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MAJOR ARTICLE

Effects of IC14, an Anti-CD14 Antibody, on Coagulation and Fibrinolysis during Low-Grade Endotoxemia in Humans

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To determine the role of CD14 in lipopolysaccharide (LPS)–induced effects on coagulation and fibrinolysis in humans, 16 healthy subjects received an intravenous injection of LPS preceded by intravenous IC14, a recombinant chimeric monoclonal antibody against human CD14, or placebo. LPS-induced coagulation activation (tissue-factor mRNA in whole blood cells and plasma concentrations of F1+2) was not influenced by IC14, whereas the antibody reduced the increase in thrombin-antithrombin complexes and soluble fibrin. LPS injection also was associated with an early activation of fibrinolysis (plasma concentrations of tissue-type plasminogen activator and plasmin-α_2–antiplasmin complexes), followed by an inhibitory response (plasminogen activator inhibitor type 1), which were attenuated by IC14. Furthermore, LPS reduced thrombin-activatable fibrinolysis-inhibitor antigen levels and increased soluble thrombomodulin levels, which were not influenced by IC14. These results suggest that different hemostatic responses during endotoxemia may proceed via CD14-dependent and -independent pathways.

Severe sepsis is associated with the activation of multiple inflammatory pathways. Activation of the coagulation system, which may ultimately lead to the clinical syndrome of disseminated intravascular coagulation, is an important manifestation of the systemic inflammatory response of the host to severe infection [1]. Many of the proinflammatory responses observed during gram-negative infection are induced by endotoxin, the lipopolysacchacaride (LPS) moiety of the outer membrane of gram-negative bacteria.

The innate recognition of many microbial pathogens is mediated by CD14, which serves as a receptor for LPS [2], for peptidoglycan [3], cell walls [4], and lipoteichoic acids [5] of gram-positive bacteria, and for lipoarabinomannan of mycobacteria [4]. After the binding of bacterial constituents by CD14, signal transduction takes place through Toll-like receptors 2 and 4 [6]. Once activated, responsive cells release proinflammatory cytokines such as tumor necrosis factor (TNF)–α, interleukin (IL)–1, and IL-6 [7]. LPS also enhances the expression of tissue factor at the surface of monocytes and endothelial cells, which is considered crucial for the activation of the coagulation system in severe sepsis [8–11]. In accordance, LPS injection

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Written informed consent was obtained from all volunteers, and the study was approved by the institutional scientific and ethics committees of the Academic Medical Center, Amerstam, The Netherlands.. The human experimentation guidelines of were followed in the conduct of the present study.

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initiates coagulation activation in normal humans and mammalian primates in vivo, which can be prevented by treatment with tissue factor pathway inhibitor [12] or an anti–tissue factor antibody [8].

Blocking of the CD14 receptor has been found to inhibit the inflammatory response to LPS (and other bacterial antigens) in animals [13, 14] and in humans [15]. Knowledge of the effect of blocking CD14 on the altered vascular hemostatic balance in LPS-induced inflammation is lacking. Therefore, in the current study, we used the well-characterized human model of endotoxemia to determine the effect of IC14, a newly developed recombinant chimeric monoclonal antibody directed against CD14, on procoagulant and fibrinolytic responses elicited by a single dose of LPS.

MATERIALS AND METHODS

Study design. The present investigation was performed simultaneously with a study examining the effect of IC14 on LPS-induced cytokine release and neutrophil activation, of which the results have been reported elsewhere [15]. Sixteen healthy, male volunteers (mean age, 23 years; range, 20–33 years) were enrolled in this double-blind, randomized placebo-controlled trial. Medical history, physical examination, routine laboratory examination, and electrocardiogram readings were all normal. Tests for human immunodeficiency virus (HIV) infection and hepatitis B and C were negative. The participants did not smoke, use any medication, have any febrile illness in the month preceding the study, and never received monoclonal antibody therapy. The subjects fasted overnight before LPS administration. Eight of the volunteers received IC14, and 8 volunteers were given placebo.

