Anticoagulants have gained increasing attention in the treatment of sepsis. This study used danaparoid to investigate the role of factor Xa in endotoxin-induced coagulation and inflammation and its effectiveness when coagulation activation has already occurred. Thirty healthy volunteers were enrolled in the randomized, placebo-controlled trial. Subjects received 2 ng/kg endotoxin and danaparoid 10 min or 3 h thereafter or placebo. Endotoxin increased prothrombin fragment 1 + 2 (F1+2) levels from 0.5 to 7.0 nmol/L at 5 h in the placebo group. Early danaparoid infusion inhibited endotoxin-induced thrombin formation: maximum F1+2 levels reached only 1.8 nmol/L (P < .01, vs. baseline or placebo). Delayed danaparoid infusion effectively blocked further thrombin formation. However, danaparoid did not alter endotoxin-induced changes in the fibrinolytic system, cytokine levels, activation of leukocytes, or tissue factor expression on monocytes. Danaparoid therefore selectively attenuates endotoxin-induced coagulopathy, even with delayed administration when coagulation activation is well under way.

Septic shock still carries a high mortality rate, despite advances in intensive-care medicine and antimicrobial therapy. The associated development of multiple organ failure is due, in part, to host inflammatory responses initiated by infection. In addition to hemodynamic instability and impairment of renal, pulmonal, and hepatic function, disseminated intravascular coagulation (DIC) is frequently encountered in patients with septic shock and endotoxemia [1]. DIC may contribute to organ failure through the occlusion of microvasculature and a consequent decrease in organ perfusion [2]. Thus, therapeutic strategies have extended beyond antimicrobial substances to immunomodulating strategies and anticoagulant regimens [3–6]. Despite promising in vitro data and animal studies, clinical trials have so far provided disappointing results. The recent paper by Bernard et al. [6] represents the first published trial that showed a survival benefit for patients with sepsis treated with an anticoagulant (i.e., drotrecogin-α [activated]), whereas, almost concurrently, antithrombin III failed [7]. As the mechanism of action with respect to the coagulation cascade of both of these naturally occurring anticoagulants is similar, studies elucidating potential anti-inflammatory capacities of anticoagulants are crucial. Coagulation activation in sepsis is tissue factor (TF)–dependent, and anti-TF strategies have been shown to be effective in experimental models of DIC [8–11].

We have recently found that hirudin, unfractionated heparin (UFH), and low-molecular-weight heparin (LMWH) differ in their capacity to inhibit lipopolysaccharide (LPS)–induced thrombin formation [11, 12]. Although it seems reasonable to assume that an intervention more upstream in the coagulation cascade (anti–factor Xa [FXa] vs. anti–factor IIa [FIIa]) could provide more benefit, it is not clear to what extent the anti-FXa activity of the latter 2 drugs contributed to their anticoagulant action. Taylor et al. [13], using inactivated FX in an animal model of lethal *Escherichia coli* sepsis, demonstrated complete inhibition of the consumption of fibrinogen by inactivated FX, whereas inflammatory, coagulant, and cell injury responses to *E. coli* were unchanged. A recent study by Senden et al. [14], however, showed that stimulation of endothelial cells with FXa induced the production of IL-6, IL-8, and macrophage chemotactic protein (MCP)–1. Such interactions of FXa with the cytokine network could lend additional importance to anti-FXa strategies. Therefore, we chose a drug with predominant anti-FXa activity, danaparoid, to clarify the role of FXa in endotoxin-induced coagulation. Danaparoid has been shown to be effective in an animal model of sepsis [15], where it inhibited the endotoxin-induced decreases in the platelet count and plasma fibrinogen level and also inhibited the endotoxin-induced increase in glomerular fibrin deposition in the kidney. Danaparoid has been widely used as an anticoagulant in patients with active or past heparin-induced thrombocytopenia...
and has an anti-FXa:anti-FIIa ratio >20:1 [16, 17]. We used a well-established model of human endotoxemia [18], which induces coagulation activation by TF induction [19, 20], to address 3 major objectives: (1) to clarify the role of anti-FXa strategies in endotoxin-triggered coagulation activation in vivo [20], (2) to determine whether danaparoid is still effective when the infusion is delayed until coagulation activation is almost maximal, and (3) to determine whether FXa inhibition would alter inflammatory responses induced by LPS.

