Experimental Endotoxemia in Humans: Analysis of Cytokine Release and Coagulation, Fibrinolytic, and Complement Pathways

By Sander J.H. van Deventer, Harry R. Büllner, Jan W. ten Cate, Lucien A. Aarden, C. Erik Hack, and Auguste Sturk

Endotoxemia was evoked by bolus injection of *Escherichia coli* endotoxin (2 ng/kg body weight) in six healthy subjects to investigate the early kinetics of cytokine release in relation to the development of clinical and hematologic abnormalities frequently seen in gram-negative septicemia. The plasma concentration of tumor necrosis factor (TNF) increased markedly after 30 to 45 minutes, and reached a maximal level after 60 to 90 minutes. In each volunteer, the initial increase of plasma interleukin 6 (IL-6) concentrations occurred 15 minutes after the initial TNF increase, and maximal IL-6 concentrations were reached at 120 to 150 minutes. A transient increase in body temperature and pulse rate occurred simultaneously with the initial TNF and IL-6 increases, whereas a significant decrease in blood pressure occurred after 120 minutes. These changes were proportional to the changes in TNF and IL-6 concentrations. Coagulation activation, as assessed by a rise of prothrombin fragments and thrombin-antithrombin III complexes, was noted after 120 minutes, in the absence of activation of the contact system. A two- to sixfold increase in the concentrations of tissue plasminogen activator (t-PA) and von Willebrand factor antigen indicated endothelial cell activation. This increase started at 120 and 90 minutes, respectively. The release of t-PA coincided with activation of the fibrinolytic pathway, as measured by plasminogen-α₂-antiplasmin complexes. The fibrinolytic activity of t-PA was subsequently offset by release of plasminogen activator inhibitor, observed 150 minutes after the endotoxin injection, and reaching a peak at 240 minutes. No complement activation was detected. These results show that in humans endotoxin induces an early, rapidly counteracted fibrinolytic response, and a more long-lasting activation of thrombin by a mechanism other than contact system activation. In addition, our data suggest that endotoxin-induced leukopenia and endothelial cell activation are mediated by TNF.

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MATERIALS AND METHODS

Study design. Six healthy, male volunteers (age 25 to 26 years) participated in the study. Informed consent was obtained from each.

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subject before the start of the study and the study was approved by the hospital ethics committee. This committee functions in accordance to the Helsinki Doctrine on Human Experimentation.

In the month before the study none of the volunteers suffered from any febrile disease, and each had hematologic indices and liver and kidney functions within the normal range. The volunteers did not take any medication during the week preceding the study, and fasted overnight before endotoxin administration. The endotoxin preparation (Escherichia coli 0113:H10 K-negative [lot EC-5], kindly provided by Dr D. Hochstein, Bureau of Biologics, Food and Drug Administration, Bethesda, MD) was dissolved in isotonic saline, and administered intravenously as a bolus injection at a dose of 2 ng/kg body weight. Body temperature was recorded orally and blood pressure was measured with a sphygmomanometer, at half-hourly intervals. The mean arterial blood pressure (MAP) was defined as the sum of the diastolic pressure and one third of the pulse pressure.

The study was supervised by two internists in a clinical research unit where standard emergency equipment was immediately available. Blood was obtained at 0, 15, 30, 45, and 60 minutes and thereafter at half-hourly intervals for a total period of 6 hours, by separate venipunctures using a 19-gauge butterfly needle.

*Endotoxin and cytokine assays.* Blood for the Limulus assay was collected in polystyrene tubes (Falcon 2063, Oxnard, CA), containing pyrogen-free heparin (Thromboliquine; Organon, Oss, The Netherlands) at a final concentration of 30 IU/mL, and the tubes were immediately immersed in melting ice. Platelet-rich plasma was prepared by centrifugation for 10 minutes at 190g (4°C) and stored at -40°C. Endotoxins were determined with the chromogenic Limulus assay as has been described. This assay has a detection limit of 3 ng/mL for standard endotoxin in blood (E coli 055:B5; Mallinckrodt Inc, St Louis, MO). Standard curves prepared with lot EC-5 showed similar sensitivity of the chromogenic Limulus assay for this endotoxin in blood.

Serum for determination of IL-1 and IL-6 was prepared by centrifugation of clotted blood for 20 minutes at 1,600g (room temperature). IL-1 was measured in the D10 assay. D10 cells were incubated with serum in the presence of IL-2 (50 U/mL). Under these conditions 1 pg of IL-1 per milliliter causes half-maximal proliferation. Standard curves were generated by incubation with recombinant IL-1α.

