SEPTIC SHOCK is a frequent underlying cause of severe progressive failure of multiple organ systems.1,2 Histologically, hypotensive bacteremia is characterized by cell damage or necrosis and vascular damage.3,4 Fibrin deposition and platelet thrombi are common findings in the microvasculature.3 Endotoxin, a major cell wall component of gram-negative bacteria, interacts with inflammatory cells, releasing endogenous mediators such as cytokines, hydrolyases, peptides, prostaglandins, and amines that contribute to the pathophysiology of septic shock.5-4

The contact system is composed of plasma factor XII (FXII), factor XI (FXI), prekallikrein (PK), and high molecular weight kininogen (HK). FXII, FXI, and PK are serine protease zymogens, and HK is a bradykinin (Bk) precursor that functions as a cofactor in most of the reactions involved in contact activation.6 This activation is initiated by binding of plasma FXII to a negatively charged surface, where autoactivation of zymogen FXII occurs, yielding activated FXII (FXIIa). A small amount of FXIIa leads to the subsequent activation of its substrates, PK, FXI, and HK, resulting in consumption and decreased plasma levels of these zymogens and the procofactor. Kallikrein, which results from PK activation, has been shown to be a chemoattractant for neutrophils, which it stimulates to aggregate and release lysosomal enzymes such as elastase. Additionally, kallikrein efficiently hydrolyzes HK to release Bk, a potent vasodilator. Kallikrein further amplifies contact activation by catalyzing the conversion of FXII to FXIIa. The major regulators of activation of this system are the naturally occurring plasma protease inhibitors, C1 inhibitor (for FXIIa and kallikrein), α2-macroglobulin (for kallikrein), and α1-antitrypsin (for FXIa).6

Mason et al6 were the first to describe decreased PK, FXII, and kallikrein inhibitor (C1-inhibitor) in human septic shock. Information is limited regarding the effect of endotoxin on human coagulation in vivo.8,10 Kimball et al9 were the first to show that Bk was increased in all subjects during administration of 3 to 5 ng/kg endotoxin, with a peak response at 1 hour. Van Deventer et al10 failed to show contact activation measuring C1 inhibitor complexes with kallikrein and FXIIa, while administering 2 ng/kg of endotoxin to their subjects. Studies in animals have shown that the contact system of blood coagulation participates in the biochemical and hemodynamic changes induced by bacterial cell products.11,13 Lerner et al14 showed a decrease in FXII to 61% 6 hours after endotoxin infusion in rabbits. Gallimore et al15 showed a decrease in PK and high molecular weight kininogen (HK) in dogs receiving endotoxin in lethal doses.

In the present study, we performed quantitative assays to determine the role of the contact system as well as to define contact activation measuring C1 inhibitor complexes with kallikrein and FXIIa, while administering 2 ng/kg of endotoxin to their subjects. Studies in animals have shown that the contact system of blood coagulation participates in the biochemical and hemodynamic changes induced by bacterial cell products.11,13 Lerner et al14 showed a decrease in FXII to 61% 6 hours after endotoxin infusion in rabbits. Gallimore et al15 showed a decrease in PK and high molecular weight kininogen (HK) in dogs receiving endotoxin in lethal doses.

The objective of this study was to determine the role of the kallikrein-kinin system in healthy humans after intravenous administration of either Escherichia coli endotoxin or saline. We studied a total of 18 healthy nonsmoking volunteers, 23 to 38 years old, in an open-label study at the Critical Care Medicine Department, Clinical Center, National Institutes of Health (Bethesda, MD) in which some of the patients served as their own controls. After baseline data collection, the subjects received intravenously either E coli endotoxin (n = 15, 4 ng/kg of body weight) or saline (n = 8, controls). Signs, symptoms, systemic blood pressure, factor XII, plasma prekallikrein (PK), factor XI (FXI), antithrombin III (AT-III), high molecular weight kininogen (HK), and α2-macroglobulin—kallikrein complexes were measured at baseline and 1, 2, 3, 5, and 24 hours after injection of either saline or endotoxin. After infusion of endotoxin, we found the functional plasma levels of FXI decreased at 2 hours (P < .05) and at 5 hours (P < .05). Functional PK was significantly depressed by 2 hours (P < .05), at 5 hours (P < .05), and at 24 hours (P < .01), whereas the PK antigen was only low at 5 hours (P < .05). These changes were accompanied by a significant increase in circulating α2-macroglobulin—kallikrein complexes at 3 hours (P < .05) and 5 hours (P < .01). No significant changes occurred in the plasma levels of factor XII or HK. We concluded that clinical response to intravenous endotoxin in healthy human volunteers is associated with activation of the kallikrein-kinin systems. Further investigation is needed with specific inhibitors of the kallikrein-kinin system to define its primary or secondary role in the endotoxin-mediated reactions.