Subjects, Materials, and Methods

Subjects. Thirty healthy volunteers whose mean age was 31 years (range, 22–44 years) and who had a mean body mass index of 22.5 (range, 17.6–26.1) were enrolled. Hereditary thrombophilia was excluded through determination of protein C, protein S, and antithrombin III plasma levels and activated protein C resistance.

Study design. An open pilot study with 4 volunteers was conducted to define dose-anti-FXa-activity relationships. The main trial was designed as a double-blind, randomized, placebo-controlled trial in 3 parallel groups.

Treatment and intervention. Two iv catheters were inserted into antecubital veins for substance administration and determination of safety parameters. Blood for coagulation parameters was obtained by de novo venipunctures at selected time points. After premedication with paracetamol (1000 mg by mouth) [21] and start of a continuous infusion of 3 mL/kg/h glucose 5% to maintain hydration and blood glucose levels, 2 ng/kg endotoxin (National Reference Endotoxin, lot G1, E. coli; United States Pharmacopeial Convention) was administered to all subjects iv. At 10 min and 3 h, 80 U/kg danaparoid (Origan, Danaparoid-Natrium 750 Anti-Faktor Xa-Einheiten; Organon) or placebo was administered intravenously, according to group allocation. Group A received danaparoid at 10 min and placebo at 3 h; in group B, placebo was given at 10 min and danaparoid at 3 h. The control group received placebo at both time points.

Coagulation analyses. Blood was collected into tubes containing 3.8% sodium citrate and centrifuged for 15 min at 2500 g. Plasma was immediately stored at −80°C until analysis. Prothrombin fragment 1 + 2 (F1+2) was determined by use of a commercially available assay (Dade Behring) [22]. Soluble fibrin (sF) was quantified using an ELA for thrombus precursor protein (American Bio-genetic Sciences). Fibrinolytic digestion of crosslinked fibrin was assessed by measuring d-dimer levels (Roche Diagnostics).

Plasma levels of TF pathway inhibitor (TFPI) were determined by use of a 2-stage chromogenic substrate assay [23]. Activation of fibrinolysis was assessed with plasmin–α2-antiplasmin (PAP) complexes (Enzymost PAP micro; Behring). Anti-FXa activity was determined on the analyzer STA (Diagnostica Stago) using a dalteparin standard. For determination of IL-6, IL-8, TNF, and MCP-1, commercially available assays were used (R&D Systems) [24, 25].

For CD11b and TF expression, blood was collected into EDTA-anticoagulated tubes. Measurements were done with a FACS Calibur flowcytometer (Becton Dickinson). The anti-CD11b antibody was phycoerythrin-labeled (Becton Dickinson), and the anti-TF antibody was fluorescein isothiocyanate–labeled (American Diagnostica) [12, 26]. After staining, whole blood was lysed (lysing solution, Becton Dickinson), centrifuged at 500 g for 5 min, washed in PBS, re centrifuged, and fixed with cell-fix (Becton Dickinson). Cell populations were identified on the basis of their characteristic forward-sideward scattergramm, which previously had been validated against simultaneous identification of monocytes with CD14 antibody. Results are presented as mean fluorescence intensity (MFI) [26] for CD11b and as percentage of TF-positive (TF+) monocytes for TF expression.

Data analysis. Because the data were nonnormally distributed, all comparisons were made by use of nonparametric tests. For descriptive statistics, data are expressed as means and 95% confidence intervals (CIs). Statistical comparisons within groups were done by the Friedman analysis of variance (ANOVA) and the Wilcoxon signed-rank test. To test changes in end points between groups, the Kruskal Wallis ANOVA, followed by the Mann Whitney U test, was used for peak levels only. The 2-sided level of significance was 5%.

Results

Results from the 4 pilot subjects showed that 80 U/kg of danaparoid did indeed result in peak anti-FXa activities of 1.2 U/mL (95% CI, 1.1–1.2 U/mL). For the main trial, anti-FXa activities achieved in the active treatment groups, A and B, can be seen in figure 1. Danaparoid infusion (80 U/kg) resulted in peak anti-FXa activities of 1.2 U/mL 10 min after infusion, irrespective of the time when danaparoid was infused.

Hemodynamic effects of endotoxin were comparable to those seen in our previous trials and did not vary between the groups; the maximum temperature was 37.5°C, mean arterial blood pressure decreased by 10%, and heart rate increased by 10 beats per minute. Peak changes were seen 4 h after endotoxin infusion.

