Elimination of Interleukin 6 Attenuates Coagulation Activation in Experimental Endotoxemia in Chimpanzees

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Summary

The role of interleukin 6 (IL-6) in the toxic sequelae of sepsis is controversial. To assess the part of IL-6 in inflammatory responses to endotoxin, we investigated eight chimpanzees after either a bolus intravenous injection of *Escherichia coli* endotoxin (n = 4; 4 ng/kg) or after the same dose of endotoxin with a simultaneous bolus intravenous injection of an anti-IL-6 mAb (30 mg; n = 4). Anti-IL-6 did not affect the induction of the cytokine network (tumor necrosis factor [TNF], soluble TNF receptors types I and II, and IL-8) by endotoxin, nor did it influence the occurrence of a neutrophilic leukocytosis and neutrophil degranulation, as monitored by the measurement of elastase-α_1-antitrypsin complexes. In contrast, anti-IL-6 markedly attenuated endotoxin-induced activation of coagulation, monitored with the plasma levels of the prothrombin fragment F1+2 and thrombin–antithrombin III complexes, whereas activation of fibrinolysis, determined with the plasma concentrations of plasmin-α_2-antiplasmin complexes, remained unaltered. We conclude that IL-6 does not have a feedback effect on the release of other cytokines after injection of endotoxin, and that it is not involved in endotoxin-induced neutrophilia or neutrophil degranulation. IL-6 is, however, an important intermediate factor in activation of coagulation in low grade endotoxemia in chimpanzees.

IL-6 is a pleiotropic cytokine that exerts multiple effects on many different types of target cells. Major biologic activities of IL-6 include induction of acute phase protein synthesis in hepatocytes, induction of the terminal differentiation of B cells, and activation of T cells (1). IL-6 is one of the cytokines that is released early in the course of systemic infection. In experimental lethal bacteremia in baboons, the appearance of IL-6 in the circulation shortly follows that of TNF (2). In addition, elevated serum concentrations of IL-6 are found in the majority of patients with sepsis and show a strong positive correlation with mortality rates (3, 4). It is uncertain, however, to what extent IL-6 is involved in tissue injury in sepsis. Passive immunization against IL-6 confers only limited protection against lethality induced by endotoxin in mice (5, 6), and administration of high doses of recombinant IL-6 does not cause hypotension or endothelial damage in laboratory animals (7). The induction of IL-6 synthesis may even serve to protect the host against the destructive potential of more toxic members of the cytokine cascade, since in vitro IL-6 suppresses endotoxin-induced production of TNF and IL-1 (8, 9). This study aimed to more specifically investigate the intermediate role of IL-6 in inflammatory responses known to contribute to organ damage in sepsis. For this purpose, chimpanzees were intravenously injected with a bolus dose of endotoxin with or without a neutralizing anti-IL-6 mAb, and sequential measurements were performed to monitor cytokine release, neutrophil activation, and activation of the coagulation and fibrinolytic systems.

Materials and Methods

Study Design. Eight healthy adult chimpanzees (Pan troglodytes) were studied. They were recruited from the Laboratory for Experimental Medicine and Surgery in Primates (LEMSIP) (New York University School of Medicine, New York). None of the animals

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had abnormalities on routine laboratory investigation. The chimpanzees were sedated with ketamin chloride, and after intubation, kept under general anesthesia with nitrous oxide and halothane until the end of the 5-h experiment. Vital functions were registered during the entire experiment. All chimpanzees received a bolus intravenous injection of purified endotoxin (lot EC-5 from *Escherichia coli* 0113, Federal Drug Administration standard preparation) at a dose of 4 ng/kg body weight. Four animals also received a bolus intravenous injection of 30 mg anti-IL-6 mAb immediately before the administration of endotoxin. Anti-IL-6 mAb CLB.IL-6/#8 is a murine IgG1 against human IL-6 that potently neutralizes IL-6 activity in biological assays (K_d 6 × 10^{-12} M) (10). CLB.IL-6/#8 does not discriminate between human and chimpanzee IL-6. The antibodies were produced by in vitro cultures and purified by protein A–Sepharose chromatography (10). The dose of endotoxin chosen has been shown to induce reproducible systemic inflammatory responses (11). Clinical signs, including fever, are minimal or absent. Control studies, i.e., without injection of endotoxin, have demonstrated that the experimental procedures themselves do not elicit changes in the inflammatory parameters under investigation (11). Venous blood samples were obtained by separate venipunctures directly before the administration of endotoxin and at 0.5, 1, 1.5, 2, 3, 4, and 5 h thereafter. All blood samples (except samples for determination of leukocyte and platelet counts) were centrifuged at 4°C for 20 min at 1,600 *g* and stored at −70°C until assays were performed. The protocol was approved by the animal health and welfare committees of LEMSIP and was conducted according to the guidelines of the American Physiological Society.