Serum IL-6 levels were measured using the B9 assay as has been reported. Briefly, serum was heated for 30 minutes at 56°C and a titration of each serum was added to 5,000 B9 cells and compared with a standard IL-6 preparation. After 3 days, proliferation was measured by [3H]thymidine incorporation. Serum IL-6 levels were expressed as nanomoles per liter. PAP complexes bound to the beads were quantitated by a subsequent assay using antiserum to rIL-6.

Blood for the TNF assay was collected in trisodiumcitrate tubes (Falcon 2063, Oxnard, CA) at a final concentration of 10 mmol/L EDTA and 0.05% (wt/vol) polybrene (final concentration), centrifuged for 30 minutes at 1,600g (4°C). The ratio of prothrombin fragments F1+2, fragments were performed in plasma anticoagulated with 38 mmol/L citric acid, 75 mmol/L sodium citrate, 136 mmol/L NaCl, 6 mmol/L EDTA, 6 mmol/L adenosine, and 25 U/mL heparin, centrifuged for 30 minutes at 1,600g (4°C). The ratio of anticoagulant to blood used was 0.2:1.0 (vol/vol). Prothrombin fragments were determined using the F1+2 assay, as has been reported.

Blood for complement studies was obtained in siliconized tubes, containing 10 mmol/L EDTA and 0.05% (wt/vol) polybrene (final concentration), centrifuged for 20 minutes at 1,600g (room temperature), and the plasma was stored at -70°C. Contact system activation was detected by measuring factor XIIa-C1-inhibitor and kallikrein-C1-inhibitor complexes as described. As little as 0.05% activation of either factor XII or prekallikrein in plasma can be detected by these assays. Modified inactive C1-inhibitor was measured with a specific monoclonal antibody (MoAb). A standard curve for complement activation was assessed by measuring C₃a-des-arg levels by a radioimmunoassay. To prevent interference of native C₃ in the assay, samples were incubated with polycethylene glycol before being tested. Details of the assay are extensively described elsewhere. Results were expressed as nanomoles C₃a-des-arg per liter.

Plasmin-α₂-antiplasmin (PAP) complexes were measured in the EDTA-polybrene plasma samples with a recently developed assay (J.P. de Boer et al, manuscript in preparation). Briefly, the plasma samples were incubated with a MoAb specifically directed against complexed α₂-antiplasmin, and which had been coupled to Sepharose beads (Pharmacia Fine Chemicals, Uppsala, Sweden). Then, the PAP complexes bound to the beads were quantitated by a subsequent incubation with a radiolabeled MoAb against plasmin. Results were related to a standard curve of pooled plasma in which all α₂-antiplasmin had complexed to plasmin on addition of urokinase, and were expressed as nanomoles per liter.

Statistical analysis. Individual minima and maxima were tested against preinfusion results with the Wilcoxon test for related samples (two-tailed p values). These values were in each case below and above the preinfusion data, respectively. Correlations were calculated as Kendall's rank correlation and tested using Kendall's test for correlation (one-tailed p values).

### RESULTS

Clinical features and hematologic responses. All subjects complained of influenza-like symptoms, such as myalgia, headache, and nausea, starting 50 to 90 minutes after the injection. Four of the six subjects developed chills 50 to 75 minutes following endotoxin challenge. Four of these subjects were subsequently noted to be sleepy, and two had mild amnesia for the period 60 to 90 minutes after the start of the study. In all subjects an increase in body temperature (mean maximal increase ± SD: 1.7 ± 0.4°C; range 0.8 to 2.3°C; Fig IA) and pulse rate (mean maximal increase ± SD: 39 ± 17 beats/min; range 16 to 42 beats/min) was recorded. A
Fig 1. Endotoxin induced effects. (A) Changes in body temperature. (B) Leukocyte counts. Error bars indicate 1 SEM, N = 6.

Decline of MAP was observed after 120 minutes in all volunteers. The lowest blood pressures were recorded 210 minutes after the bolus endotoxin injection (mean maximal decrease ± SD: 25.0 ± 8.2 mm Hg), coinciding with the maximum increase in body temperature. The time course of these changes in body temperature, MAP, and pulse rates were remarkably similar in all six subjects.

Leukocyte counts initially decreased in all volunteers, reaching levels as low as 2.3 to 5.0 x 10^9/L (3.7 ± 1.0 x 10^9/L [mean ± SD]) 60 minutes after endotoxin injection, and subsequently rapidly increased, reaching a zenith of 8.4 to 15.1 x 10^9/L (10.6 ± 2.7 x 10^9/L [mean ± SD]) (Fig 1B), with a marked neutrophilia. No significant changes in platelet counts occurred during the 6-hour study period. We attempted to measure platelet activation by the assay of plasma β-thromboglobulin and platelet factor 4 levels. Despite the use of the appropriate collection medium, ie, citrate/theophylline/adenosine/dipyridamole, the results of the assays showed that extensive platelet activation had occurred in vivo due to the simultaneous blood collection of the other assays. Thus, the effect of the endotoxin administration on platelet activation could not be investigated (data not shown).