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the sequence of events that occurs in response to intravenous administration of endotoxin. In addition, we compared the data with previously reported effects of endotoxin (on the same group of normal volunteers) upon the cardiovascular and fibrinolytic systems as well as on the neutrophil function.

MATERIALS AND METHODS

Subjects and protocol. Eighteen healthy nonsmoking volunteers, 23 to 38 years old, participated in the study. The current investigation was performed simultaneously with an evaluation of the effects of endotoxin on hemodynamic and fibrinolytic systems. The results of these parts of the study have been published elsewhere.16,17 Five of the subjects underwent repeat control studies without endotoxin 3 months later. The protocol was approved by the Institutional Review Board on Human Experimentation at the National Institutes of Health, and written consent was obtained from all subjects.

Blood collection. Blood samples were obtained through the indwelling radial artery catheter at 1, 2, 3, and 5 hours after infusion of endotoxin or normal saline. Samples obtained 24 hours after the infusion were drawn from a peripheral vein. All samples were collected in plastic syringes and immediately transferred to plastic tubes containing 3.8% sodium citrate (9:1, vol:vol) that were kept on ice. The samples were centrifuged at 2,500 rpm at 4°C for 10 minutes, and the top two-thirds of the plasma layer was removed and stored at −70°C until the time of assay.

Endotoxin preparation. Purified lipopolysaccharide from Escherichia coli (US Standard Reference Endotoxin, lot EC-5, Bureau of Biologics, Food and Drug Administration, Bethesda, MD) was supplied as a sterile dry powder containing 1 mg (≈10,000 U) of endotoxin, which was reconstituted before infusion with 5 mL of sterile water.

Assays of coagulation and contact activation. Antithrombin III (AT-III) was measured by a functional microplate assay using the Coatest Antithrombin Kit (Kabi Pharmacia, Franklin, OH).18 The assay measured the extent of hydrolysis of a chromogenic substrate, S-2328, by the residual thrombin after inhibition of a fixed amount of thrombin by the inhibitor in the plasma sample. C1 inhibitor was measured by the inhibition of plasma kallikrein using an amidolytic assay as previously described,19 with the following modifications. The plasma was methylene treated as described and kallikrein was added to the methylene-treated plasma as per the referenced method to form the reaction mixture. The assay was adapted to a microplate by the addition of 10 μL of reaction mixture at precise time intervals (0.5, 1, 1.5, 2 and 4 minutes) to a microplate well containing 190 μL of 50 mmol/L Tris-Cl, 0.1% polyethylene glycol, 2 mmol/L S-2302, pH 7.9. Each assay point was allowed to incubate with substrate for 10 minutes before addition of 50 μL of acetic acid to stop the cleavage of amidolytic substrate. The absorbance was read at 405 nm and the apparent rate constant was determined as described19 and converted to micromers per milliliter of C1 inhibitor. PK levels were determined by a functional assay, using the chromogenic substrate, S-2302 (Pro-Phe-Arg-p-nitroanilide).20 Plasma was depleted of proteolytic inhibitors by incubation with dilute acid for 15 minutes. Zymogen PK in the plasma samples was then activated by incubation with plasma PK activator (Kabi) containing HK-activated FXII and ellagic acid. S-2302 was subsequently added to each sample, and the extent of hydrolysis of the substrate was proportional to the amount of PK in the plasma samples.

FXI functional activity was determined by a method developed in this laboratory.21 In brief, plasma FXI was activated by kaolin (0.1 μg/mL) for 50 minutes at 23°C after acid treatment. This reaction was performed in the presence of soybean trypsin inhibitor (1.6 μmol/L) to inhibit plasma kallikrein generation during the activation step. Corn trypsin inhibitor (0.01 μg/mL) prevented hydrolysis of the chromogenic substrate S-2366 by FXIIa, and after 10 minutes of incubation, the chromogenic substrate was added. HK and FXII coagulant activities were determined by a slight modification of the method described elsewhere.22 One hundred microliters of FXII-deficient plasma (George King Biomedical, Inc, Overland Park, KS), or total kininogen-deficient plasma (Williams plasma), 100 μL of 20 mmol/L Tris (hydroxymethyl)-aminomethane (Tris) HCl, pH 7.4, containing 0.15 mol/L NaCl, 100 μL of kaolin (5 mg/mL in saline), and 100 μL of 0.2% bovine albumin were mixed together. Normal pooled human plasma (10, 5, 2, and 1 μL) was added and incubated at 37°C for 8 minutes. Coagulation time was determined after the addition of 100 μL of 30 mmol/L CaCl2. This procedure was used to generate a standard curve (log-log relationship). Samples for analysis were assayed under the same conditions, using 10 μL of the human plasma sample, and data were expressed as a percentage of normal pooled human plasma. One unit is defined as the amount of activity in 1 mL of normal pooled human plasma.

