further.

Mean values in CFU/mL and standard errors (SE) for bacterial counts of the three groups (controls, hirudin treated, LMWH treated mice) in PF, blood and liver are shown for male and female mice in Table 1. The figures show, that pre-treatment with hirudin and LMWH in males leads to $10^6 - 10^7$ fold higher bacterial counts in PF compared to the control group ($p = 0.0002$), $10^4$ to $10^5$ fold higher counts in blood ($p = 0.002$), and comparable values for the liver. Thus, thrombin inhibition led to significantly attenuated bacterial clearance. In female mice, the respective values revealed 10- to 100-fold higher bacterial counts in PF for the hirudin group. Quite surprisingly, however, $10^6$ fold lower counts for the LMWH group ($p = 0.0001$), $10^2$ to $10^3$ fold lower values in blood for the LMWH group, and $10^2$ to $10^3$ for the liver were found.

<table>
<thead>
<tr>
<th>Males n = 8</th>
<th>PF</th>
<th>Blood</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>$2.6 \times 10^4 \pm 1 \times 10^7$</td>
<td>$7.6 \times 10^4 \pm 5 \times 10^4$</td>
<td>$2.1 \times 10^6 \pm 2 \times 10^6$</td>
</tr>
<tr>
<td>LMWH</td>
<td>$1.9 \times 10^{12} \pm 8 \times 10^{12}$</td>
<td>$2.6 \times 10^{10} \pm 3 \times 10^{10}$</td>
<td>$4.4 \times 10^5 \pm 3 \times 10^5$</td>
</tr>
<tr>
<td>Hirudin</td>
<td>$4.5 \times 10^{13} \pm 2 \times 10^{13}$</td>
<td>$9.9 \times 10^8 \pm 4 \times 10^8$</td>
<td>$8.2 \times 10^7 \pm 5 \times 10^6$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Females n = 8</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>$1.5 \times 10^{10} \pm 6 \times 10^9$</td>
<td>$1.8 \times 10^7 \pm 1 \times 10^5$</td>
<td>$6.7 \times 10^5 \pm 3 \times 10^5$</td>
</tr>
<tr>
<td>LMWH</td>
<td>$5.0 \times 10^9 \pm 5 \times 10^4$</td>
<td>$1.7 \times 10^7 \pm 1.5 \times 10^3$</td>
<td>$3.0 \times 10^3 \pm 1 \times 10^2$</td>
</tr>
<tr>
<td>Hirudin</td>
<td>$8.6 \times 10^{11} \pm 6 \times 10^{11}$</td>
<td>$9.4 \times 10^7 \pm 9 \times 10^7$</td>
<td>$7.5 \times 10^6 \pm 7 \times 10^6$</td>
</tr>
</tbody>
</table>

**Table 1** Pre-treatment with hirudin and LMWH in males leads to $10^6 - 10^7$ fold higher bacterial counts in PF compared to the control group ($p = 0.0002$), $10^4$ to $10^5$ fold higher counts in blood ($p = 0.002$), and comparable values for the liver.

**Cytokine response in blood**

In the acute experiment, 24 hours after bacterial inoculation, IL-6 levels in plasma of male mice were 1000 pg/mL in control mice, 1800 pg/mL in the hirudin group, and 1400 pg/mL in the LMWH group, reflecting the presence of more bacteria in the thrombin inhibitor treated groups. In females, control mice had higher levels (1800 pg/mL) than males, whereas the hirudin group reached slightly lower levels (1400 pg/mL), and the LMWH treated group showed significantly
lower levels with 500 pg/mL. TNFα levels were much lower at 24 hours, but showed a pattern that was comparable to that of IL-6 (see Figure 1).

Figure 1. IL-6 response to bacterial challenge in mice pretreated with hirudin or LMWH as compared to non-anticoagulated controls.
**Thrombin-Antithrombin-Complexes**

Mean TAT levels in plasma were 5 ng/mL in control mice, 6.5 ng/mL in hirudin treated mice, and 5.5 ng/mL in the LMWH group. No significant differences were seen between groups, as shown for male mice in Figure 2. Thus, systemic activation of the coagulation system was not observed in the infected mice.