The study drug, IC14, was supplied by ICOS. IC14 is a recombinant chimeric (murine/human) monoclonal antibody that recognizes human CD14. The murine parent is an antibody designated 28C5 [14, 16]. It is secreted from CHO cells as an $L_2H_{2\gamma 4}$ immunoglobulin. CHO cells were grown in bioreactors, and the sterile harvest fluids were collected. IC14 was isolated from the sterile harvest fluids using affinity, ion-exchange, and hydrophobic interaction chromatography steps. A dose of 1 mg/kg in a solution of 150 mL 0.9% wt/vol NaCl was administered intravenously over 1 h through a 0.22-μm low-protein binding filter. The placebo solution consisted of the dilution fluid and was administered in an identical manner. Of note, an irrelevant isotype matched control antibody would have been a better control for IC14; however, such an antibody suitable for administration to humans was not available. The *Escherichia coli* LPS preparation used in the present study, lot G (VSP), was administered intravenously over 1 min at a dose of 4 ng/kg, 2 h after the initiation of the IC14 or placebo infusion. The study was performed in a special research unit under the continuous supervison of physicians, with emergency and resuscitation equipment immediately available.

Assays. Blood samples were obtained from an intravenous canula before the infusion of IC14 or placebo ($t = -2$ h), at the end of the infusion of IC14 or placebo ($t = -1$ h), immediately before LPS injection ($t = 0$ h), and at 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, and 21 h thereafter. Blood for coagulation and fibrinolysis assays was collected in siliconized vacutainer tubes (Becton Dickinson) containing 0.105 *M* sodium citrate; the ratio of anticoagulant to blood was 1:9 (vol/vol). The other assays were conducted in EDTA-anticoagulated plasma. All blood samples (except samples for leukocyte counts and differentials) were centrifuged at 2000 *g* for 20 min at 4°C, and plasma was stored at −20°C until assays were performed. Leukocyte counts, differentials, platelets, and mean platelet volume (MPV) were assessed by a STKR Coulter counter. Plasma concentrations of prothrombin fragment F1+2 and thrombin-antithrombin complex (TATc) were measured by ELISAs (Behringwerke), and soluble fibrin was measured by spectrophotometric assay (Berichrom FM; Dade Behring).

For determination of tissue factor messenger RNA expression, total blood RNA was isolated and amplified by nucleic acid sequence-based amplification (NASBA), followed by an electrochemiluminescence (ECL)–based detection system, as described elsewhere [17]. In brief, for the NASBA, 100 μL of blood was mixed with 900 μL NASBA lysis buffer (50 m*M* Tris-HCl [pH 6.4], 20 m*M* EDTA, 1.3% [wt/vol] Triton X-100, and 5.25 *M* guanidine thiocyanate). After the addition of positive control RNA, total nucleic acids were isolated from whole blood, according to the method of Boom et al. [18]. Five microliters of nucleic acid solution was used in the NASBA reactions, which were carried out as described elsewhere [17]. Amplified RNA was detected using a 1-step probe hybridization method, followed by detection and quantitation in an ECL reader. Tissue factor was expressed as number of tissue-factor molecules per monocyte.

ELISAs were used to measure plasma concentrations of tissue-type plasminogen activator (tPA) (Asserachrom tPA; Diagnostic Stago), plasmin-α_2–antiplasmin complexes (PAPc) (Enzygnost PAP micro; Behring Diagnostics), and plasminogen activator inhibitor type 1 (PAI-1) antigen (Monozyme).

Thrombin-activatable fibrinolysis inhibitor (TAFI) antigen levels in plasma were determined as described elsewhere [19]. Plasma levels of soluble thrombomodulin were assayed by EIA from Diagnostica Stago (Chausson).

Statistical analysis. Values are given as mean ± SE. Differences between IC14 and placebo treatments were tested by analysis of variance (ANOVA) for repeated measures using SPSS for Windows. Changes of parameters in time were tested using 1-way ANOVA. A 2-sided $P < .05$ was considered to be significant.

