

REVIEW

The cytokine-mediated imbalance between coagulant and anticoagulant mechanisms in sepsis and endotoxaemia

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Abstract. Disseminated intravascular coagulation (DIC) is a frequently occurring complication of sepsis and may contribute to multiple organ failure. More insight into the pathogenesis of this derangement of the coagulation system is necessary to develop more effective therapeutic strategies for this condition. Recently, more detailed knowledge on the pathogenetic pathways involved in DIC has been obtained by the study of models of experimental bacteraemia and endotoxaemia in human subjects and non-human primates. The mechanisms that lead to activation of coagulation, potentiated by the simultaneous depression of physiological inhibitory systems and to impaired function of the fibrinolytic system, are outlined in this review. In addition, the mediatory role of various cytokines in the derangement of coagulation is discussed.

Keywords. Blood coagulation, cytokine, disseminated intravascular coagulation, endotoxin, fibrinolysis, lipopolysaccharide, sepsis.

Introduction

Severe systemic infection may be accompanied by serious haemodynamic, metabolic and inflammatory derangements, ultimately resulting in septic shock and multiple organ failure [1]. Simultaneously occurring derangement of the coagulation system leads to the deposition of microvascular thrombi in various organs [2,3], which may contribute to the pathogenesis of the multiple organ failure. As outlined below, the derangement of the coagulation system comprises enhanced activation of coagulation, depression of inhibitory mechanisms of coagulation and inhibition of the fibrinolytic system. Secondly, depletion of platelets and coagulation proteins, due to the extensive and ongoing activation of the coagulation system (and possibly also

by impaired liver synthesis and by activated protease-mediated destruction of coagulation factors), may induce severe bleeding complications. The resulting clinical picture of the simultaneous occurrence of (microvascular) thrombosis and bleeding is called disseminated intravascular coagulation (DIC) [4]. At present, no specific and effective therapy for DIC is available. The primary treatment of DIC in septic patients should be directed at the underlying disease (e.g. by antibiotics or surgical intervention), but clinically important thrombus formation and the simultaneous occurrence of bleeding may require additional supportive measures. In order to develop effective therapeutic strategies that target the coagulation system in sepsis, it is important to determine the mechanisms involved in the development of coagulation disorders associated with sepsis.

Models of experimental bacteraemia or endotoxaemia and activation of coagulation

Experimental models may be useful to analyse these pathogenetic mechanisms of disseminated intravascular coagulation. Models of experimental bacteraemia or endotoxaemia can be divided into *in vitro* and *in vivo* models. The *in vitro* models often consist of cell culture systems in which, for example, cultures of human endothelial cells can be exposed to bacterial endotoxin. These simplified systems can provide adequate answers on the significance of molecular processes at the surface of the endothelial cell, but the main problem of these *in vitro* models is the difficulty of translating the results to the *in vivo* situation. One obvious confounding factor is the lack of possible interactions with other mediators, which cannot be taken into account. In *in vitro* systems this is not a theoretical concern, as it has repeatedly been found that inflammatory mediators may have additive or synergistic effects that markedly differ from their individual biological activities [5].

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Experimental studies performed in smaller animals such as the rat and the rabbit do not have this disadvantage but nevertheless are difficult to compare with the human situation. For instance, the dose of endotoxin per kilogram that is necessary for the induction of a septic syndrome in an animal such as the rat is many times higher than needed to induce sepsis in humans [6]. Primate animal models, such as baboons or chimpanzees, appear to be more suitable for studying the pathogenetic mechanisms involved in the development of the septic syndrome. In many studies baboons (*Papio cynocephalus cynocephalus*) have been used to investigate the efficacy of potential therapeutic substances by assessing their ability to improve the survival of a lethal *Escherichia coli* infusion [2,3]. In such animal models, the effect of the therapeutic intervention on the coagulopathy induced by septicaemia can be defined using well-defined end points, such as the amount of fibrinogen consumption and fibrin deposition [7,8]. However, these models are not sufficiently specific to analyse incisively the involvement of individual coagulation and fibrinolytic factors or inhibitors in the pathogenesis of DIC.