Assays. Serum levels of CLB.IL-6/#8 were determined by ELISA using recombinant human IL-6 as capturing agent and peroxidase-conjugated rat mAb against mouse κ L chain as detecting agent. Serum IL-6 concentrations were measured with ELISA (12) and with the B9 bioassay (13). The capture antibody in the ELISA is mAb CLB.IL-6/#16. Binding of IL-6 to this antibody is not affected by the presence of as much as 10 μg/ml of CLB.IL-6/#8 because these antibodies recognize independent epitopes (10). However, there is some interference with the polyclonal second antibodies used in the ELISA (data not shown), leading to a slight underestimation of IL-6 levels. The B9 assay is disturbed by the presence of excessive amounts of CLB.IL-6/#8. However, we have shown previously that another mAb to IL-6 (CLB.IL-6/#14) can compete with mAb CLB.IL-6/#8 for IL-6 binding (10). As CLB.IL-6/#14 is non-neutralizing, addition of an excess (10 μg/ml) of CLB/IL-6/#14 to the B9 assay allows quantitation of IL-6, even in the presence of mAb CLB.IL-6/#8 (14). TNF serum levels were determined with an immuno radiometric assay (IRMA) (Medgenix, Fleurus, Belgium). The serum concentrations of the type I and II soluble receptors for TNF (sTNFR[1] type I and type II) were measured with enzyme-bound immunological assays (Hoffmann La Roche Ltd., Basel, Switzerland) (15, 16). IL-8 serum levels were determined with an ELISA (17). Leukocyte and platelet counts were determined in blood anticoagulated with 0.38 mM EDTA using flow cytometry. Blood for the measurement of elastase-α_1-antitrypsin complexes and plasmin-α_2-antiplasmin (PAP) complexes was collected in siliconized vacutainer tubes (Becton Dickinson, Plymouth, UK) to which 0.05% wt/vol hexadimethrine bromide (Polybrene; Janssen Chimica, Beerse, Belgium) and 10 mM EDTA were added to prevent in vitro complex formation. The plasma concentration of elastase-α_1-antitrypsin and PAP complexes were measured with RIAs (18, 19). Blood (9 vol) for the measurement of the prothrombin fragment F1+2 and thrombin–antithrombin III (TAT) complexes was collected in syringes containing 3.2% sodium citrate (1 vol). The plasma levels of F1+2 and TAT complexes were determined with ELISAs (Behringwerke AG, Marburg, Germany).

In Vitro Tissue Factor Expression on Monocytes. To study the role of IL-6 on the induction of tissue factor expression on monocytes, (heparinized) whole blood from human volunteers was incubated in vitro with endotoxin (lot EC-5 from *E. coli* 0113, 1 or 10 ng/ml) in the presence or absence of anti-IL-6 mAb (CLB.IL-6/#8, 30 μg/ml). After the incubation, erythrocytes were lysed and leucocytes were pelleted and subsequently washed. Aliquots of cells were incubated with 10 μg/ml antitissue factor mAbs, kindly provided by Dr. T. Edgington, Scripps Research Institute, La Jolla, CA) or control IgG1 mAb (Becton Dickinson Immunocytometry Systems, San Jose, CA). After washing, the cells were incubated with goat anti–mouse Ig antibody conjugated to PE (DAKO, Glostrup, Denmark). The expression of tissue factor was analyzed by immunocytometry using a FACScan® (Becton Dickinson Immunocytometry Systems) (20). Tissue factor expression on monocytes was expressed as the increment in mean fluorescent intensity (MFI) as compared with the IgG1 controls. Mean values (and SD) of six experiments in each group are given.

