The Proinflammatory Cytokine Response to Coagulation and Endotoxin in Whole Blood

By Kirk Johnson, Lucien Aarden, Yoon Choi, Els De Groot, and Abla Creasey

Acute inflammatory illnesses, including the sepsis syndrome, often include a component of coagulation. A human whole blood culture system was developed so that the relationship between coagulation activation and cytokine responses in the presence or absence of lipopolysaccharide (LPS) could be evaluated. In the absence of LPS stimulation, coagulation activation resulted in a novel pattern of cytokine production. During a 4-hour culture of coagulating blood, significant production of interleukin-8 (IL-8; >2,000 pg/mL) was observed, whereas other proinflammatory cytokines including IL-1β, IL-6, or tumor necrosis factor α were undetectable or less than 35 pg/mL. The cytokine profile was distinct from that of fully anticoagulated, LPS-stimulated blood, which showed levels of all the indicated proinflammatory cytokines ≥2,000 pg/mL over the same time period. Over 24 to 48 hours, the coagulation-induced cytokine response was characterized by marked and sustained IL-8 production, limited IL-6 generation (with kinetics delayed relative to IL-8), and minimal or undetectable tumor necrosis factor α levels. The magnitude of the whole blood IL-8 response correlated with the level of coagulation activation as determined by measurement of thrombin-antithrombin III complex formation. The combined stimuli of coagulation activation and LPS challenge induced a synergistic enhancement of IL-8 production but not of IL-6. Coagulation-induced cytokine production and the synergistic production of IL-8 by coagulation and LPS could be attenuated by hirudin or tissue factor pathway inhibitor (TFPI). Studies to elucidate mechanisms implicated (1) the TFPI third Kunitz and carboxy-terminus as important structural components for TFPI regulation of coagulation activation and (2) thrombin as a candidate mediator of the mononuclear cell cytokine response to coagulation activation. In summary, a unique aspect of the cross-talk between the coagulation and cytokine cascades in whole blood is shown with the identification of IL-8 as a key proinflammatory participant.

The Proinflammatory Cytokine Response to Coagulation and Endotoxin in Whole Blood

The observation of coagulation activation after induction of acute inflammatory reactions has been well documented. For example, the infusion of bacteria or endotoxin into humans and animals induces cytokine production from activated cells as well as cascades of complement and coagulation activation and fibrinolysis. A dominant pathway for coagulation activation during acute inflammation involves the extrinsic pathway and is driven by the exposure of the blood compartment to tissue factor that acts as a cofactor for the factor VIIa-dependent conversion of factors X to Xa and IX to IXa. In inflammatory conditions, direct vascular injury may reveal constitutively expressed tissue factor on subendothelial tissues. Alternatively, endotoxin and proinflammatory cytokines such as tumor necrosis factor α (TNFa) or interleukin-1β (IL-1β) may efficiently induce tissue factor activity on the surface of endothelial cells and monocytes. Although tissue factor-driven coagulation is likely predominant, activation of the contact or intrinsic pathway may play a role in certain inflammatory conditions including severe bacterial infection. Coagulation activation ultimately results in thrombin formation that may positively feed back on the cascade and facilitate clot formation via fibrinogen conversion to fibrin and the activation/aggregation of platelets. Although coagulation generally benefits the host, both localized thrombosis and disseminated intravascular coagulation (DIC) may occur in some situations and contribute to host morbidity or mortality.

The deleterious effects of coagulation in some inflammatory states has prompted the evaluation of various anticoagulants for efficacy in diseases ranging from postangioplasty stenosis to sepsis. One attractive candidate is the natural extrinsic pathway regulator, the tissue factor pathway inhibitor (TFPI). TFPI is a Kunitz-type serine proteinase inhibitor that directly inhibits factor Xa and inhibits the VIIa/TF complex in an Xa-dependent manner. Although TFPI is present in plasma at a concentration of ∼2 nmol/L, primarily bound to lipoproteins, the inhibitor may also be localized via glycosaminoglycans to the endothelium. TFPI associated with the endothelium may be important in local regulation of coagulation and may contribute to systemic anticoagulation when released on heparin administration. Recently, we have evaluated the potential benefit of TFPI in a baboon model of Escherichia coli sepsis with DIC. Unlike some other anticoagulants tested in the baboon model, TFPI attenuated coagulation and provided a significant survival benefit even when administered as long as 2 to 4 hours after initiation of the E coli infusion. Moreover, TFPI reduced the levels of IL-6, a cytokine that contributes to the acute phase response and may represent a prognostic marker of sepsis. Altered cytokine production by TFPI was not limited to IL-6 because levels of other proinflammatory cytokines, including IL-8, were also shown to be downregulated in the baboon model in subsequent studies.