To study the specific mediators and mechanisms involved in the coagulative response to septicaemia, it is necessary to measure either peptides liberated from the coagulation factor zymogens during their activation (activation peptides) or complexes between activated coagulation factors and their natural inhibitors. Because activated coagulation factors in plasma have a very short half-life, direct measurements of these factors during coagulation activation is not feasible. In our experience the chimpanzee is the only animal in which the specific human assays for these activation peptides and protease complexes can be employed [9]. Because the response of the chimpanzee to an infusion of a low dose of endotoxin is identical to the human response and the coagulation system of chimpanzees is virtually identical to the human system, the chimpanzee seems to be a very suitable animal model for the study of coagulopathies associated with sepsis [9,10]. By the administration of monoclonal antibodies and other preparations directed against the activity of specific human coagulation factors or inhibitors this model has yielded more insight in the pathogenesis and potential therapeutic approaches in endotoxaemia.

Apart from the animal studies, infusion of low doses of endotoxin into healthy volunteers appears to be a safe and very useful model for studying coagulation activation during endotoxaemia in humans. This model can give more insight into the early dynamics and route of coagulation activation during human endotoxaemia. Finally, clinical studies in septic patients may be helpful, although these studies are difficult to interpret because of the already late stages of coagulation activation at presentation, which in many cases have already proceeded to full-blown diffuse intravascular coagulation (DIC).

Like most host responses, the derangement of coagulation is initially triggered by micro-organisms and their products, but mediators, in particular cytokines, produced by the hosts in response to these agents play a

significant role in the development of these processes. Cytokines are low molecular weight proteins that are released by a number of cells such as mononuclear cells, lymphocytes and fibroblasts [11,12]. An increasing number of cytokines have been identified and elevated levels of a significant number of these mediators can be detected in tissue or serum of patients with sepsis, indicating a potential mediatory role for each of these cytokines [11,13]. In order to determine which cytokines are most important for the disturbances in the systems of coagulation and fibrinolysis, suitable experimental models are also required. Recently, a number of studies were performed in which limited amounts of specific cytokine preparations were infused in human subjects (cancer patients or healthy volunteers). These experiments made it possible to study the effects of individual cytokines on the systems of coagulation and fibrinolysis (see below). In addition, blocking monoclonal antibodies directed against specific cytokines may be helpful in determining which cytokine plays a significant role in the pathogenesis of endotoxin-induced activation of coagulation.

In conclusion, it appears that, besides clinical studies in septic patients, studies in primates such as baboons or chimpanzees, as well as experimental studies in healthy volunteers, are particularly useful in the investigation of the haemostatic imbalance in sepsis. In this review we will focus on recent insights into the pathogenesis of the coagulative derangement during this experimental bacteraemia or endotoxaemia.

Activation and inhibition of coagulation during endotoxaemia

The intravenous administration of (low-dose) endotoxin to human subjects or primates elicited a marked coagulant response, as reflected by elevations in plasma level of prothrombin activation fragment F1+2, thrombin-antithrombin (TAT) complexes and fibrinogen activation peptide FpA, with peaks between 3 and 5 h after the injection [10,14]. These studies demonstrated that the thrombin generation observed is mediated by activation of factor X, measured as an increase in factor X activation peptide, preceding the conversion of prothrombin to thrombin. Factor X, the first factor of the common pathway, can be activated either by the contact system-mediated pathway of coagulation (intrinsic pathway) or by the (extrinsic) tissue factor/factor VIIa-mediated route. Although indicators of contact activation can be detected in a number of patients with sepsis [15], it has been shown in models of experimental endotoxaemia that markers for activation of the contact system (factor XIIa-C1 inhibitor complexes and kallikrein-C1 inhibitor complexes) remain unchanged [10], indicating that the contact system is not involved in the initiation of coagulation activation during sepsis. In studies of bacteraemic baboons it has indeed been shown that administration of factor XIIa-blocking monoclonal antibodies can not prevent activation of coagulation [16]. Thus, it has been postulated that thrombin generation is mediated