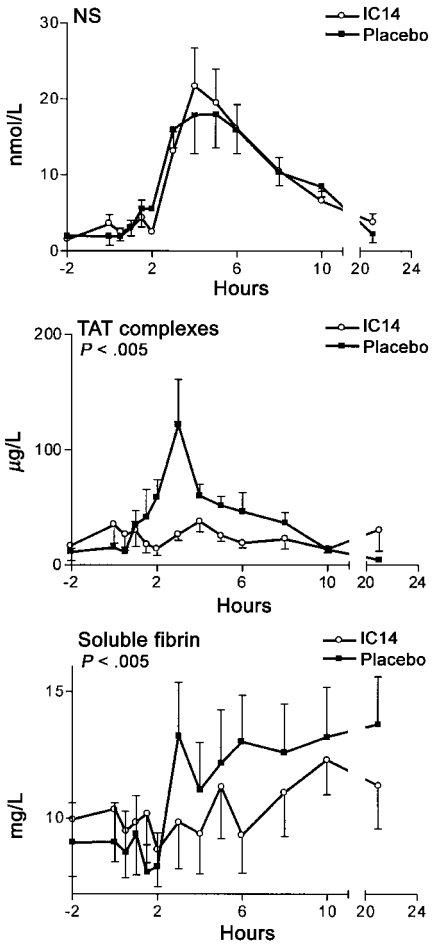

Figure 1. Mean (± SE) plasma concentrations of F1+2, thrombin-antithrombin (TAT) complexes, and soluble fibrin. IC14 (1 mg/kg; 8 subjects) or placebo (8 subjects) was given intravenously from −2 to −1 h, relative to endotoxin injection (4 ng/kg; $t = 0$ h). *P,* difference between IC14 and placebo; NS, not significant for difference between IC14 and placebo.

RESULTS

Activation of coagulation. LPS injection was associated with activation of the coagulation system, as reflected by rises in the plasma concentrations of the prothrombin fragments F1+2, TATc, and soluble fibrin (all $P < .001$). F1+2 increased from 1.98 ± 0.13 at baseline to 18.01 ± 4.45 nmol/L after 5 h, TATc from 11.3 ± 3.0 to 121.6 ± 39.4 μg/L after 3 h, and soluble fibrin from 9.0 ± 1.6 to 13.3 ± 2.1 mg/L after 3 h (figure 1). IC14 treatment did not significantly influence the LPS-induced increase of F1+2, but, surprisingly, did decrease TATc formation (peak value, 37.8 ± 9.0 μg/L, $P = .002$) and soluble fibrin production (peak value, 11.2 ± 2.1 mg/L $P = .004$) compared with placebo.

Tissue factor mRNA. Because tissue factor is considered to be the key mediator of LPS-induced coagulation activation [8–12], we were interested in the effect of IC14 on tissue-factor expression. For this, we quantified tissue factor mRNA molecules in whole-blood cells by NASBA and ECL for 4 subjects treated with LPS and placebo and for 5 subjects treated with LPS and IC14 (figure 2). Because monocytes represent the cells in peripheral blood that express tissue factor and because LPS injection induced a transient monocytopenia (figure 2), we expressed our results as number of tissue factor molecules per 10^6 monocytes (see Materials and Methods) [17]. In accordance with our previous findings [17], the number of tissue factor molecules per 10^6 monocytes increased after LPS injection, reaching a peak after 2 h ($1.6 \times 10^6 \pm 0.8 \times 10^6$ cells; $P < .05$ vs. baseline; figure 2). In subjects treated with IC14, the increase in the number of tissue factor molecules per 10^6 monocytes was delayed and was lower than that in subjects treated with LPS only (peak, $0.6 \times 10^6 \pm 0.2 \times 10^6$ cells at 3 h). However, the difference between the IC14 and placebo group did not reach statistical significance. Notably, the transient increase in TF mRNA molecules expressed per 10^6 monocytes paralleled

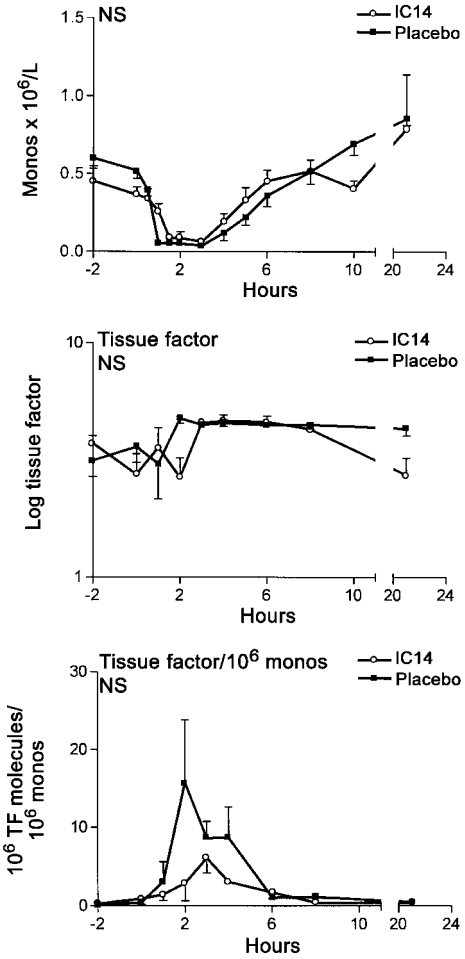

Figure 2. Mean (± SE) nos. of monocytes and tissue-factor molecules/ 10^6 monocytes. IC14 (1 mg/kg; 5 subjects) or placebo (4 subjects) was given intravenously from −2 to −1 h, relative to endotoxin injection (4 ng/kg; $t = 0$ h). NS, not significant for difference between IC14 and placebo.