The observation of regulated cytokine production by TFPI in an animal model of bacterial sepsis with DIC prompted us to investigate mechanisms of cross-talk between coagulation and acute inflammation in a controlled fashion. The objective was to evaluate the effect of coagulation activation, in the presence or absence of lipopolysaccharide (LPS), on leukocyte cytokine production in an ex vivo model system. Accordingly, we have used whole blood cultures whereby...
coagulation activation could proceed in the presence or absence of LPS and the cytokine response could be monitored. A unique profile was observed involving IL-8; i.e., coagulation efficiently induced IL-8 production, and the combination of coagulation activation plus LPS produced significantly more IL-8. Addition of TFPI or other anticoagulants to the whole blood cultures abrogated IL-8 production induced by either coagulation alone or the combination of coagulation activation and LPS. Mechanistic studies implicated thrombin as a candidate mediator of the proinflammatory cytokine program.

MATERIALS AND METHODS

Reagents. LPS (E coli Rc) and cycloheximide were obtained from Sigma (St Louis, MO). Recombinant TFPI was expressed in E coli and purified and refolded as previously described.34 The TFPI was formulated at 11 mg/mL in 2 mol/L urea, 20 mmol/L sodium phosphate (pH, 7.2), and 0.14 mol/L NaCl. Because TFPI concentrations in the current experiments were ≤20 μg/mL, the excipient was diluted sufficiently so that no vehicle effect was ever observed under any experimental condition. TFPI lacking the third Kunitz and carboxy (C)-terminus (TFPI,336) was generously provided by Dr Tom Girard (Monsanto/Searle, St Louis, MO) and was prepared as described.35 Recombinant yeast hirudin and purified human a-thrombin were obtained from American Diagnostica (Greenwich, CT), and heparin was purchased from ESI (Cherry Hill, NJ). The purified coagulation factors, prothrombin and factor Xa, were obtained from Haematologic Technologies (Essex Jct, VT).

Whole blood cultures. Venous blood from normal healthy donors was collected directly into clinical heparin or EDTA (K3) vacutainers (Becton Dickinson, Rutherford, NJ). Alternatively, venous blood was collected through 18-gauge needles into sterile polypropylene syringes and was immediately transferred into microtiter wells containing indicated concentrations of various additives including LPS, heparin, hirudin, TFPI, and so on. In some cases, blood was collected into special vacutainers in which sterile hirudin had been pre aliquoted to provide a final concentration in blood of 50 to 60 U/mL. Blood collected into hirudin-vacutainers was quickly transferred into polypropylene tubes before addition into culture wells.

Whole blood was cultured in 48- or 96-well microtiter plates (Corning, Corning, NY) at a volume of 1 mL/well or 0.2 mL/well, respectively, in a humidified atmosphere of 5% CO2 at 37°C. Whole blood was cultured either undiluted or at a final dilution of 1:10 in RPMI 1640 + 0.1% low-endotoxin fetal calf serum (FCS; Hyclone, Logan, UT). The coagulation-induced cytokine response and the outcome of combined coagulation and endotoxin stimuli described herein was observable in cultures of either undiluted or 1:10 diluted blood. The harvest of supernatants was performed by transferring culture plates onto ice, rimmng any clots that formed, and spinning the plates at 500g for 1 minute at 4°C. Cell-free supernatants were harvested into microtiter wells containing ESB stop buffer (10 mmol/L EDTA, 100 μg/mL soybean trypsin inhibitor, and 10 mmol/L benzamidine) and stored at −20°C until analysis of soluble inflammatory mediator levels.

Peripheral blood mononuclear cell (PBMC) cultures. Whole blood collected into EDTA vacutainers was layered over a leukocyte separation gradient (neutrophil isolation medium [NIM]; Cardinal Assoc, Santa Fe, NM) at a mixture of 7 to 8 mL blood onto 5 mL NIM medium in 15-mL polystyrene tubes. The tubes were spun at 350g for 30 minutes, and the mononuclear cell layer was isolated as the top band in the gradient. After saline washes, PBMCs were cultured at ≈1 × 10^6 cells/well in RPMI/0.1% FCS as described above for whole blood cultures. Notably, the low concentration of FCS in the PBMC assay medium precluded a robust endotoxic response.

Cytokine analyses. Sandwich enzyme-linked immunosorbent assays (ELISAs), sensitive to 1 to 10 pg/mL, were used to measure supernatant levels of human TNFa, IL-6, and IL-8 from duplicate cultures as previously described.37-39 IL-1β was measured with the Quantikine IL-1β ELISA according to the manufacturer’s instructions (R & D Systems, Minneapolis, MN). A strong correlation between antigen levels and bioactivity of the cytokines measured in blood samples has been previously documented.31-33 Factor VIII/antithrombin factor inhibition by TFPI versus TFPI,336. The relative Vlla/TF inhibitory activity of full-length TFPI versus TFPI lacking the third Kunitz domain and C-terminus (TFPI,336) was measured in a multisite chromogenic end point assay as described by Girard et al.36 Based on the combination of data from three separate assays, the following 50% inhibitory concentration (IC50) values for the Xa-dependent inhibition of factor Vlla/TF were determined for the two TFPI preparations: TFPI = 5 ± 1 ng/mL; TFPI,336 = 3 ± 1 ng/mL.