Statistical Analysis. All values are give as means ± SEM. Changes within and differences between the two treatment groups were tested by analysis of variance. *p*-values below 0.05 were considered statistically significant.

Results

IL-6 and Anti-IL-6. In the chimpanzees treated with endotoxin alone, a transient rise in serum IL-6 concentrations was observed, peaking after 2 h (165 ± 54 pg/ml; $p < 0.05$; Fig. 1). Administration of anti-IL-6 mAb CLB.IL-6/#8 led to relatively stable serum levels of the mAb throughout the 5-h experiment (8.4 ± 0.4 μg/ml after 5 min; 7.6 ± 0.7 μg/ml after 1 h; and 7.4 ± 1.2 μg/ml after 5 h). In the animals treated with CLB.IL-6/#8, the initial increase in IL-6 levels as measured by ELISA was similar to that recorded in the chimpanzees injected with endotoxin alone (Fig. 1). However, IL-6 did not decrease towards the end of the experiment. Peak IL-6 levels were found after 4 h (1357 ± 298 pg/ml; $p < 0.05$ versus endotoxin alone). These high concentrations were most likely due to impaired clearance of IL-6 complexed with CLB.IL-6/#8 (21), since in the B9 bioassay a corresponding high degree of IL-6 activity could be detected after addition of an excess (10 μg/ml) of the non-neutralizing anti-IL-6 mAb CLB.IL-6/#14, causing IL-6 to dissociate from the in vivo–administered neutralizing mAb CLB.IL-6/#8 (1810 ± 499 pg/ml after 5 h; Fig. 1).

Other Cytokines. Endotoxin-induced marked increases in the serum concentrations of TNF, both types of sTNFRs, and IL-8 (Figs. 2 and 3). Peak levels of TNF and its soluble receptors were measured after 1.5 h (TNF, 285 ± 75 pg/ml; sTNFR type I, 2.8 ± 0.5 ng/ml; sTNFR type II, 6.5 ± 1.1 ng/ml; all $p < 0.05$). Maximal IL-8 concentrations were found after 3 h (294 ± 161 pg/ml; $p < 0.05$).

The simultaneous injection of anti-IL-6 did not affect these endotoxin-induced responses (Figs. 2 and 3). Peak concen-

[1] *Abbreviations used in this paper:* CLB, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service; MFI, mean fluorescence intensity; PAP, plasmin-α_2-antiplasmin; sTNFR, soluble receptor for TNF; TAT, thrombin–antithrombin complex.

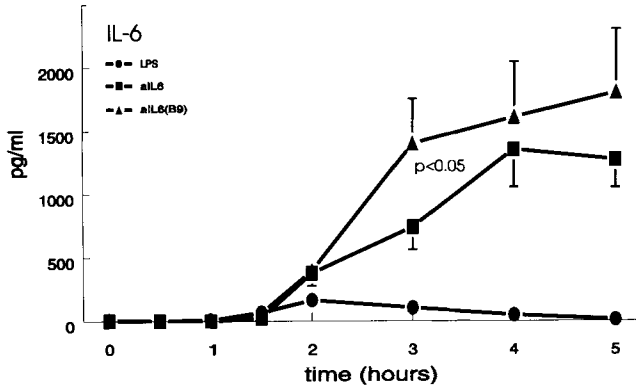

Figure 1. Mean (± SEM) serum concentrations of immunoreactive IL-6 after a bolus intravenous injection of *E. coli* endotoxin (4 ng/kg) with (*squares*; $n = 4$) or without (*circles*; $n = 4$) a simultaneous bolus intravenous injection of the neutralizing anti-IL-6 mAb CLB IL-6/#8 (30 mg). p-value indicates the difference between the two treatment groups. Curve with triangles indicates IL-6 activity in the chimpanzees treated with CLB IL-6/#8 as measured in a modified B9 assay, i.e., in which an excess (10 μg/ml) of the nonneutralizing anti-IL-6 mAb CLB IL-6/#14 was added to dissociate mAb CLB.IL-6/#8 from IL-6.

trations of TNF were 181 ± 17 pg/ml ($p = 0.21$ versus endotoxin alone), of sTNFR type 1, 2.5 ± 0.3 ng/ml ($p = 0.55$ versus endotoxin alone), of sTNFR type II, 5.8 ± 0.7 ng/ml ($p = 0.71$ versus endotoxin alone), and of IL-8, 203 ± 84 ng/ml ($p = 0.29$ versus endotoxin alone). The fact that the mean levels of TNF and IL-8 were higher in the chimpanzees treated with endotoxin alone was caused by one animal in this group that showed very high cytokine levels compared with all other chimpanzees studied.