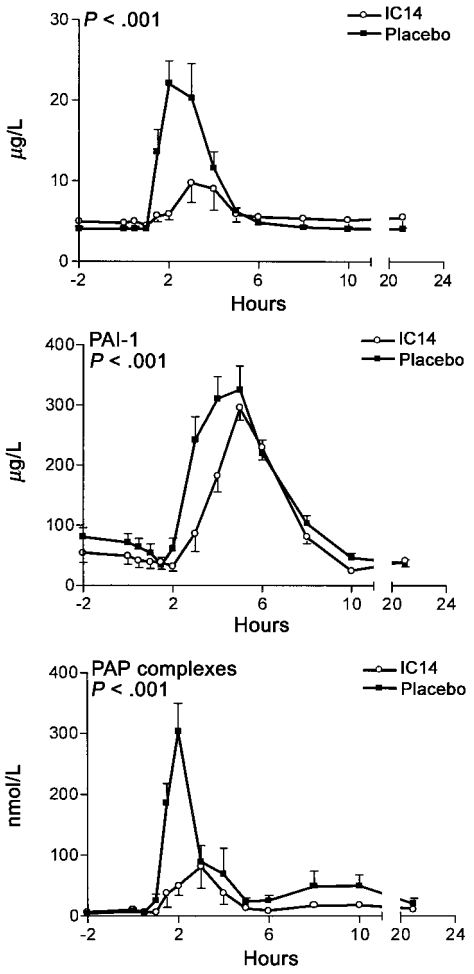

Figure 3. Mean (± SE) plasma concentrations of tissue-type plasminogen activator (tPA), plasminogen activator inhibitor type 1 (PAI-1), and plasmin-α_2–antiplasmin (PAP) complexes. IC14 (1 mg/kg; 8 subjects) or placebo (8 subjects) was given intravenously from −2 to −1 h, relative to endotoxin injection (4 ng/kg; $t = 0$ h). *P,* difference between IC14 and placebo.

the transient decrease in monocyte counts (figure 2), confirming our previous results [17].

Activation and inhibition of fibronolysis. Injection of LPS was associated with an early activation of the fibrinolytic system, as indicated by a transient increase in the plasma concentrations of tPA, peaking after 2 h from 4.0 ± 0.0 at baseline to 22.0 ± 2.9 µg/L ($P < .001$; figure 3, *upper panel*). This profibrinolytic response was followed in time by an increase in the plasma levels of PAI-1, peaking after 5 h from 81 ± 16 to 326 ± 40 µg/L, ($P < .001$; figure 3, *middle panel*). The transient generation of plasmin was confirmed by an increase in the plasma concentrations of PAPc, peaking after 2 h from 4.1 ± 0.7 to 302.3. ± 47.6 nmol/L ($P < .001$; figure 3, *lower panel*). IC14 treatment slowed down and reduced activation of the fibrinolytic system, as indicated by delayed and markedly diminished increases in the plasma levels of both tPA (peak at 3 h, 9.8 ± 2.4 µg/L; $P < .001$) and PAPc (peak at 3 h, 81.1 ± 35.3 nmol/L, $P < .001$). In addition, IC14 delayed and modestly attenuated the increase in plasma PAI-1 levels (peak at 5 h, 295 ± 20 µg/L, $P < .001$).

TAFI levels. TAFI has been implicated as an important link between coagulation and fibrinolysis [20], and we therefore determined the effect of LPS and IC14 treatment on the plasma concentration of TAFI. After infusion of LPS, TAFI antigen levels decreased from 84.7% ± 4.3% at $t = -2$ h to 68.3% ± 2.1% at 10 h ($P < .001$). IC14 treatment did not influence the LPS-induced decrease in TAFI antigen levels (figure 4).