Coagulation activation. Aside from the gross observation of clot formation, measurement of the extent of coagulation activation was performed quantitatively via immunodetection of thrombin:antithrombin (TAT) complexes as an indication of thrombin generation. In separate experiments not presented, the correlation of TAT levels and the IL-8 response were validated with alternative measurements of coagulation activation including prothrombin fragment F1.2 levels and fibrinopeptide A (FPA) levels using immunnoassays according to the manufacturer’s protocols (Baxter Diagnostics [Deerfield, IL] and Diagnostica [Stago, France], respectively). Supernatants were also routinely analyzed for chromogenic activity against various substrates including Spectrozyme Xa and TH (thrombin; American Diagnostica) for correlation with the above metrics as well as to confirm activity of purified factors added to isolated PBMC cultures including prothrombin, a thrombin, and factor Xa, hirudin, and TFPI.

RESULTS

Coagulation of whole blood induces a selective cytokine response. Experiments with whole blood were performed in microplate wells in which the blood was cultured either undiluted or at a 1:10 final dilution. All phenomena described were observable regardless of the dilution format. In some settings, an undiluted format was preferable to maintain the normal plasma balance of coagulation factors and their inhibitors and to measure protease:inhibitor complexes. In other settings, a diluted format was preferable to reduce the amount of donor blood required or to facilitate the dilution of a titratable anticoagulant present during blood collection.

To investigate the potential induction of proinflammatory cytokines by coagulation activation, venous blood collected into a polypropylene tube was immediately transferred to microtubes and cultured at 37°C. At various times, the supernatants from duplicate wells were harvested into a stop buffer, and the levels of proinflammatory cytokines were determined by sensitive sandwich ELISA. When cultured in this manner, blood from healthy donors coagulated at ≈30 minutes of culture. A singular cytokine response was observed on analysis of supernatants: for IL-1β, IL-6, IL-8, and TNFa; only IL-8 was reproducibly detected at significant levels in the first 4 hours of culture. As indicated in Fig 1, IL-8 became detectable at 30 minutes of culture, with marked
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Whole blood from healthy donors was collected into polypropylene tubes and immediately cultured undiluted in the absence of TFPI-anticoagulated cultures were undetectable or 550 pg/mL at 4 hours and analyzed for the indicated cytokines. Results represent the mean of duplicate determinations from at least two experiments with different donors.

Throughout the 4-hour culture period, the levels of IL-1β, IL-6, and TNFα were undetectable or less than 35 pg/mL. Mean IL-8 levels with standard error are shown for the control group. IL-8 levels overlaid by heparin but containing optimal concentrations of LPS (1 ng/mL, see Fig 2A) produced ≥2,000 pg/mL of IL-1β, IL-6, IL-8, and TNFα within the same 4-hour time frame in which "coagulation" blood showed significant levels of only IL-8 (Fig 1B).

LPS-induced cytokine production and the effect of TFPI in heparin-anticoagulated whole blood. The possibility that TFPI may directly affect LPS-induced cytokine production, independent of its anticoagulant activity, was tested in cultures of whole blood collected in a high concentration (50 U/mL) of the anticoagulant heparin. As shown in Fig 2A, the production of IL-8 (as well as other cytokines such as IL-6 and TNFα) peaked at 1 ng/mL LPS. Accordingly, the effect of TFPI on IL-8 produced in response to 1 ng/mL LPS was evaluated in the heparin-anticoagulated blood cultures. TFPI at concentrations ranging from 0.01 to 2 µg/mL had no significant effect on LPS-induced cytokine production (Fig 2B). Consistent with the depicted IL-8 levels, the IL-6 and TNFα response was similarly unaltered by TFPI. Although the physiological concentration of TFPI in the plasma of normal individuals averages about 0.1 µg/mL (≈2 nmol/L), TFPI may directly affect LPS-induced cytokine production, independent of its anticoagulant activity, was tested in cultures of whole blood collected in a high concentration (50 U/mL) of the anticoagulant heparin. As shown in Fig 2A, the production of IL-8 (as well as other cytokines such as IL-6 and TNFα) peaked at 1 ng/mL LPS. Accordingly, the effect of TFPI on IL-8 produced in response to 1 ng/mL LPS was evaluated in the heparin-anticoagulated blood cultures. TFPI at concentrations ranging from 0.01 to 2 µg/mL had no significant effect on LPS-induced cytokine production (Fig 2B). Consistent with the depicted IL-8 levels, the IL-6 and TNFα response was similarly unaltered by TFPI. Although the physiological concentration of TFPI in the plasma of normal individuals averages about 0.1 µg/mL (≈2 nmol/L), TFPI was tested at higher concentrations because TFPI levels of 2 to 3 µg/mL were used in the aforementioned animal studies.44–46 A trend towards slightly reduced cytokine production was observed only at very high concentrations of 10 to 50 µg/mL TFPI in which IL-6 and IL-8 levels were reduced about 25% (Fig 2B and data not shown). Therefore, TFPI has a minimal effect on LPS-induced cytokine responses in whole blood when coagulation is effectively curtailed by heparin.