![TAT levels in male TMpro/pro mice](image)

**Figure 2** TAT levels as a marker of activated coagulation were comparable between groups

**Histological analysis of liver and lungs**

Male mice treated with hirudin had more fibrin deposition, thrombosis formation, and tissue necrosis in the liver than controls and mice treated with LMWH. Male control mice had more granulocyte influx in the liver than those in the comparison groups. All control mice, independent from their gender, had more thrombosis formation and fibrin deposition in the spleen than those receiving either hirudin or LWMH. All mice showed equal signs of pulmonary inflammation (Table 2)
Table 2 Thrombosis and in the spleen and signs of inflammation in the lungs were most pronounced in control mice.

**Discussion**

In this report we demonstrate that prothrombotic mice react with impairment of their host defense towards inoculation of Gram-negative bacteria, when pretreated with the direct thrombin inhibitor hirudin or with the indirect thrombin inhibitor LMWH, although less distinctly in the latter case. These results support the hypothesis that a disturbance of the coagulation system influences host defense. Surprisingly though, in particular in the light of recent clinical success with the coagulation inhibitor activated protein C, thrombin inhibition seems to favor sepsis rather than prevent it.

In our model, TM<sup>−/−</sup> mice are prothrombotic because of their inability to bind TM to thrombin, which leads to impairment of the anticoagulant protein C pathway<sup>9</sup>. In this situation, the amount of thrombin present in the blood of these animals should be higher than in normal mice. In addition to its role in coagulation thrombin is known to possess pro-and anti-inflammatory effects<sup>10</sup>. By the administration of a direct thrombin inhibitor in this situation, it may be that the inflammatory activity of thrombin is inhibited, resulting in an attenuated host defense reaction. This appears to be the case in males treated with hirudin and less pronounced in the heparin group. In contrast, female mice reacted differently with slightly increased bacterial counts after hirudin treatment, but significantly lower levels after LMWH administration.
At present we have no reasonable explanation to offer for the strikingly opposite results in the LMWH treated female mice. Experimental error is an unlikely explanation, because the experiments were done on the same day with the same bacterial suspensions. Moreover, the preliminary survival experiment suggests impaired host defense in LMWH treated female mice when a large dose of E coli is administered, which is in keeping with what was observed in the male animals. Possibly the susceptibility of mice to E coli under LMWH treatment follows a bell-shaped curve with improved defense at low doses and impaired host defense at higher doses of live bacteria. Clearly further experimentation with varying doses of bacteria and anti-coagulants are necessary to clarify this issue.

Whatever the interpretation, the male mice seemed much more susceptible than the female mice. The existence of a gender dimorphism in autoimmune responses and host defense in septic patients has been noted before\(^1\),\(^12\). One explanation for this gender difference is the presence of androsterones in males, inhibiting immune functions, while oestrogens exhibit immunoprotective properties\(^13\). As recently reported, IL-6 contributes essentially more in female than in male mice to the stimulation of the adrenal response to stress, for example bacterial challenge, resulting in enhanced glucocorticoid levels\(^14\). In our experiment, female controls had indeed higher levels of IL-6 than males, leading perhaps to amplified stimulation of the adrenal response to bacterial stress.

Regarding the influence of antithrombotic agents on the immune system, hirudin and LMWH, have been shown to display anti-inflammatory effects in various settings of infection or inflammation\(^10\),\(^15\),\(^16\). For neither of the two substances has there been any evidence for the presence of a direct bactericidal effect\(^17\). Mostly, influx of inflammatory cells was reduced by the administration of LMWH or hirudin\(^18\). However, data for hirudin in this respect are controversial, as no evidence was found for its ability to reduce thrombin induced leukocyte infiltration in myocardial tissue\(^19\), but on the other hand, its ability of suppressing the invasion of inflammatory cells in the rat cerebral glia was shown in a cerebral ablation model\(^20\). For LMWH, there seems to be a direct relation between TNF expression and LMWH, with the latter displaying an inhibitory effect on TNF expression without affecting mast cell numbers or degranulation in asthmatic reactions to allergen challenge\(^21\).

In conclusion, the data presented here indicate that anticoagulant treatment attenuates host defense against a localized infection with E coli. Future research should be aimed at elucidating why thrombin inhibition by way of activated protein C improves outcome in sepsis, and thrombin inhibition through hirudin or LMWH seems to favor impairment of host defense.
Acknowledgments

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