by the (extrinsic) tissue factor/factor VIIa-dependent pathway. This hypothesis has been confirmed by several experiments in primates in which the coagulant response upon bacteraemia or endotoxaemia could be completely blocked by the simultaneous administration of monoclonal antibodies that are able to inhibit tissue factor activity [10,17]. In these experiments a single bolus injection of the anti-tissue factor antibody prevented the endotoxin-induced generation of thrombin, as reflected by unchanged plasma levels of prothrombin activation fragment F1+2 and complexes between thrombin and antithrombin III. Similarly, in another series of experiments it was shown that Fab fragments of monoclonal antibodies with potent factor VIIa-inhibiting properties could completely block the endotoxin-induced activation of coagulation [18].

As shown above, the contact system of coagulation does not play a significant role in the initiation of endotoxin-induced activation of coagulation. However, in septic patients low plasma levels of factor XII and prekallikrein and high plasma levels of complexes between kallikrein and C1 inhibitor or α_2 -macroglobulin and between factor XIIIa and C1 inhibitor can be detected [15,19,20]. These findings indicate that the contact system is activated in septic patients, although not involved in coagulation activation. Blocking the contact activation system in baboons by a factor XIIIa-inhibiting antibody resulted in a reduction in the *E. coli*-induced irreversible and lethal hypotension. In these studies the initial *E. coli*-induced hypotension, occurring 90 min after the *E. coli* infusion, was not prevented by inhibition of the contact system but the secondary and lethal hypotension, occurring approximately 180 min after the infusion of *E. coli*, was not present in the baboons treated with the contact system-inhibiting antibody. These observations suggest an important role for the contact activation system in the haemodynamic derangements of septic patients [15]. This effect is probably mediated by the generation of kinins, such as bradykinin, during activation of the contact system.

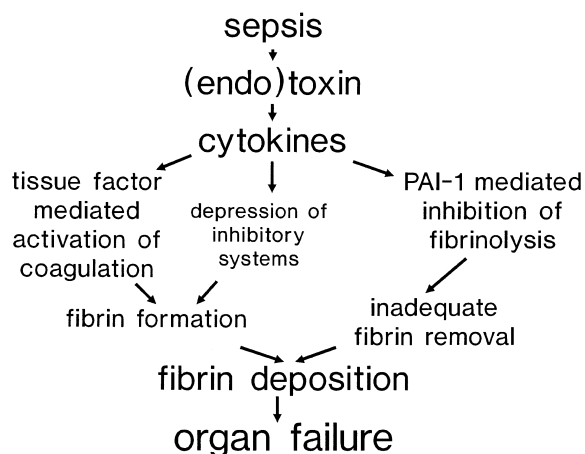


Figure 1. Schematic representation of the pathogenetic pathways of the derangement of coagulation and fibrinolysis in patients with sepsis.

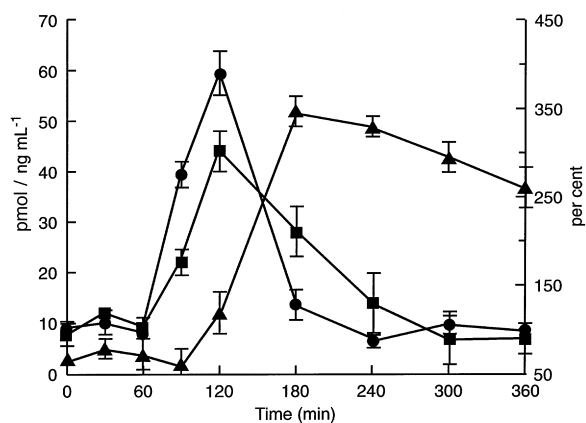


Figure 2. Effect of an intravenous injection of *E. coli* endotoxin (4 ng kg^{-1}) at $t=0$ min in six human subjects on parameters of fibrinolysis. Plasminogen activator activity (●, right axis) increases, leading to the generation of plasmin, as reflected by enhancement of plasma levels of complexes between plasmin and α_2 -antiplasmin (■, left axis, nmol L^{-1}). After an initial delay, plasminogen activator inhibitor type 1 (▲, left axis, ng mL^{-1}) sharply increases, resulting in a complete and sustained shut-down of plasminogen activator activity and plasmin generation.