Endotoxemia and induction of cytokines. Transient endotoxemia was detected 5 to 15 minutes after administration. Peak circulating endotoxin concentrations were reached at 5 minutes, and ranged from 7 to 13 ng/L (Fig 2).

Circulating IL-1 was not detected in any of the subjects during the entire study period. TNF concentrations were initially below the limit of detection (10 ng/L) and gradually increased after 30 to 45 minutes, reaching a peak at 60 to 90 minutes. Maximal levels ranged from 68 to 1,374 ng/L (Fig 2). Peak TNF levels correlated significantly with the highest body temperature (R = .73; P < .05) as well as with the highest IL-6 levels (R = .73; P < .05). In each volunteer, the initial IL-6 increase was 15 minutes delayed compared with the initial TNF increase (Fig 2). Baseline IL-6 concentrations ranged from 6 to 11 U/mL. A rise in IL-6 levels was noted starting 45 to 60 minutes after endotoxin injection. The appearance of IL-6 coincided with the development of chills. Peak levels of IL-6 were reached at 120 to 150 minutes, and ranged from 72 to 2,820 U/mL (Fig 2). The peak IL-6 concentration correlated significantly with the maximum increase in body temperature (R = .73; P < .05). The peak levels of TNF correlated significantly with the decrease in leukocyte numbers observed at 60 minutes after endotoxin infusion (R = .87; P = .01). Also, peak levels of IL-6 correlated with this drop in leukocyte numbers, but this correlation was not significant (R = .70; P > .05).

Endothelial cell response. In five subjects vWFAg release in blood was detected after 120 to 150 minutes and was maximal after 180 minutes. The levels remained elevated during the further 3 hours of follow-up (Fig 3). T-PA levels increased in five subjects starting 90 minutes after endotoxin injection, and reached a zenith at 120 to 180 minutes (Fig 3) (33.2 ± 16.2 pg/L [mean ± SD]). t-PA levels subsequently decreased, but had not reached baseline values at 6 hours. The sixth subject (with the lowest IL-6 and TNF responses) had only minor changes in vWFAg and t-PA levels. The appearance of vWFAg was delayed in each volunteer as compared with t-PA. The first increase in PAI levels was observed after 150 minutes, ie, when t-PA levels had already reached a zenith (Fig 3). PAI levels peaked at 240 minutes (30.6 ± 6.9 µg/L [mean ± SD]), ie, 120 minutes after the t-PA peak. Peak levels of vWF antigen correlated significantly with both TNF as well as with IL-6 peak levels (R = .69; P = .05 for both correlations). Also, peak levels of
t-PA correlated significantly with peak levels of both cytokines \((R = .87; P = .01\) for both correlations).

Response of the coagulation, fibrinolytic, and complement pathways. An increase in prothrombin fragment levels (mean ± SD at 0, 120, and 240 minutes: 0.88 ± 0.30, 1.63 ± 0.65, 3.52 ± 1.50 nmol/L, respectively), indicative of activation of the common pathway of coagulation, was first observed at 120 minutes and was further increased at 240 minutes. Unfortunately, no blood samples for \(F_1\_2\) determinations were obtained at later timepoints (Fig 4). TAT complexes were also first observed at 120 minutes (mean ± SD at 0 and 120 minutes were 4.8 ± 2.8 ng/L and 8.1 ± 6.5 ng/L, respectively). The complexes were maximally increased at 150 minutes (11.7 ± 6.9 ng/L [mean ± SD]), and then gradually decreased to virtually normal values (5.6 ± 2.0 ng/L [mean ± SD] at 360 minutes). Activation of the coagulation system by the contact system was estimated by the measurement of factor XIIa-C1-inhibitor complexes, kallikrein-C1-inhibitor complexes, and modified inactive C1-inhibitor. No changes from the baseline levels of these parameters could be observed (data not shown). There was also no evidence for complement activation, as measured by \(C\alpha\_a\_des-arg\) levels (Table 1).