Coagulation activation and endotoxin stimulation synergize for IL-8 production. Considering the results from the sepsis/DIC animal model and those described above, it was of interest to evaluate the IL-8 response and its potential regulation by TFPI after combined stimulation by coagulation and LPS. In preliminary experiments, it was determined that venous blood could be collected in vacutainers in the presence of the thrombin inhibitor, hirudin (final concentration, 50 U/mL), and cultured so that the generation of thrombin would exceed the inhibitory capacity of hirudin and allow clotting in the culture wells at ≈1 hour ("Low Hirudin" control). Accordingly, whole blood collected in vacutainers containing hirudin or heparin (fully anticoagulating, 50 U/mL) was transferred to culture wells containing media alone or LPS and/or TFPI. Analysis of supernatants collected at 2 hours of culture indicated that coagulation activation or
LPS stimulation resulted in IL-8 production of \( \approx 500\) pg/mL (Fig 3, shaded “low hirudin” or “LPS + heparin” bars). As expected, the addition of TFPI prevented IL-8 production caused by coagulation but did not significantly reduce LPS-induced IL-8 release (Fig 3, black). Interestingly, the combination of LPS treatment and coagulation resulted in a greater-than-additive enhancement of IL-8 to the level of \( \approx 3,000\) pg/mL (Fig 3, “LPS + Low Hirudin”, right shaded bar). TFPI addition inhibited the coagulation component of the synergistic response resulting in the production of \( \approx 650\) pg/mL IL-8.

Selective cytokine production after activation by coagulation and LPS. The marked induction of IL-8 by coagulation activation and LPS prompted analysis of the influence of coagulation on LPS-induced IL-6 and TNFα responses. Whole blood collected in low hirudin concentrations was diluted in assay medium and cultured in the presence of 1 ng/mL LPS. The low hirudin controls coagulated at approximately 1 hour of culture, whereby culturing in the presence of 5 U/mL heparin or 10 pg/mL TFPI prevented coagulation. As indicated in Fig 4A, the magnitude of IL-6 produced in whole blood on LPS stimulation was approximately the same in coagulating or anticoagulated cultures. In contrast, LPS-stimulated TNFα release was substantially and reproducibly lower in the coagulating cultures (Fig 4B). Anticoagulation by heparin or TFPI relieved an apparent inhibitory influence of coagulation activation on LPS-induced TNFα production (Fig 4B).

Importance of the third Kunitz and C-terminus for TFPI regulation of coagulation-induced IL-8 production. It has been documented that the third Kunitz and C-terminus of TFPI contribute significantly to Xa inhibition.\(^{26,39}\) To better understand the coagulation mechanisms operative in the whole blood cultures and to correlate TFPI structural domains with IL-8 regulation, full-length and truncated TFPI (TFPI\(_{1-166}\)) were compared in cultures of coagulation-induced IL-8 production. As indicated in the Materials and Methods section, the two TFPI preparations were compared for VIa/TF inhibitory activity, with the results indicating that TFPI\(_{1-166}\) was of equal or greater potency when compared with full-length TFPI. IL-8 levels, measured after 20 hours of culturing diluted whole blood in the absence (control) or presence of various concentrations of TFPI or TFPI\(_{1-166}\) are indicated in Table 1. Full-length TFPI significantly reduced coagulation-induced IL-8 production at 1 μg/mL and completely prevented IL-8 induction at 10 μg/mL. In contrast, TFPI\(_{1-166}\) minimally inhibited IL-8 production. Accordingly, the TFPI third Kunitz and C-terminus are important struc-
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Therefore, coagulation-induced cytokine production in whole blood is characterized by a strong IL-8 response, with minimal TNFα production and IL-6 generation at low levels and quite delayed relative to that of IL-8.

When cultured in the presence of 1 ng/mL LPS, the expected (Fig 3) synergistic enhancement of IL-8 production by coagulation and endotoxin was observed throughout the culture period (Fig 5B). IL-8 levels were first detected in the coagulating low-hirudin control at 2 hours and steadily increased to approximately fivefold greater levels than those observed in the absence of LPS. TFPI reduced the generation of IL-8 at all time points in a dose-dependent manner. Therefore, TFPI inhibition of coagulation-induced IL-8 production in whole blood is not because of an alteration of kinetics.