Figure 1. Anti–factor Xa activities after administration of danaparoid (80 U/kg); 30 healthy volunteers were challenged with lipopolysaccharide (LPS; 2 ng/kg) at 0 h. Group A (▲, n = 10) received danaparoid at 10 min and group B (●, n = 10) at 180 min after LPS challenge. A control group of 10 volunteers is depicted as squares.
Figure 2. Lipopolysaccharide (LPS)–induced changes in prothrombin fragment 1 + 2 (F1+2) levels (upper panel), soluble fibrin (thrombus precursor protein [TpP]) (middle panel), and D-dimer (bottom panel) in 30 healthy volunteers. All subjects were challenged with 2 ng/kg LPS at 0 h. Group A (▲, n = 10) received 80 IU/kg danaparoid 10 min after LPS infusion, and group B (●, n = 10) received 80 IU/kg danaparoid 180 min after LPS. The control group (□, n = 10) received placebo. Data are means and 95% confidence intervals. *Significant difference from that of placebo (P < .05).

Prothrombin F1+2. In the placebo group, LPS infusion increased F1+2 levels from a baseline of 0.5 nmol/L (95% CI, 0.5–0.6 nmol/L) to a maximum of 7.0 nmol/L (95% CI, 4.3–9.7 nmol/L) at 5 h (figure 2). In group A, which received danaparoid 10 min after LPS, maximum levels were only 1.8 nmol/L (95% CI, 1.2–2.3 nmol/L; P < .05, vs. baseline and vs. placebo). In group B, where the infusion of danaparoid was delayed until 3 h after LPS infusion, F1+2 levels increased similar to the placebo group up to 3 h (peak values at 3 h, 4.0 nmol/L; 95% CI, 1.7–6.2 nmol/L; P < .05, vs. baseline and vs. placebo). As soon as danaparoid was infused, F1+2 levels decreased rapidly, consistent with a short in vivo half-life of thrombin generation [27].

sF. Consistent with previous trials, sF increased from 1.3 μg/mL (95% CI, 1.0–1.6 μg/mL) to 9.0 μg/mL (4.7–13.3 μg/mL) at 8 h after LPS infusion in the placebo group (figure 2). In subjects treated with danaparoid at 10 min, maximum levels at 8 h were 3.0 μg/mL (1.3–4.7 μg/mL; P < .05, vs. baseline and vs. placebo). Subjects in group B, who received danaparoid at 3 h, reached maximum levels at 6 h, with 2.7 μg/mL (1.2–4.2 μg/mL; P < .05, vs. baseline and vs. placebo).

D-dimer. In subjects on placebo D-dimer increased from 0.19 ng/mL (95% CI, 0.09–0.29 ng/mL) to a maximum of 0.71 ng/mL (95% CI, 0.46–0.96 ng/mL) at 8 h and remained at that level until 24 h (figure 2). In group A, treated with danaparoid at 10 min after LPS infusion, D-dimer levels increased only to 0.39 ng/mL (95% CI, 0.20–0.58 ng/mL; P < .05, vs. baseline and vs. placebo). D-dimer levels in group B increased in parallel with placebo until 3 h, when a maximum of 0.53 ng/mL (95% CI, 0.79–0.26 ng/mL) was reached. Thereafter, no further increase occurred, and levels decreased after 8 h. Because of the earlier decline of D-dimer levels with danaparoid, the difference to placebo was most evident 24 h after LPS infusion in both danaparoid-treated groups (P < .05, vs. placebo).

PAP. PAP increased from 184 μg/L (95% CI, 131–237 μg/L) to 1103 μg/L (95% CI, 720–1486 μg/L) at 2 h (n = 30). No difference between the groups was seen (P > .05; figure 3).

TFFP. No changes in TFFP were seen in subjects on placebo after LPS infusion. Danaparoid treatment induced a short lasting increase from 0.97 μg/L (95% CI, 0.73–1.21 μg/L) to 1.43 μg/L (95% CI, 1.14–1.72 μg/L) at 60 min in the early
Interleukin (IL)–6 and macrophage chemotactic protein (MCP)–1 levels in 30 healthy volunteers after infusion of 2 ng/kg lipopolysaccharide (LPS) with and without danaparoid. Group A (●, n = 10) received 80 IU/kg danaparoid 10 min after LPS infusion, and group B (○, n = 10) received 80 IU/kg danaparoid 180 min after LPS. A control group (□, n = 10) received placebo. Data are means and 95% confidence intervals.