In four of the six individuals, a marked increase in PAP complexes was initially observed at 90 minutes, reached its zenith at 120 minutes, and was still increased at 150 minutes. In one other individual, PAP complexes were slightly increased at only 150 minutes. In the sixth individual, who had the lowest peak values of TNF and IL-6 and also hardly showed an increase in vWFAg, no increase in PAP complexes was observed. The peak values of PAP complexes correlated significantly with peak values of TNF \((R = .82, P < .05)\). Although peak levels of PAP complexes also correlated positively with peak levels of t-PA and IL-6, neither of these correlations was significant \((R = .71\) and \(R = .60, P > .05\), respectively).

Table 1. \(C\alpha\_a\_des-arg\) Levels during experimental endotoxemia in humans

<table>
<thead>
<tr>
<th>Minutes After Infusion</th>
<th>Mean (nmol/L)</th>
<th>SEM (nmol/L)</th>
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<tbody>
<tr>
<td>0</td>
<td>2.2</td>
<td>0.3</td>
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<tr>
<td>15</td>
<td>2.2</td>
<td>0.5</td>
</tr>
<tr>
<td>30</td>
<td>2.0</td>
<td>0.6</td>
</tr>
<tr>
<td>60</td>
<td>1.6</td>
<td>0.2</td>
</tr>
<tr>
<td>120</td>
<td>2.0</td>
<td>0.3</td>
</tr>
<tr>
<td>240</td>
<td>1.5</td>
<td>0.2</td>
</tr>
<tr>
<td>360</td>
<td>1.3</td>
<td>0.2</td>
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Normal 95 percentile range of the assay is ≤5 nmol/L.27
DISCUSSION

Gram-negative sepsis is a leading cause of morbidity and mortality in hospitalized patients. Despite the introduction of potent new antibiotics, the mortality rate of approximately 30% to 50% has remained virtually constant in the last decade. Many of the clinical symptoms and hematologic abnormalities of the gram-negative septic syndrome are believed to be initiated by endotoxin-induced release of cytokines. Indeed, it has been shown that in febrile patients endotoxemia precedes the development of the septic syndrome. Moreover, various clinical studies have documented the presence of elevated levels of TNF, IL-1, IL-6, and IL-10 in sepsis. Relatively little is known about the early kinetics of cytokine release on endotoxin stimulation in humans. Michie et al reported release of TNF into the circulation 60 to 180 minutes after the infusion of 4 ng endotoxin/kg body weight, ie, an endotoxin dose twice higher than that used in the present study. These investigators were unable to detect IL-1 in the circulation, and did not report data on IL-6. In another investigation, peak IL-6 levels were observed 2 to 4 hours following endotoxin injection in healthy volunteers. The present study confirms that after brief experimental endotoxemia, TNF is rapidly released into the circulation. In addition, we demonstrated release of IL-6, which appeared to be delayed approximately 15 minutes as compared with TNF. The systemic registration of release of these cytokines coincided with changes in vital signs, ie, an increase in body temperature and pulse rate, but the moderate decrease in MAP occurred later. Although peak plasma endotoxin levels were comparable in all subjects, large differences in the blood concentrations of TNF and IL-6 were observed. The concentrations of both cytokines correlated significantly with the change in body temperature \( (P < .05) \), and the subjects with the highest levels experienced the largest changes in body temperature, pulse rate, and subjective discomfort. Interestingly, and in agreement with Michie et al, IL-1, another endogenous pyrogen, was not detected in the present investigation. These observations therefore suggest that TNF, as well as IL-6, are the major circulating mediators of lipopolysaccharide toxicity, and that interindividual differences in macrophage responsiveness may determine the severity of clinical symptoms. The temporal difference between the appearance of TNF and IL-6 may indicate that TNF stimulates the release of IL-6 from the monocyte/macrophage compartment or possibly from the endothelial cells. This hypothesis is supported by data from a recent study which showed that in a lethal bacteremia model in baboons treatment with anti-TNF antibodies partially blocked the IL-6 response.