The IL-8 response to coagulation activation in isolated PBMC cultures. To better understand the cell type(s) and blood factors responsible for the IL-8 responses documented above, cultures of isolated mononuclear cells were performed. The addition of recalcified autologous plasma to PBMCs cultured in assay medium resulted in tremendous IL-8 production over that of the background level (Fig 6). TFPI addition prevented clotting and reduced IL-8 production in the cultures. The addition of fresh autologous serum to the PBMC cultures supported IL-8 production to approximately one fifth the level of recalcified plasma and was not affected by TFPI (Fig 5). Analysis for IL-6 and TNFα levels resulted in similar observations as those observed in the whole blood cultures; ie, TNFα levels were <25 pg/mL, and the IL-6 levels increased to less than one tenth the magnitude observed for IL-8 (plasma = 1.494 ± 216 pg/mL IL-6; plasma+TFPI, serum, and serum+TFPI = <200 pg/mL IL-6). Therefore, the results with PBMC cultures implicated active coagulation factors, such as thrombin, as mediators of a unique mononuclear cell cytokine response.

Correlation between thrombin generation and IL-8 production in whole blood. Mediation of the IL-8 response by coagulation cascade proteases such as thrombin was implicated from several observations including (1) maximal IL-8 production coincident with maximal coagulation activation (ie, clotting) and (2) enhanced IL-8 production in PBMC cultures with recalcified plasma versus fresh serum. Quantitative metrics of coagulation activation were used to directly explore the relationship between coagulation and cytokine production. Undiluted whole blood cultured for 4 to 5 hours in various concentrations of TFPI were analyzed for IL-8 levels and indices of thrombin levels (TAT complexes) and thrombin activity (FPA levels; data not shown). As indicated in Fig 7, the addition of TFPI produced a dose-dependent

Table 1. Regulation of Coagulation-Induced IL-8 Production in Whole Blood: Functional Significance of the TFPI Third Kunitz and C-Terminus

<table>
<thead>
<tr>
<th>Addition</th>
<th>IL-8 (pg/mL)</th>
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<tbody>
<tr>
<td>Control</td>
<td>1,028 ± 263</td>
</tr>
<tr>
<td>1 μg/mL TFPI</td>
<td>309 ± 64</td>
</tr>
<tr>
<td>10 μg/mL TFPI</td>
<td>7 ± 3</td>
</tr>
<tr>
<td>1 μg/mL TFPI,160</td>
<td>1,180 ± 235</td>
</tr>
<tr>
<td>10 μg/mL TFPI,160</td>
<td>747 ± 243</td>
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The cytokine response to purified coagulation factors. Significant contribution of Xa-driven coagulation activation in the cultured whole blood was implicated by the differential activity of full-length versus C-terminal–truncated TFPI in coagulation and the IL-8 response (Table 1). Accordingly, the cytokine-inducing activity of α-thrombin and factor Xa as candidate mediators of coagulation-induced IL-8 responses was evaluated. Thrombin and factor Xa have shown biological activity in cultures of purified mononuclear cells including the induction of chemotaxis and IL-6 production by thrombin and the costimulation of proliferation and enhanced LPS-induced IL-1 activity by thrombin and Xa.40-42 As indicated in Fig 8A, α-thrombin stimulated the production of significant levels of IL-8 in isolated PBMC cultures. The dose-response curve indicated stimulation of IL-8 above the background level at <5 nm and with marked cytokine stimulation at thrombin concentrations >200 nmol/L.

Similar dose-response studies with isolated PBMCs were performed with factor Xa. Preliminary studies with a chromogenic substrate validated the activity of the factor Xa and its inhibition by TFPI. Factor Xa at concentrations from 10 to 500 nmol/L failed to induce cytokine production despite significant recovery of chromogenic activity from the PBMC supernatants (Fig 8B and data not shown).

A direct effect of TFPI on thrombin activity was ruled out because TFPI did not affect the dose-response for a thrombin-induced IL-8 production (Fig 8B). Moreover, TFPI at concentrations >10 μg/mL did not affect thrombin activity against chromogenic substrates.

The possibility of endotoxin contamination of thrombin as a major contributor to the IL-8 response was ruled out by additional experiments. First, the magnitude of thrombin-induced IL-8 was not increased when the PBMCs were cultured in sufficient FCS to support a robust LPS response (IL-8 levels of 41 ng/mL in 0.1% FCS v 35 ng/mL in 5% FCS). Second, the dose-response to α-thrombin was not significantly shifted when the cultures contained 5% FCS and 5 μg/mL anti-CD14 F(ab')2, which attenuates an LPS re-

Figure 5. Time course of coagulation- and endotoxin-induced IL-8 production and its inhibition by TFPI. Whole blood was collected, diluted 1:10, and cultured as described in the legends to Figs 3 and 4. Supernatants were harvested at indicated time points and analyzed by ELISA for IL-8 levels. (A) Dose-dependent inhibition by TFPI of IL-8 production induced by coagulation activation throughout a 48-hour culture period. (B) Inhibition by TFPI of IL-8 levels induced by the combination of coagulation (control) and LPS. Data represent the mean ± SE of the combined data from two experiments with different donors.

decrease in the generation of thrombin as measured by TAT complex formation (Fig 7, ○). The curve resulting from plotting the levels of IL-8 detected at each concentration of TFPI (ie, at each level of TAT formation) was parallel to the TAT curve, with a slight shift to the right. Although IL-8 production was stimulated by relatively low levels of coagulation activation (TAT ≤100 ng/mL), tremendous IL-8 production occurred when coagulation was pronounced (TAT >10 μg/mL; FPA >10 ng/mL). Therefore, the whole blood IL-8 response was incrementally related to the magnitude of coagulation activation, as indicated by direct correlation with levels of TAT complexes.