Soluble thrombomadulin. Plasma concentrations of soluble thrombomodulin increased from 40.18 ± 3.52 µg/L at baseline to 50.14 ± 4.58 µg/L at 2 h and to 64.45 ± 5.04 µg/L at 24 h ($P < .001$; figure 5). IC14 treatment did not influence the release of soluble thrombomodulin in plasma (figure 5).

Platelets. Injection of LPS induced a modest but significant decrease in platelet counts from $230 \times 10^9 \pm 23 \times 10^9$ platelets/L to $192 \times 10^9 \pm 22 \times 10^9$ platelets/L after 1.5 h ($P < .05$). Treatment with IC14 did not alter this LPS response. MPV did not change in either group (data not shown).

DISCUSSION

Sepsis is associated with excessive activation of a number of host mediator systems, including the cytokine network, leukocytes, coagulation, and fibrinolysis, each of which can contribute to the development of tissue injury [21]. Binding to CD14 is considered to be the common pathway to induction of the host response to a variety of microbial pathogens. Several studies have shown that absence or blocking of CD14 protected animals from LPS-induced cytokine release and toxic effects [13, 14, 22]. The present study is the first to describe the effect of an anti-CD14 antibody on alterations in the hemostatic

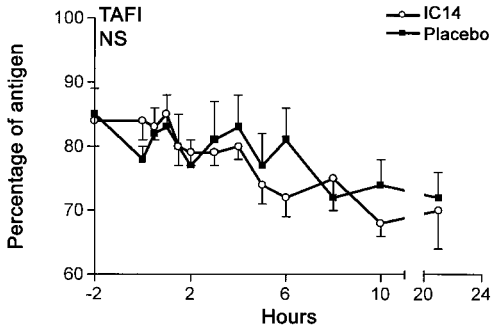

Figure 4. Mean (± SE) plasma concentrations of thrombin-activatable fibrinolysis inhibitor (TAFI). IC14 (1 mg/kg; 8 subjects) or placebo (8 subjects) was given intravenously from −2 to −1 h, relative to endotoxin injection (4 ng/kg; $t = 0$ h). NS, not significant for the difference between IC14 and placebo.

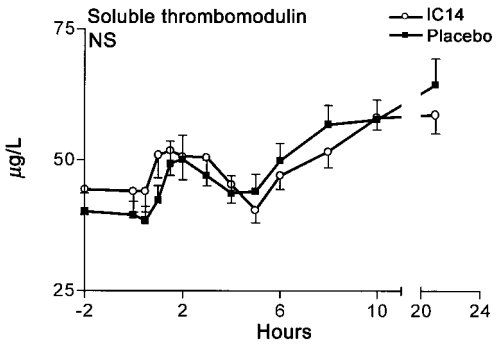

Figure 5. Mean (± SE) plasma concentrations of soluble thrombomodulin. IC14 (1 mg/kg; 8 subjects) or placebo (8 subjects) was given intravenously from −2 to −1 h, relative to endotoxin injection (4 ng/kg; $t = 0$ h). NS, not significant for the difference between IC14 and placebo.

mechanism induced by LPS in vivo. IC14 treatment did not reduce coagulation activation, as indicated by the unchanged LPS-induced increases in tissue-factor mRNA in monocytes and plasma levels of prothrombin fragment F1+2. However, LPS effects on thrombin release were inhibited by IC14, as indicated by reduced plasma concentrations of TATc and soluble fibrin. In addition, IC14 attenuated the fibrinolytic response.

Activation of coagulation in sepsis and after LPS administration is mediated by the exposure of tissue factor to circulating blood [23, 24]. It is assumed that tissue factor normally is not expressed on cells in direct contact with blood, but tissue factor may become expressed on intravascular cells (mainly monocytes and endothelial cells) by the action of inflammatory stimuli, including LPS [25]. Largely on the basis of in vitro data, both monocytes and endothelial cells are assumed to be the sites of induced intravascular tissue-factor expression. Monocytes express membrane-bound CD14, whereas endothelial cells, that lack CD14 on their surface, putatively are stimulated via LPS-soluble CD14 complexes or by LPS indirectly, via products of myeloid cells like cytokines [2, 16, 26]. The presence of soluble or membrane-bound CD14 is crucial for LPS-induced tissue factor expression by monocytes as well as by endothelial cells [27, 28] We have shown elsewhere that IC14 infusion virtually completely blocked CD14 on circulating monocytes [15], yet IC14 only tended to diminish the increase in tissue factor mRNA in blood cells, without influencing the initiation of the coagulation system as represented by the plasma concentrations of the prothrombin fragments F1+2. The fact that the decrease in TF expression after IC14 treatment did not reach statistical significance may be because of the relatively large interindividual variation and the limited number of subjects investigated. However, we cannot exclude the possibility that LPS-induced tissue factor expression and subsequent coagulation activation may use CD14-independent mechanisms and/or require lower LPS concentrations. In support of the former possibility are earlier findings of CD14 independent pathways for LPS-induced activation of monocytes in vitro [29, 30] and for LPS in mice in vivo [22]. With regard to the latter possibility, it should be noted that most, but not all, monocyte CD14 binding sites were occupied by IC14 [15]. Indeed, monocyte saturation was 94.9% ± 1.4% for the first 6 h after the completion of the IC14 infusion.