During gram-negative sepsis, particularly in the course of meningococcemia, abnormalities of coagulation and fibrinolysis are frequently observed. Approximately 30 to 45 minutes after the endotoxin-induced release of TNF into the circulation, a steep increase in the plasma t-PA concentration was noted. In agreement with a recent study in humans, we observed release of PAI 150 minutes following the endotoxin bolus injection. In vitro endotoxin and TNF both stimulate the release of PAI by endothelial cells, but decrease the synthesis of t-PA. Therefore it has been recently hypothesized that the endotoxin-induced t-PA increase in humans may be induced by thrombin formation, as thrombin stimulates the synthesis of both t-PA and PAI by endothelial cells. Endotoxin-induced thrombin formation might be mediated by TNF, which reduces transcription of the thrombomodulin gene, and, in addition, causes internalization of thrombomodulin expressed on the endothelial cell surface. However, in our study, the increase in t-PA levels was not preceded by thrombin formation as measured by the F1+2 and TAT assays. Therefore, other mechanisms are possibly responsible for the endotoxin-induced rise in t-PA levels in humans. The significant correlations of t-PA peak levels to both IL-6 and TNF peak levels suggest that this release is directly or indirectly linked to the release of these cytokines. The increase in PAP complexes induced by endotoxin is in agreement with the findings of Suffredini et al. However, peak levels of PAP complexes (in four volunteers) at 90 and 120 minutes preceded the maximal t-PA level that occurred at 120 minutes or later in each subject. Thus, during the initial phase of its release t-PA was able to activate plasminogen, subsequently being effectively inhibited by PAI. The vWFAG increase was noted 30 to 60 minutes after the t-PA increase. This observation could indicate that the regulation of t-PA and vWFAG release is different, that released vWFAG is initially rapidly consumed, or that different endothelial cell populations are responsible for release of t-PA and vWFAG in vivo. Clarification of these hypotheses will have to await additional in vivo and in vitro studies.

Activation of the coagulation pathway was clearly shown at 120 minutes by both the F1+2 and the TAT assays. However, using very sensitive assays, activation of the contact system of coagulation could not be shown in this investigation. This result is in contrast to observations in septic patients. We presume that coagulation activation during low level endotoxemia in the present study was either induced directly, via the extrinsic coagulation pathway, or via expression of procoagulants on endothelial cells, monocytes, or macrophages. Our attempts to measure platelet activation by analysis of plasma β-thromboglobulin and platelet factor 4 levels were unsuccessful. The possibility of procoagulant activity expression on platelets can thus not be supported or excluded. Despite the sensitivity of our assays in-vitro, we cannot exclude the possibility that some activation of the intrinsic pathway had occurred and contributed to the coagulation abnormalities during the observation period. First, although in normal plasma C1 esterase-inhibitor is the main inhibitor of both factor XIIa and of Kallikrein in vitro, it is not known whether in vivo other inhibitors may contribute to the inhibition of both contact phase proteases and thus decrease the sensitivity of our assays (which are dependent on complex formation with C1-esterase inhibitor) in vivo. Second, there are, to our knowledge, no data on the extent of activation of the intrinsic pathway that is necessary to generate thrombin in vivo. Apparently, more studies are needed to resolve this issue.

Complement activation has been observed in the majority of patients with septicemia. In contrast, during the
present experimental low-level endotoxemia, complement activation was not detected. The reason for this absence of activation remains uncertain. Complement activation may require higher endotoxin levels, or bacterial components other than endotoxins may be responsible for complement activation in vivo. In vitro studies do not show that this apparent lack of complement activation was due to insensitivity of the assay for C3a, for instance due to an interference by native C3.27 Moreover, with the same assay we could readily detect activation of complement in several disease states, including sepsis3 and during the development of a sepsis-like syndrome during immunotherapy with recombinant IL-2.48,50 Thus, experimental endotoxemia either induced only a low degree of complement activation, and then the C3a generated might have escaped detection by binding to cellular receptors,52 or did not induce activation at all. Complement activation in animals induces a rapid decrease of circulating neutrophils by aggregation of these cells and adherence to endothelial cells.32 This result is due mainly to a rapid binding of C3a to cellular receptors on neutrophils (for this reason we did not attempt to measure C3a in this study).53 However, we do not believe that the observed decrease in neutrophil numbers during experimental endotoxemia in this study was due to complement activation. This decrease only occurred after 60 minutes, whereas in the case of complement activation the decrease in neutrophils occurred within minutes after administration of the activator.52 Also in support of our assumption is a recent study in which the decrease of neutrophils during experimental endotoxemia in rats was unaffected by the presence of anti-C3a antibodies.53 The significant correlation of the percental decrease in neutrophils with the peak levels of TNF instead suggests involvement of cytokines in this phenomenon. In particular, TNF potently induces the expression of the neutrophil adhesion molecule endothelial leukocyte adhesion molecule-1 (ELAM-1) on endothelial cells.54

In summary, experimental endotoxemia in humans induces release of the macrophage-derived cytokines TNF and subsequently IL-6. The release of cytokines was followed by the activation of the common pathway of the blood coagulation cascade in the absence of contact system activation. An initial fibrinolytic response was counteracted by the subsequent release of PAI. At the low endotoxin dose administered, we did not observe activation of the complement system. These data show that low-level endotoxemia can induce long-lasting thrombin generation by a mechanism other than contact system activation. Finally, our data suggest that TNF is an important mediator of endotoxin-induced coagulation activation.

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