Besides the tissue factor-mediated activation of the coagulation system, the procoagulant state is further promoted by impaired function of physiological inhibitory mechanisms. Antithrombin III, the main inhibitor of thrombin and factor Xa, is rapidly consumed by the ongoing thrombin formation, resulting in (very) low levels of this protease inhibitor [4]. The importance of sufficient plasma levels of antithrombin III has been shown in experimental and clinical studies [21,22]. In addition, a significant down-regulation of the protein C–protein S system may occur. Protein C and protein S are plasma proteins with anticoagulant functions. Protein C is an important inhibitor of factor Va and factor VIIIa and is activated by complex formation of thrombin with an endothelial cell surface protein, thrombomodulin (TM). The anticoagulant capacity of protein C is enhanced by its natural co-factor, protein S. The down-regulation of thrombomodulin during sepsis, which is probably caused by cytokines [in particular tumour necrosis factor (TNF)], results in diminished protein C activity and may enhance the procoagulant state [23,24]. It has indeed been shown that treatment with (activated) protein C concentrate can reduce the coagulopathy in bacteraemic baboons [25]. In plasma, 60% of the co-factor protein S is complexed to a complement regulatory protein, C4b-binding protein (C4bBP). The anticoagulant capacity of protein C is enhanced by the free fraction of protein S. Taylor [26] proposes that increased plasma levels of C4bBP may result in a relative protein S deficiency as a consequence of the acute-phase reaction in inflammatory diseases, thus contributing further to the procoagulant state during sepsis. In support of this hypothesis, it has been shown that the infusion of C4bBP in combination with a sublethal dose of *E. coli* into baboons resulted in a lethal response with severe organ damage due to diffuse intravascular coagulation

[27]. Finally, an important physiological inhibitor of tissue factor activity, tissue factor pathway inhibitor (TFPI), has recently been discovered, and (dis)regulation of this inhibitor may potentially be an important factor as well [28–30].

In conclusion and summarized in Fig. 1, bacteraemia or endotoxaemia causes a tissue factor-mediated activation of coagulation which is potentiated by depression of the physiological inhibitory mechanisms of coagulation and may lead to deposition of fibrin in the vasculature.

Activation and inhibition of fibrinolysis during endotoxaemia

Endotoxaemia also affects the fibrinolytic system, as originally described by Suffredini *et al.* [31] and later confirmed by other investigators [32]. As shown in Fig. 2, endotoxaemia is followed by a rapid enhancement of plasminogen activator activity, owing to increases in tissue-type plasminogen activator and urokinase-type plasminogen activator. The increase in plasminogen activator activity leads to the generation of plasmin, as indicated by an increase in the plasma level of plasmin- α_2 -antiplasmin (PAP) complexes. Approximately 1 h after the increase in plasminogen activator, rapidly increasing plasma levels of plasminogen activator inhibitor type 1 (PAI-1) result in complete suppression of plasminogen activator activity and plasmin generation, and the fibrinolytic system remains completely shut off for several hours after the administration of endotoxin [32].

Comparing these results with the findings of studies on endotoxin-induced coagulation activation or endotoxin administration [10,14] it can be concluded that at the time of maximal thrombin generation (i.e. 4 h after endotoxaemia) fibrinolysis has already been shut down. This leads to a remarkable imbalance between the coagulation and fibrinolytic system, resulting in a net procoagulant state.