The cytokine response to purified coagulation factors. Significant contribution of Xa-driven coagulation activation in the cultured whole blood was implicated by the differential activity of full-length versus C-terminal–truncated TFPI in coagulation and the IL-8 response (Table 1). Accordingly, the cytokine-inducing activity of α-thrombin and factor Xa as candidate mediators of coagulation-induced IL-8 responses was evaluated. Thrombin and factor Xa have shown biological activity in cultures of purified mononuclear cells including the induction of chemotaxis and IL-6 production by thrombin and the costimulation of proliferation and enhanced LPS-induced IL-1 activity by thrombin and Xa.40-42 As indicated in Fig 8A, α-thrombin stimulated the production of significant levels of IL-8 in isolated PBMC cultures. The dose-response curve indicated stimulation of IL-8 above the background level at <5 nm and with marked cytokine stimulation at thrombin concentrations >200 nmol/L.

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Figure 6. TFPI inhibits coagulation-induced IL-8 production in a reconstituted system of isolated PBMCs and recalcified autologous plasma. Isolated PBMCs were cultured with autologous recalcified plasma or autologous fresh serum in the absence or presence of 10 μg/mL TFPI. Culture supernatants were harvested at 24 hours, and IL-8 levels were quantitated by ELISA. Data are presented as the mean ± SE of combined results from three experiments with different donors.
response by preventing cellular stimulation by LPS–LPS-binding protein (LPS-LBP) complexes (data not shown). Accordingly, the results with titrated hirudin (Figs 3 and 4), the correlation of TAT levels and the IL-8 response (Fig 7), and the thrombin effect on PBMC IL-8 production (Fig 8A) implicate thrombin as a prime candidate for contributing to coagulation-induced cytokine production in whole blood.

Many of thrombin's diverse activities, including the enhancement of LPS-induced monocyte IL-1 activity, are dependent on its catalytic activity. Accordingly, we examined the effect of prothrombin and hirudin-inhibited α-thrombin on IL-8 production by PBMCs. Although both prothrombin and α-thrombin induced IL-8 release, the stimulation by equimolar α-thrombin was consistently greater than three-fold that observed by prothrombin. A component of the low IL-8 response to prothrombin could be related to some thrombin contamination or generation during cell culture. Low-level endotoxin contamination of prothrombin contributing to some "tickling," even in the absence of serum, was not excluded by rigorous testing as performed for α-thrombin. The addition of hirudin at concentrations sufficient to inhibit thrombin's chromogenic activity abrogated the induction of IL-8 by α-thrombin. Therefore, hirudin-interaction regions on thrombin, including the catalytic site, were important for inducing the mononuclear cell cytokine response.

DISCUSSION

The specific aim of the experiments described herein was to evaluate the proinflammatory cytokine response to the individual or combined stimuli of coagulation and LPS, the active component of endotoxin, in human whole blood. Insight from such a system should contribute to a mechanistic understanding of the influence of coagulation activation on acute proinflammatory cascades. Although cytokine responses to LPS have been evaluated in whole blood and isolated mononuclear cell cultures, the regulation of proinflammatory cytokine production after coagulation activation or the combination of coagulation and LPS in human whole blood has not been reported. Establishing a whole blood culture system was deemed essential as an ex vivo model for studying leukocyte inflammatory mediator responses for at least two reasons. First, multiple blood cell types are capable of responding to LPS and coagulation factors so that the ultimate cytokine response may be influenced by potential cross-talk between cells in a physiological milieu. Second, whole blood cytokine responses to stimuli such as LPS may more accurately reflect the in vivo situation than

![Fig 7. Relationship of thrombin generation, as measured by TAT complex formation, and IL-8 release in whole blood. Undiluted fresh whole blood was cultured with indicated final concentrations of TFPI for 4 hours. Supernatants were harvested and analyzed for mediator levels. TAT complex formation (in micrograms per milliliter; ○) is indicated on the left side. Upper left arrow indicates TAT formation in coagulating control cultures containing no TFPI. Right side of figure represents IL-8 levels (in picograms per milliliter; □) at each concentration of TFPI. Results are mean values with SD less than 20% from a single experiment, representative of three experiments with different donors. IL-8 production in control cultures (No TFPI) was 1980 ± 380 pg/mL.](#)