IL-6 and MCP. IL-6 levels increased from 2 pg/mL to 858 pg/mL at 180 min and returned to baseline levels at 360 min (figure 4). No difference between the groups was observed. MCP-1 increased from baseline values of 145 pg/mL to a maximum of 15,995 pg/mL 6 h after LPS stimulation. No difference between the groups was seen.

IL-8 and TNF-α. IL-8 levels increased from 4 pg/mL to a maximum of 602 pg/mL at 120 min. No difference between the study groups was observed.

Leukocytes. After LPS infusion, neutrophils in peripheral blood fell from a baseline of 3.4 × 10⁷ cells/L (95% CI, 3.0 × 10⁷–3.8 × 10⁷ cells/L) to a nadir of 0.9 × 10⁷ cells/L (95% CI, 0.7 × 10⁷–1.1 × 10⁷ cells/L) at 80 min after LPS, followed by a leukocytosis with a peak of 8.3 × 10⁷ cells/L (95% CI, 7.6 × 10⁷–8.9 × 10⁷ cells/L) at 6 h. No differences were observed between the groups.

Monocyte counts in peripheral blood fell from 0.33 × 10⁷ cells/L (95% CI, 0.27 × 10⁷–0.39 × 10⁷ cells/L) before LPS infusion to 0.03 × 10⁶ cells/L (95% CI, 0.01 × 10⁵–0.05 × 10⁶ cells/L) at 2 h, and they returned to normal (0.3 × 10⁵–0.5 × 10⁵) by 6 h. This could be observed in all 3 groups (P > .05).

Leukocyte activation and monocyte TF expression. Expression of CD11b on neutrophil surfaces was 186 MFI (95% CI, 134–238 MFI) before LPS in all groups (figure 5). As expected [26], LPS infusion increased CD11b levels to 597 MFI (95% CI, 491–703 MFI, measured at 8 h; P < .05, vs. baseline, P > .05, between groups).

At baseline, 4% (95% CI, 3%–6%) of circulating monocytes expressed TF on their surface. Eight hours after LPS, 11% (95% CI, 9%–13%) of monocytes were TF⁺ (P < .05 versus baseline). There was no difference between the groups for both parameters (P > .05).

**Discussion**

In the present study, we investigated the effect of danaparoid-sodium on coagulation activation and inflammation during experimental human endotoxemia. Danaparoid was chosen because of its predominant anti-FXa activity. Although the precise
mechanism of action is not clear, its high ratio of anti-FXa: anti-FIIa activity (>20:1) is assumed to be related to the differences in half-lives of anti-FXa and anti-FIIa activities (20 h and 90 min, respectively) [28].

Previously published data from our group [11] that compared UFH with LMWH have raised questions about a central role of FXa for the effects of anticoagulants in endotoxin-induced coagulation activation. In addition, FXa might represent an important link between coagulation and inflammation [14].

After infusion of endotoxin into healthy volunteers, F$_{1+2}$ increased >13-fold in subjects on placebo. When danaparoid was infused 10 min after LPS infusion, this increase was only 2.7-fold. Similarly, high increases in F$_{1+2}$ levels were seen after administration of dalteparin [11], lepirudin [12], or low-dose TFPI [19] in the same model. In contrast, UFH [11] and high-dose TFPI [19] completely abolished thrombin formation. We used 6400 U of danaparoid in the present trial, which is currently only recommended in the setting of cardiac bypass surgery. Although we have not fully reached the initial anti-FXa activities of the UFH trial, anti-FXa activities were in the “supratherapeutic range” for any standard indication of danaparoid [28], up to 6 h after administration. Hence, even very high doses of a relatively selective anti-FXa anticoagulant cannot fully suppress thrombin generation during endotoxemia. We therefore postulate that thrombin formation after LPS-induced TF activation is not completely dependent on FXa. The TF/VIIa complex, in addition to activating FXa, is also considered to generate minute quantities of FIXa. It can be assumed that TFPI also blocks this generation of IXa, which could explain its superior efficacy in the present model of endotoxin-induced coagulation activation. Alternatively, danaparoid may not inhibit the small quantities of platelet-bound FXa generated in this protected environment.

In addition, leukocytes and platelets become activated during endotoxemia. As a consequence, leukocyte-platelet aggregates form and CD11b expression is enhanced [26]. Because CD11b has been described as an alternative receptor for FXa [29–31], this mechanism could represent an alternative pathway of coagulation induction in endotoxemia.