Neutrophil Counts and Degranulation. Neutrophil counts and the plasma concentrations of elastase-α_1-antitrypsin complexes, indicative of degranulation of neutrophils, are given in Fig. 4. Endotoxin elicited a profound leukocytosis, reaching a summit at the end of the observation period (from 7.5 ± 0.4 to 26.7 ± 1.0 × 10^9/liter; $p < 0.05$), which was the result of a marked neutrophilia (from 4.4 ± 0.2 to 24.6 ± 1.0 × 10^9/liter after 5 h; $p < 0.05$). In addition, endotoxin provoked a rise in elastase-α_1-antitrypsin complexes from 10.8 ± 4.0 to 20.3 ± 5.3 ng/ml after 5 h ($p < 0.05$). None of these responses was significantly affected by administration of anti-IL-6.

Coagulation and Fibrinolysis. Endotoxin administration resulted in an activation of the common pathway of coagulation, as reflected by rises in the plasma levels of the prothrombin fragment F1+2 and TAT complexes (Fig. 5). F1+2 increased from 0.77 ± 0.24 to 4.75 ± 1.43 nmol/liter after 4 h ($p < 0.05$), and TAT complexes increased from 8.6 ± 2.1 to 68.8 ± 8.9 ng/liter after 4 h ($p < 0.05$). Endotoxin induced a more rapid activation of the fibrinolytic system, as indicated by a transient rise in the plasma levels of PAP complexes from 7.9 ± 0.6 to 38.2 ± 15.7 nmol/liter after 3 h ($p < 0.05$; Fig. 5).

The simultaneous administration of anti-IL-6 markedly attenuated the endotoxin-induced activation of coagulation, whereas fibrinolytic activation was not affected (Fig. 5). Peak

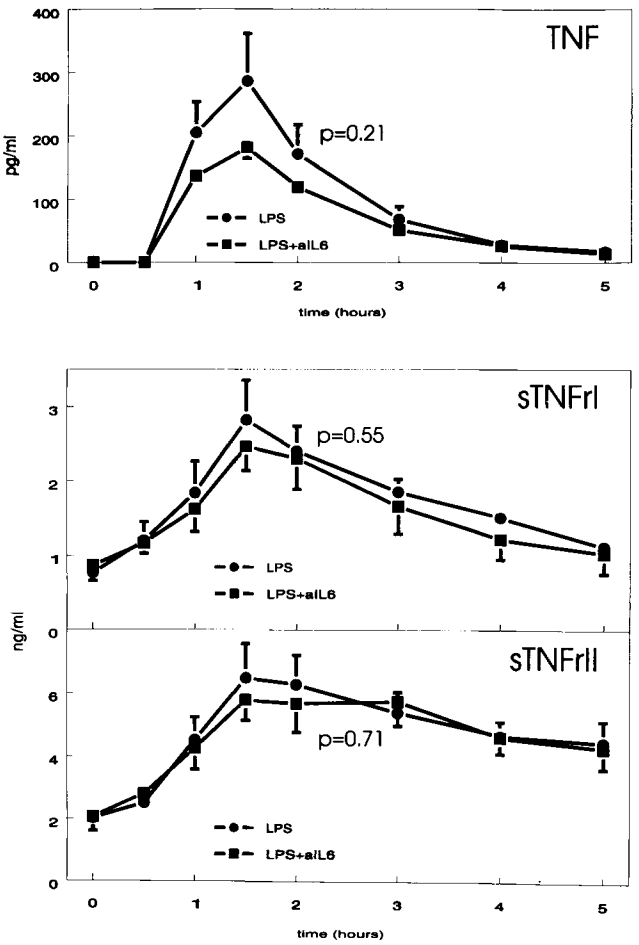

Figure 2. Mean (± SEM) serum concentrations of TNF and the soluble TNF receptors types I and II after a bolus intravenous injection of *E. coli* endotoxin (4 ng/kg) with (*squares*; $n = 4$) or without (*circles*; $n = 4$) a simultaneous bolus intravenous injection of the neutralizing anti-IL-6 mAb CLB.IL-6/#8 (30 mg). p = values indicate difference between the two treatment groups.