![Fig 8. Dose-response and structural requirement of α-thrombin-induced IL-8 production by PBMCs. PBMCs were cultured with purified coagulation factors for 20 to 24 hours, and supernatants were analyzed for cytokine levels. (A) The dose-response of thrombin-induced IL-8 production is shown. Data represent the mean ± SE of combined data with different donors from three experiments. (B) Effect of purified coagulation factors (10 μg/mL TFPI; 200 U/mL hirudin; 500 nmol/L α-thrombin, Xa, prothrombin) on IL-8 production by PBMCs. Data points are representative of mean ± SD of two separate experiments. Thrombin-induced IL-8 production is significantly inhibited by the thrombin inhibitor, hirudin, but not by TFPI.](#)
would cultures of isolated cell types (DeGroot et al, unpublished observations).

A novel cytokine response to coagulation activation and the combination of coagulation and endotoxin was observed in cultured whole blood. A figurative representation of the phenomena is shown in Table 2. Analysis of levels of the proinflammatory cytokines IL-6, IL-8, and TNFα after LPS challenge in fully anticoagulated blood resulted in significant production of TNFα, IL-6, and IL-8 (in order of increasing peak time point and concentration) as documented in vivo and in vitro (Creasey et al, Carr et al, Deforge et al, and De Groot et al, unpublished observations). However, when blood was cultured so that coagulation could proceed, a response dominated by IL-8 production and dependent on the level of coagulation activation was observed. In undiluted coagulating whole blood, IL-8 levels became detectable at 30 minutes of culture and steadily increased throughout a 24-hour culture period. Evidence for active synthesis of IL-8 was derived from the observation that (1) IL-8 levels in coagulating blood were markedly reduced in cycloheximide-containing cultures and (2) IL-8 levels observed over a 2- to 3-hour culture period of coagulating blood significantly exceeded the level of IL-8 detected in lysed blood. A further distinction of the coagulation-driven cytokine response in comparison with that of the endotoxin-stimulated mediator production (in anticoagulated blood) was that coagulating blood did not show detectable levels of IL-6 or TNFα within the same time frame in which levels ≥2 ng/mL were observed in LPS-stimulated cultures. Coagulation-induced IL-6 production was consistently of a lower magnitude and of delayed kinetics relative to those of the IL-8 response and those of LPS-driven IL-6 production (in anticoagulated blood), thus suggesting that IL-6 production in coagulating whole blood may be influenced by IL-8 generation.

The proinflammatory cytokine response to endotoxin was qualitatively and quantitatively influenced by clotting. When a coagulating culture was costimulated with LPS, the combined stimuli of coagulation activation and LPS resulted in synergistic IL-8 release. Paradoxically, coagulation activation concurrent with LPS stimulation tended to reduce TNFα levels, whereas IL-6 release was not significantly altered relative to the responses in anticoagulated endotoxin-containing cultures. The mechanism for the differential response of coagulation on LPS-stimulated cytokine production (ie, amplification of IL-8 release but not of TNFα) is unclear.

In the current study, the natural inhibitor of the extrinsic coagulation pathway, TFPI, was evaluated for its ability to regulate inflammatory mediator production after cell stimulation by coagulation and/or endotoxin. In anticoagulated blood cultures, TFPI showed minimal regulation of an LPS-induced response except at high concentrations (10 to 50 μg/mL), in which a slight inhibition of LPS-induced IL-6 and IL-8 production was observed. In contrast, TFPI represented an effective inhibitor of coagulation and the subsequent IL-8 response to coagulation activation. Accordingly, synergistic IL-8 production induced by the combination of coagulation plus endotoxin was abrogated by TFPI. Because the coagulation-induced cytokine response could be easily attenuated by appropriate concentrations of heparin, hirudin, or TFPI, participation of direct signaling of IL-8 production by VIIa/TF activity was not implicated.

A comparative analysis of the ability of full-length versus C-terminus–truncated TFPI (TFPII, (late) to modulate coagulation and the subsequent IL-8 response in whole blood provided mechanistic insights. In the absence of anticogulants, the cultured whole blood clotted at ≈30 minutes in the culture wells. Contact activation likely contributed to initiation of a coagulation cascade involving the intrinsic pathway (Furie and Furie, Mann et al, Davie et al, Esmon, Maynard et al, and K. Johnson, unpublished observations). Consistent with such a scenario was the observation that TFPI lacking the third Kunitz and C-terminus (TFPII, (late) showed significantly reduced potency as compared with that of full-length TFPI in attenuating coagulation and the subsequent IL-8 response. Based on relative VIIa/TF inhibitory activity (TFPII, (late) =TFPI) and the known significance of the third Kunitz and C-terminus of TFPI to Xa inhibition, coagulation in the whole blood cultures (without added LPS) involved intrinsic pathway-driven Xa generation. In LPS-stimulated whole blood, a greater contribution of the extrinsic pathway to coagulation activation and the IL-8 response was implied from observations of increased TFPI potency for the regulation of TAT and IL-8 (K. Johnson, unpublished observations).