PK antigen was measured by a sandwich-type enzyme-linked immunosorbent assay (ELISA) using two mouse monoclonals against the heavy chain of PK. These two monoclonal antibodies (MoAbs) have previously been shown to have nonoverlapping binding sites.21 Microplate wells were coated with the first monoclonal, 13G11, at a concentration of 5 μg/mL in 0.166 mol/L H3BO4, 0.125 mol/L NaCl, pH 8.5 overnight at 23°C. The following day, the microplate wells were blocked with 0.5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS)-Tween for 1 hour at 23°C. Samples were diluted in PBS-Tween + BSA and incubated in the microplate wells for 2 hours at 23°C. The second monoclonal used, 10B6, was directly conjugated with alkaline phosphatase by the method of Voller et al.24 This conjugated monoclonal was diluted in PBS-Tween + BSA to approximately 2 μg/mL and incubated in the wells for 2 hours. Finally, substrate (p-nitrophenyl phosphate, 1.5 mg/mL) was added to the microplate wells, and the absorbance read at 405 nm.

α2-Macroglobulin–kallikrein (α2M-Kal) complexes were determined with a sandwich ELISA using Fab fragments of a polyclonal antibody to α2M as the capturing antibody, and a unique MoAb to kallikrein heavy chain (13G11) to detect kallikrein complexed with α2M.21 The lower detection limit of the assay is approximately 5 nmol/L when the blood is processed at 23°C.22

Statistics. Results are presented as mean ± SD. The zero time point was designated 100% (for PK and FXI) to decrease statistical variability and to eliminate the influence of small differences at baseline between the groups, which could bias comparisons made at subsequent time points. Absolute values are given for the α2M-Kal complexes. A multiple-comparison procedure, the Bonferroni-t-test,24 was used, which is based on the t-test and includes an appropriate correction for the multiple comparisons.

RESULTS

Functional PK levels were significantly lower in the endotoxin group as compared with controls at 2 hours after infusion (P < .05) with a slight recovery by 3 hours. The concentration of functional PK decreased further and remained low throughout the rest of the experimental protocol (5 and 24 hours, P < .05 and P < .01, respectively, Fig 1), suggesting that this contact phase protein may serve as an indicator of contact activation by endotoxin.

PK antigen levels were significantly decreased (Fig 2) only
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140
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140
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Fig 1. Time course of plasma PK functional values after injection of endotoxin. The functional assay was performed with a chromogenic substrate as outlined in Materials and Methods.

during the 5-hour time interval (P < .05), paralleling the decrease in the functional levels at the same time interval. The ratio of PK function to antigen did not differ significantly for the endotoxin group when compared with the saline group.

FXI levels were significantly decreased (Fig 3) by the 2-hour time interval (P < .05). Like the PK levels, a recovery was observed by the 3-hour time interval, with a subsequent decrease in the circulating levels by the 5-hour interval (P < .01, Fig 3). Unlike the PK levels, the FXI levels were found normal by 24 hours after infusion of endotoxin.

There were no significant differences between functional levels of FXII, HK, C1 inhibitor, or AT-III in the endotoxin group compared with the saline control (data not shown).

The concentration of $\alpha_{2}$-M-Kal complexes was elevated fourfold in the endotoxin-treated group by 3 hours (P < .05) and fivefold by 5 hours (P < .01), with a decrease to normal in the circulating levels of complexes by 24 hours (Fig 4). All patients receiving endotoxin but none receiving saline showed elevated $\alpha_{2}$-M-Kal complexes at 3 hours (range, 6.9 to 23 nmol/L) and at 5 hours (range, 11.4 to 17.5 nmol/L).

DISCUSSION

Our observations show that within 2 hours after the administration of endotoxin to normal humans, contact activation is initiated. PK and FXI decrease, and by 5 hours reach a nadir that is accompanied by a fivefold increase in $\alpha_{2}$-M-Kal complexes. The latter increase in the enzyme-inhibitor complexes confirms the activation of kallikrein by endotoxin in vivo. Only PK functional levels remain significantly low at 24 hours, possibly reflecting its longer half-life or depressed hepatic synthesis. The concentrations of FXII necessary are very small compared with the concentrations of the zymogen, and thus no significant change in FXII coagulant activity was observed.