In contrast to its lack of inhibitory influence on the initiation of coagulation activation (F1+2 levels reflecting prothrombin activation), IC14 inhibited LPS-induced increases in thrombin levels (as measured by TATc) and action (as indicated by soluble fibrin), possibly by binding of thrombin to another molecule, thereby hampering its action. A possible candidate is thrombomodulin, which binds thrombin and alters its catalytic specificity, thereby reducing its ability to cleave fibrinogen but similarly increasing its capacity to activate circulating protein C. Although the LPS-induced increase in the plasma concentrations of soluble thrombomodulin was not influenced by IC14, it can be hypothesized that membrane bound thrombomodulin expression is increased, because TNF-mediated down-regulation of membrane-bound thrombomodulin expression [31, 32] and cleavage by proteases, such as elastase, are probably reduced after IC14 infusion [15]. This may result in increased thrombin binding to thrombomodulin, leading to less binding to antithrombin, as reflected by a decrease in TATc and decreased soluble fibrin levels. We cannot exclude that thrombin bound to other molecules such as α_2-macroglobulin or membrane bound protease receptors; however, the change in the coagulation system, reflected by decreased plasma levels of TATc and soluble fibrin, suggests that thrombomodulin is the most likely candidate [33].

LPS-induced fibrinolysis, as measured by PAPc, tPA, and the fibrinolysis inhibitor PAI, was markedly inhibited by IC14. Fibrinolytic changes during sepsis and endotoxemia are largely mediated by TNF [34–36]. Because IC14 treatment nearly completely inhibited TNF release [15], the inhibition of fibrinolysis at least in part is secondary to TNF blockade.

TAFI down-regulates the fibrinolytic system and plays an important role in the susceptibility of a clot for lysis. In a rabbit arterial thrombolysis model and a rabbit jugular vein thrombolysis model, inhibition of TAFI by specific inhibitors enhanced the tPA-induced lysis of a thrombus [37, 38]. In inflammatory diseases, TAFI has been found to correlate with markers of the acute-phase response such as C-reactive protein and haptoglobin [39]. Although C-reactive protein increased 100-fold after administration of LPS [15], TAFI levels decreased significantly in the same subjects, which suggests that TAFI is not an acute-phase response marker during low-grade endotoxemia. By the design of our study, the etiology of the decrease in TAFI cannot be clarified. It seems unlikely that increased levels of plasmin may have inactivated TAFI [40, 41] for 2 reasons. First, plasmin has been found to severely reduce TAFI

activity, with only a slight decrease in TAFI antigen levels [42], and, in the present study, measurement of both parameters in 2 subjects showed a high correlation (data not shown), confirming earlier findings [43]. Second, no difference between TAFI levels could be detected in subjects receiving IC14 or placebo treatment, although plasmin levels, as measured by PAPc, were decreased in the IC14-treated subjects.

The vascular endothelium likely contributes significantly to LPS-induced hemostatic alterations in healthy humans. An important limitation of our study is that no direct information was obtained on the effect of IC14 on activation of coagulation and other responses at the surface of endothelial cells. We have demonstrated elsewhere that IC14 exhibited an inhibitory effect on endothelial cell activation, as indicated by reduced release of von Willebrand factor antigen and soluble E-selectin [15].

Our results suggest that CD14 plays a more important role in certain hemostatic changes induced by intravenous LPS than in other hemostatic responses—that is, both coagulation and anticoagulation were attenuated by IC14, whereas the generation of thrombin (as measured by prothrombin fragments F1+2) and TAFI were not influenced. These data suggest that the activation of the coagulation system by LPS may proceed via CD14-dependent and -independent pathways.

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