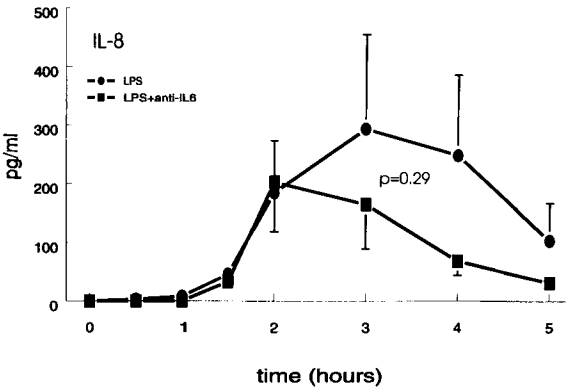

Figure 3. Mean (± SEM) plasma concentrations of IL-8 after a bolus intravenous injection of *E. coli* endotoxin (4 ng/kg) with (*squares*; $n = 4$) or without (*circles*; $n = 4$) a simultaneous bolus injection of anti-IL-6 mAb CLB.IL-6/#8 (30 mg). p-value indicates the difference between the two treatment groups.

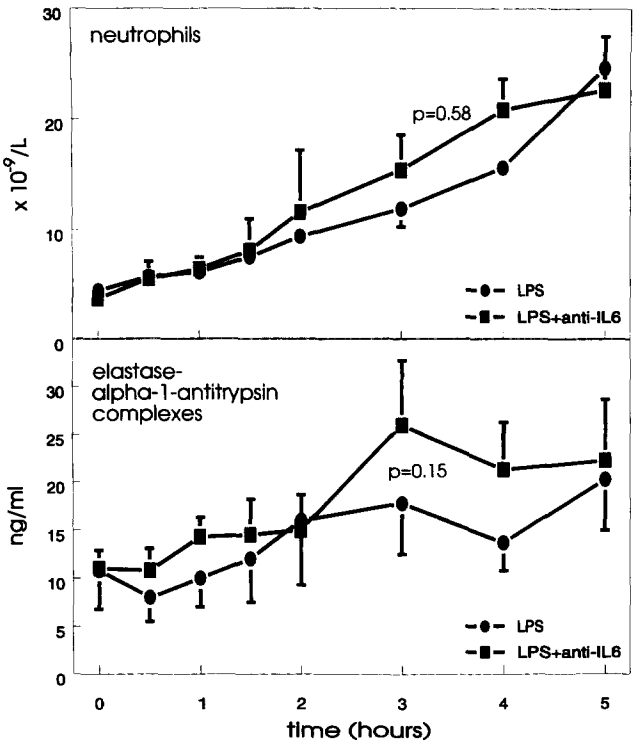

Figure 4. Mean (± SEM) neutrophil counts and plasma concentrations of elastase-α_1-antitrypsin complexes after a bolus intravenous injection of *E. coli* endotoxin (4 ng/kg) with (*squares*; $n = 4$) or without (*circles*; $n = 4$) a simultaneous bolus intravenous injection of anti-IL-6 mAb CLB.IL-6/#8 (30 mg). *p*-values indicate the difference between the two treatment groups.