With regard to the higher potency of UFH to inhibit LPS-induced thrombin formation, 3 mechanisms may play a role. UFH has repeatedly been shown to release TFPI into circulation [11, 23]. However, the degree of TFPI release was similar after UFH and dalteparin infusion, although dalteparin, similar to danaparoid, did not completely blunt thrombin formation. Thus, we believe that the additional anti-FIIa activity of UFH might confer higher efficacy in this model.

Alternatively, because heparin blunts the LPS-induced increase in TF$^+$ monocytes, this might well contribute to the superior potency of heparin in endotoxemia. As opposed to UFH [11], danaparoid did not block TF expression on monocytes (figure 5): TF$^+$ monocytes more than doubled in all 3 groups at 8 h. Because hirudin blunted the LPS-induced increase in TF$^+$ monocytes [12], this finding supports the concept that anticoagulants with a higher anti-FIIa:anti-FXa ratio or platelet-inhibitory effect [32] more potently inhibit TF expression in vivo.

In all previous studies that have tested anticoagulants in this model of coagulation activation, anticoagulants were infused shortly after LPS administration. This might not adequately reflect a clinical situation, where therapeutic interventions are usually started at a time when coagulation activation is already evident. Hence, the second major aim of the present trial was to investigate whether delayed anticoagulant treatment can still influence thrombin generation and subsequent fibrin formation. Therefore, our study design included a treatment arm in which danaparoid was delayed until 3 h after LPS infusion. Figure 2 clearly demonstrates that thrombin formation already decreases within 1 h of late danaparoid infusion. This was also reflected in a substantial reduction of the generation of sF and D-dimer, compared with placebo. These data, in a model of endotoxin-induced coagulation activation, demonstrate that delayed anticoagulant treatment is still able to inhibit further activation. This is in good agreement with the observation that recombinant human activated protein C has a beneficial impact on d-dimer levels in patients with overt DIC [6].

With regard to potential mechanisms of action of danaparoid, our results demonstrate that danaparoid induces only a minor (40%) and short-lived (<3 h) release of TFPI into circulation. In contrast, UFH and dalteparin increased TFPI levels by 100%–150% for at least 4–6 h [11]. Given that the infusion of low-dose TFPI, which increased TFPI levels 3-fold, did not fully block thrombin formation [19], it is unlikely that TFPI release by danaparoid constitutes a relevant mechanism of its action.

Effective anticoagulation with danaparoid did not alter the activation and subsequent shut down of fibrinolysis. PAP levels were comparable in all 3 groups. The same has been shown elsewhere for lepirudin [12] and TFPI [19]. This finding confirms the existing evidence that the activation of fibrinolysis is regulated independently from coagulation in the human endotoxin model [33].

Finally, a recent study showed that 10–80 nmol of FXa stimulated the production of IL-6, IL-8, and MCP-1 ~2–3-fold [14]. Similarly, thrombin enhanced 2-fold the expression of IL-8 and MCP-1 in ischemia reperfusion injury in rabbit coronary arteries [34]. This effect was inhibited by hirudin but not by defibrinogenation, which indicates a direct proinflammatory mechanism of thrombin. Such direct effects of thrombin and FX are mediated by so-called protease-activated receptors [35, 36], which have recently been subject of thorough reviews [37, 38]. In contrast, inhibition of FXa by danaparoid in our trial did not alter the LPS-induced increase in IL-6, IL-8, and MCP-1 concentrations. Notably, similar to danaparoid, effective anticoagulation with TFPI or heparins did not alter release of IL-6, IL-8, or MCP-1 into circulation [39, 40]. This discrepancy in results could possibly
be due to different behavior of endothelial cells in vitro, compared with in vivo. Furthermore, LPS challenge induced an increase in IL-6, IL-8, and MCP-1 levels by 2–3 orders of magnitude. Thus, the marked LPS-induced release of IL-6 and MCP-1 may mask any contribution of FXa or thrombin to the generation of these cytokines in vivo. Hence, our model does not argue against clinically relevant proinflammatory effects of thrombin or FX in other disease states, such as arterial thrombosis.

In summary, the current study demonstrates the anticoagulant efficacy of danaparoid in coagulation activation. It further proves the effectiveness of delayed anticoagulant treatment in the LPS-induced coagulation activation model in humans. Danaparoid decreased thrombin formation after LPS infusion to healthy humans, without influencing fibrinolytic or cytokine responses.

References