Interestingly, inhibition of the coagulant response upon endotoxaemia does not affect activation and inhibition of the fibrinolytic system. In the studies mentioned earlier, in which endotoxin-induced activation of coagulation could be completely blocked by the administration of anti-tissue factor or anti-VIIa monoclonal antibodies, fibrinolytic parameters remained unaffected [10,18]. Also, the administration of the specific thrombin inhibitor hirudin could not prevent the endotoxin-induced activation and inhibition of fibrinolysis [32]. These observations suggest that endotoxin-induced effects on coagulation and fibrinolysis are regulated independently and that earlier hypotheses of thrombin-mediated activation or inhibition of fibrinolysis (partly based on *in vitro* studies) may not be correct [33–35].

In conclusion, at the time of ongoing activation of coagulation and after an initial and short-lasting activation, the fibrinolytic system appears to be completely shut down as a result of bacteraemia or endotoxaemia (Fig. 1). This blockade is due to increased plasma levels

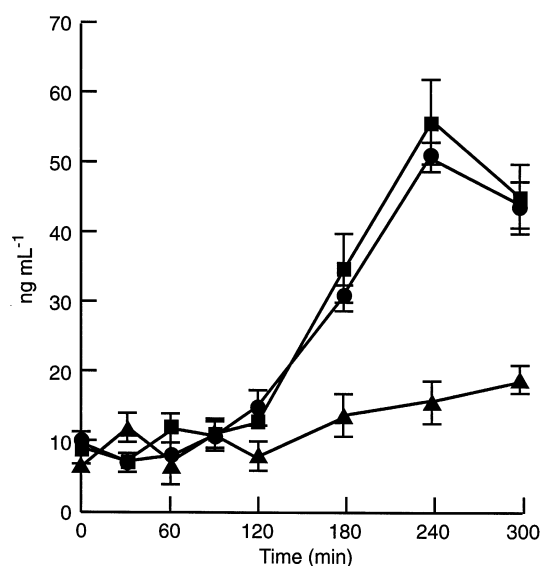


Figure 3. Effect of the inhibition of TNF or IL-6 on endotoxin-induced generation of thrombin in chimpanzees ($n=6$). *E. coli* endotoxin (4 ng kg^{-1}) was injected at 0 min. Thrombin generation is reflected by plasma concentrations of thrombin-antithrombin (TAT) complexes. The co-infusion of anti-TNF monoclonal antibodies (\bullet , 15 mg kg^{-1} , bolus i.v., obtained from Bayer, Wuppertal, Germany) or anti-IL-6 monoclonal antibodies (\blacktriangle , 30 mg kg^{-1} , bolus i.v., obtained from Dr L. Aarden, CLB, Amsterdam, The Netherlands) is compared with the administration of endotoxin alone (\blacksquare).

of PAI-1 and may be responsible for inadequate fibrin removal in DIC.

Mediatory effects of cytokines

Laboratory and clinical evidence indicates that the toxic effects of endotoxin are mediated by cytokines, some of which are detectable in septic patients, such as TNF- α , interleukin (IL) 1 and IL-6. Experimental endotoxaemia results in the transient enhancement of serum levels of several cytokines [14,36–38]. Consecutively, serum levels of TNF first become detectable, peaking at 90 min after the infusion of endotoxin and thereafter gradually declining. This peak is followed by an increase in circulating levels of IL-6 and IL-8, peaking at 120 min after the infusion of endotoxin, suggesting a possible role for these proteins in the development of the septic syndrome as well. In studies of experimental *E. coli* sepsis in baboons, IL-1 (which is not detectable in experimental endotoxaemia in humans or chimpanzees) was also increased [39].