The cytokine response to coagulation activation observed in whole blood was confirmed and extended in cultures of PBMCs whereby more mechanistic analyses were pursued. A proinflammatory cytokine response, similar in nature to that described for whole blood, was observed with PBMCs incubated with autologous recalcified plasma but not with serum, implicating mediation by active proteases versus inactive protease-serpin complexes. Consistent with documented biological activities, α-thrombin was subsequently identified as a candidate mediator of the coagulation-driven IL-8 response. First, IL-8 production directly correlated with TAT generation in whole blood and the cytokine response was attenuated by addition of the thrombin inhibitor, hirudin. Second, α-thrombin induced a significant IL-8 response in isolated mononuclear cells, with a dose-response similar to that extrapolated from analysis of TAT generation in whole blood. Finally, α-thrombin induction of IL-8 production by PBMCs appeared dependent, at least in part, on catalytic activity because prothrombin failed to mimic the α-thrombin response and hirudin could inhibit

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<th>Stimulus</th>
<th>IL-8</th>
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<tr>
<td>LPS</td>
<td>+</td>
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<tr>
<td>Coagulation</td>
<td>+</td>
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<td>-</td>
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<td>Coagulation + LPS</td>
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Relative cytokine response to indicated stimuli in undiluted whole blood (≤5 hours) or 1:10 diluted blood (≤16 hours).

Abbreviations: +, cytokine level >500 pg/mL (see Table 1 and Figs 1-8 for actual values); −, undetectable cytokine level (<20 pg/mL); + (late), detectable cytokine level but after indicated time frame; ++++, synergistic increase in cytokine level.
thrombin-induced IL-8 release. Interestingly, addition of high concentrations of factor Xa with significant recovery of postculture chromogenic activity failed to result in significant activation of IL-8 production by PBMCs. Although synergistic IL-8 production by combinations of Xa and thrombin in preliminarily PBMC experiments was not observed, the possibility of positive cooperativity between coagulation factors in whole blood responses could not be ruled out.

A significant body of evidence has accumulated describing the regulation of leukocyte functional responses by thrombin including monocyte proliferation and chemotaxis. The current results are consistent with a scenario in which the generation of thrombin in whole blood, at sufficiently high concentrations, can result in activation of blood monocytes leading to the synthesis and release of IL-8 (and delayed production of IL-6). Accordingly, the costimulation of IL-8 production in whole blood by coagulation activation and endotoxin highlights IL-8 as a key participant in the cross-talk between the coagulation and cytokine cascades. Whereas coagulation activation and endotoxin can be individually manipulated in vitro, an endotoxin response in vivo would generally include (or induce) a coagulation component. The accumulation of significant levels of proinflammatory cytokines including IL-6, IL-8, and TNFα in human and animal sepsis20-22,24,25,36-38 has been interpreted to result, at least in part, from endotoxin-induced cytokine release by monocytes and endothelial cells. Additional contributions from other inflammatory cascades including coagulation and complement and cross-talk or feedback loops between inflammatory mediators and effectors all contribute to the systemic inflammatory response syndrome typical of sepsis.20-22,24,25,36-38 Based on the current results and proposed model, the ability of TFPI to downregulate IL-8 levels in baboon sepsis with DIC might, in part, be explained by attenuation of synergistic IL-8 production by the combination of coagulation and endotoxin. The observation that TFPI did not affect levels of TNFα in the baboon model would be consistent with the current results showing minimal contribution of TNFα in the whole blood coagulation-induced cytokine response. Finally, the current results would suggest that the ability of TFPI to downregulate IL-6 levels in the baboon sepsis model may be dually related to abrogation of delayed monocyte IL-6 production by coagulation activation as well as potential inhibition of thrombin-induced endothelial cell IL-6 production. Accordingly, the role of IL-8 as a protagonist in the proinflammatory merge of cascades should continue to be evaluated in vitro and in vivo.

ACKNOWLEDGMENT

The authors are grateful for critical review of the manuscript by Drs E. Hack and P. Jansen (Central Laboratory of the Red Cross [CLB], Amsterdam, The Netherlands), B. Zimmerman and T. Gesner (Chiron, Emeryville, CA), and T. Girard (Searle, St Louis, MO). G. Broze, Jr (Washington University, St Louis, MO) and the staff of the Autoimmunity Department at the CLB offered helpful suggestions and insights. H. Chin is acknowledged for administrative assistance. This work is dedicated to the memory of Dr K.A. Johnson (University of California, San Francisco, CA).

REFERENCES


The proinflammatory cytokine response to coagulation and endotoxin in whole blood

K Johnson, L Aarden, Y Choi, E De Groot and A Creasey