The temporal relationship of these responses to endotoxin can be related to previously published observations for the same or similar subjects describing hemodynamics and fibrinolytic responses to endotoxin. Endotoxin administration to normal humans results in a hyperdynamic cardiovascular state characterized by an elevated cardiac index and heart rate and by a decreased mean arterial pressure (MAP) and systemic vascular resistance. In retrospect, the initiation of this hyperdynamic cardiovascular response was associated with a decrease in functional levels of PK (2 hours), indicative of kallikrein generation. Activation of PK to kallikrein results in the generation of Bk from HK. Bk is one of the mediators of the pain and increased capillary permeability characteristic of the inflammatory response. We have recently shown, in an experimental model of lethal bacteremia (baboons), a significant correlation (Spearman R value of .929) between the decline in the levels of HK and the development of irreversible hypotension. In the current study, the small amount of kallikrein generated is
not sufficient to detectably alter the concentration of HK or C1 inhibitor. A modest decrease of 10% of HK would result in a concentration of 65 nmol/L Bk, which can contribute to the observed hemodynamic changes.

Previous work has shown that a small dose of endotoxin promotes plasminogen activation and its subsequent inhibition in normal subjects. Tissue plasminogen activators were found to be 7 times higher when compared with control values by 3 hours. We detected c,M-M-Kal complexes by 3 hours, indicating formation of the active enzyme, kallikrein, and its subsequent inhibition. The pathophysiologic role of intrinsic plasminogen activators as opposed to tissue plasminogen activators and urokinase is unclear. In vitro, kallikrein converts plasminogen to plasmin in an apparently stoichiometric reaction. FXIa, FXIIIa, and FXIIa also convert plasminogen to plasmin. However, the activity of activated FXI or FXIIIa in plasma is calculated to be only 5% that of kallikrein. Recently, kallikrein has been shown to convert single-chain urokinase to the two-chain enzyme, which might account for enhanced fibrinolysis. Whatever the mechanism, it is possible that contact activation may enhance the fibrinolytic system, which may lower the risk of fibrin deposition during endotoxia.

Neutrophil activation with release of the neutral protease, elastase, occurs by 3 hours after endotoxin administration. Preliminary data has shown that neutrophils are primed in vivo to produce enhanced amounts of superoxide after endotoxin administration to humans. The priming of neutrophils occurred by 4 hours, but not at 15 minutes after endotoxin administration. Increased levels of c,M-M-Kal complexes, which result from kallikrein generation due to contact activation, become significant at 3 hours. Because kallikrein can induce neutrophil degranulation, this result suggests that contact activation contributes to the activation and priming of neutrophils in vivo after endotoxin administration.

Recently, it has become apparent that many of the biologic effects of endotoxin are mediated by cytokines, mainly synthesized and released by macrophages, monocytes, and endothelial cells, including tumor necrosis factor (TNF), interleukin-1 (IL-1), IL-6, and IL-8. Michie et al reported release of TNF into the circulation after infusion of endotoxin but were unable to detect IL-1 in circulation and did not measure IL-6. Fong et al detected IL-6 in plasma, and Van Deventer et al confirmed that both TNF and IL-6 are released after endotoxin administration in human volunteers. In another study, the levels of cytokines correlated with fever. Furthermore, 30 to 45 minutes after endotoxin-induced release of TNF into the circulation, a steep increase in the plasma tissue plasminogen activator (t-PA) concentration was noted, followed by a decrease in MAP. In vitro, endotoxin and TNF both stimulate the release of plasminogen activator inhibitor (PAI) by endothelial cells, but decrease the synthesis of t-PA.

Activation of the contact system in this study occurred within 120 minutes after the peak concentration of TNF (60 minutes). In agreement with the study of Van Der Poll et al, we found that in patients receiving a high dose of TNF as part of a protocol for cancer treatment (unpublished results), no activation of the contact system was detected even with sensitive techniques such as measurement of c,M-M-Kal complexes. These results suggest that TNF by itself cannot support contact activation and that endotoxin or other cytokines may be required for initiation of contact activation.

It has recently been shown that TNF primes human neutrophils for enhanced superoxide production in vitro, and kallikrein, which stimulates neutrophil degranulation, may enhance the effects. We conclude that experimental endotoxia in humans induces activation of the contact system. The activation of the contact system was found to be temporally associated with the hemodynamic changes induced by endotoxia, as well as with changes in the neutrophil function and fibrinolytic system. The results indicate that a low dose of endotoxin can induce a prolonged activation of the contact system. Studies with contact system inhibitors are required to prove that it is an important mediator of the changes induced by the administration of endotoxin. Our most recent finding that an MoAb to FXII, which blocks contact activation, ameliorates secondary hypotension in vivo in septic baboons, supports this proposition.

ACKNOWLEDGMENT

The authors thank Rita Stewart for manuscript preparation.

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Activation of the kallikrein-kinin system after endotoxin administration to normal human volunteers

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