Subsequent studies have focused on the roles of the above-mentioned cytokines in the pathogenesis of derangements of coagulation and fibrinolysis. As TNF is the first cytokine to appear in the circulation after infusion of bacteria or endotoxin and exerts potent procoagulant effects *in vitro*, it was initially thought that activation of coagulation was mediated by TNF. TNF- α is a pluripotent proinflammatory cytokine with a molecular mass of 17 kD. The principal cellular sources for TNF are blood monocytes and tissue macrophages,

although a large variety of other cell types can synthesize this cytokine [40]. *In vitro* studies in human umbilical vein endothelial cells have revealed that TNF is able to influence coagulant activity by stimulation of the production and surface expression of tissue factor and inhibition of anticoagulant mechanisms, particularly the protein C/S system (as outlined above) [23,24,41]. The hypothesis that TNF plays an important role in the induction of coagulation activation *in vivo* was strengthened by studies in which cancer patients or healthy human volunteers were injected with purified recombinant TNF [42,43]. Following the injection of TNF the observed activation of the coagulation system was virtually identical to the endotoxin-induced effects on coagulation. Pentoxifylline, a xanthine oxidase inhibitor that interrupts 'immediate early' gene activation by monocytes, appeared to be able to inhibit the activation of coagulation [10], which was interpreted as another indication of the pivotal mediatory role of TNF in the procoagulant response to bacteraemia or endotoxaemia. However, in studies using two different types of monoclonal antibody directed against TNF activity, it became clear that the endotoxin-induced increase in TNF could be completely abolished whereas activation of coagulation was unchanged (Fig. 3) [44,45]. Also, in baboons infused with a lethal dose of *E. coli*, treatment with an anti-TNF antibody had little or no effect on fibrinogen consumption [46]. Moreover, these observations made it necessary to reconsider the role of TNF as principal mediator of endotoxin-induced activation of coagulation when it became clear that the inhibiting effect of pentoxifylline might be due rather to a direct tissue factor-inhibiting effect or to an inhibitory effect on IL-6 [47]. In subsequent studies the role of IL-6 was investigated. IL-6 is a glycoprotein of 23–30 kD (the heterogeneity in size is related to the differential extent of glycosylation). In comparison with other cytokines, IL-6 has been reported most consistently in the circulation of septic patients. It was shown that infusion of a monoclonal anti-IL-6 antibody resulted in the complete abolishment of endotoxin-induced activation of coagulation in chimpanzees (Fig. 3) [48]. In addition, recent studies in cancer patients receiving recombinant IL-6 indicate that thrombin is indeed generated following the injection of this cytokine [49]. Thus, although TNF can initiate coagulation activation, these data suggest that IL-6 rather than TNF is relevant as a mediator for the induction of the procoagulant response in sepsis. This is a remarkable observation in view of the results from *in vitro* studies. However, recent studies seem to confirm the link between IL-6 and haemostasis, and it has been postulated that this interaction is regulated at the level of tissue factor gene induction or gene transcription, with a potential regulatory role for tissue factor pathway inhibitor (TFPI) [50].

As far as fibrinolysis is concerned, most evidence points in the direction of a principal mediatory role for TNF. Infusion of TNF in human subjects elicits an identical biphasic pattern of initial activation and subsequent inhibition of plasmin generation as compared

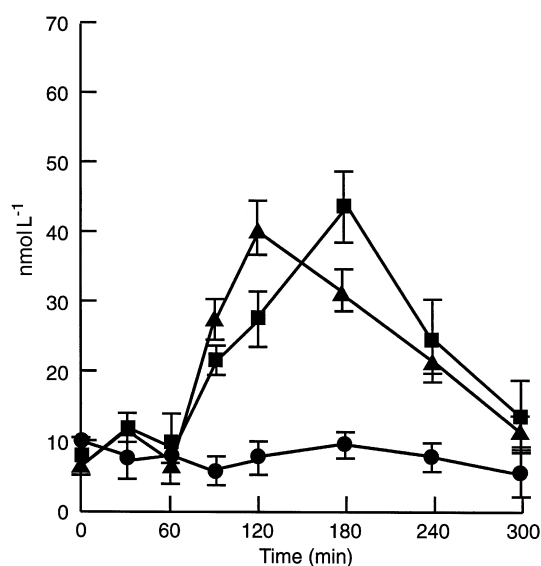


Figure 4. Fibrinolytic response upon the infusion of endotoxin in chimpanzees ($n = 6$) and modulation of the effect by antibodies directed against TNF or IL-6. *E. coli* endotoxin (4 ng kg^{-1}) was injected at 0 min. Plasmin generation is reflected by plasma concentrations of plasmin-antiplasmin (PAP) complexes. The co-infusion of anti-TNF monoclonal antibodies (\bullet , 15 mg kg^{-1} , bolus i.v., obtained from Bayer, Wuppertal, Germany) or anti-IL-6 monoclonal antibodies (\blacktriangle , 30 mg kg^{-1} , bolus i.v., obtained from Dr L. Aarden, CLB, Amsterdam, The Netherlands) is compared with the administration of endotoxin alone (\blacksquare).

with the fibrinolytic response to bacteraemia or endotoxaemia [51]. The time difference between endotoxin-induced changes in coagulation as compared with the TNF-induced kinetics coincided with the 90-min delay in the appearance of TNF after the injection of endotoxin. Studies in which TNF was blocked by monoclonal antibodies showed that the fibrinolytic response upon the administration of endotoxin was completely absent, whereas blocking IL-6 activity did not have any effect on endotoxin-induced activation and inhibition of fibrinolysis (Fig. 4) [32,44,48].

The mechanisms underlying the *in vivo* effects of TNF on the fibrinolytic system are not completely clear. In cultures of human endothelial cells, plasminogen activators and inhibitor production are stimulated by TNF *in vitro* by enhancing their respective gene expression [52,53]; however, the TNF-induced changes in these systems are detected only after several hours, and can therefore not properly explain the rapid *in vivo* fibrinolytic response upon endotoxin and TNF. The rapidity with which the fibrinolytic system is activated following endotoxaemia and the appearance of TNF is more suggestive of a release of stored plasminogen activators, probably from the vascular endothelium. An additional source of PAI-1 may be the release from endotoxin-activated platelets, in which large quantities of this protease inhibitor are stored.

The roles of IL-1 and IL-8 in the induction of the coagulant and fibrinolytic responses are less clear. Preliminary results from experiments in chimpanzees in

which IL-8 was blocked by specific monoclonal antibodies did not reveal any effect of IL-8 on activation and inhibition of the system of coagulation and fibrinolysis (unpublished data). *In vitro* studies with IL-1 have shown a significant procoagulant effect of this cytokine [54], and *in vivo* blockade of IL-1 activity by the administration of a recombinant IL-1 receptor antagonist partly attenuated the coagulative abnormalities in a baboon sepsis model [55]. In accordance with this, treatment of septic patients with recombinant IL-1 receptor antagonist for 72 h is associated with decreases in the plasma concentrations of TAT and PAP complexes, and of t-PA and PAI-1 [56]. However, many of the endotoxin-induced effects on coagulation and fibrinolysis occur well before IL-1 becomes detectable in the circulation, hence the significance of IL-1 for the derangement of coagulation and fibrinolysis remains to be elucidated.

Conclusion

The use of experimental models of bacteraemia, endotoxaemia and cytokinaemia in humans and non-human primates has partly elucidated the pathogenetic pathways that are involved in the development of disseminated intravascular coagulation in septic patients. Activation of coagulation appears to be mediated by the tissue factor/factor VIIa-dependent route and is potentiated by impaired function of the physiological inhibitory mechanisms of coagulation. Simultaneously, the fibrinolytic system is briefly activated but thereafter completely suppressed due to elevated levels of PAI-1. The effects on coagulation and fibrinolysis appear to be mediated by different cytokines, IL-6 being most relevant for activation of coagulation and TNF playing a pivotal role in the induction of the fibrinolytic responses. The significance of a number of other cytokines in the regulation of coagulation and fibrinolysis in sepsis remains to be established. In anticipation of results of clinical trials, it can be hypothesized that specific blockade of selected coagulation or fibrinolytic proteins or interference with cytokine production and action might be a rational therapeutic option for the derangement of coagulation in sepsis.

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