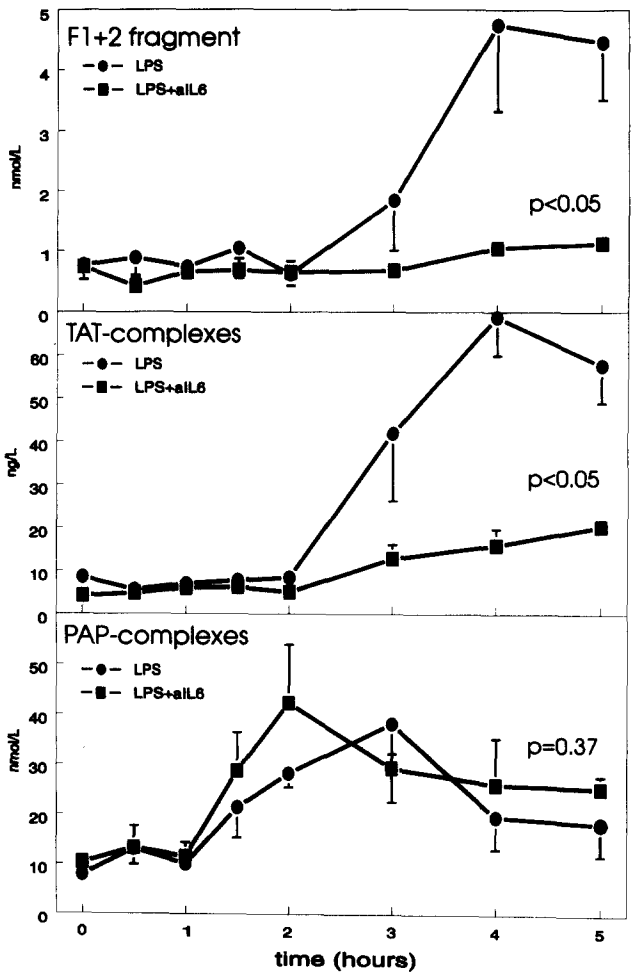

Figure 5. Mean (± SEM) plasma concentrations of F1+2, thrombin-antithrombin III complexes, and plasmin-α_2-antiplasmin complexes after a bolus intravenous injection of *E. coli* endotoxin (4 ng/kg) with (*squares*; $n = 4$) or without (*circles*; $n = 4$) a simultaneous bolus intravenous injection of anti-IL-6 mAb CLB.IL-6/#8 (30 mg). *p*-values indicate the difference between the two treatment groups.

plasma levels of F1+2 and TAT complexes were found after 5 h (1.13 ± 0.13 nmol/liter and 20.3 ± 1.7 ng/liter, respectively; both $p < 0.05$ versus endotoxin alone). Maximal concentrations of PAP complexes were detected after 2 h (42.3 ± 11.6 nmol/liter; $p = 0.37$ versus endotoxin alone).

Platelet counts did not change in either group (data not shown).

In Vitro Tissue Factor Expression on Monocytes. Incubation of whole blood with endotoxin (1 or 10 ng/ml) resulted in a significant tissue factor expression on monocytes (mean MFI, 8.6 [SD 0.7] and mean MFI, 17.2 [SD 2.3] after 1 and 10 ng/ml endotoxin, respectively). Endotoxin-induced tissue factor expression on monocytes appeared not to be inhibited by anti-IL-6 mAbs. The mean MFI after 1 ng/ml endotoxin plus anti-IL-6 mAb (30 μg/ml) was 10.5 (SD 1.5) (not significant as compared with 1 ng/ml endotoxin alone), and the mean MFI after 10 ng/ml endotoxin plus anti-IL-6 mAbs (30 μg/ml) was 16.4 (SD 3.4) (not significant as compared with 10 ng/ml endotoxin alone).

Discussion

Sepsis leads to the activation of a series of host mediator systems, including the cytokine network, neutrophils, and the hemostatic mechanism, each of which may contribute to the eventual tissue injury and organ damage. This study aimed to assess the role of IL-6 in the early phases of this sepsis cascade. It was demonstrated that treatment with anti-IL-6 mAb in low grade endotoxemia in chimpanzees did not affect cytokine and leukocyte responses, whereas this intervention strongly inhibited coagulation activation.

Endotoxin-induced IL-6 activity was neutralized by the simultaneous administration of the anti-IL-6 mAb CLB.IL-6/#8 (10), of which stable high serum levels were achieved throughout the experiment. Immunoreactive IL-6 was higher in the animals treated with CLB.IL-6/#8, because of the formation of IL-6–mAb complexes that have a far longer serum half-life than free IL-6. IL-6 activity was effectively blocked in the chimpanzees treated with CLB.IL-6/#8, considering the large excess of antibody that was injected compared with the amount of IL-6 produced and the potency of the antibody to neutralize IL-6 activity in vitro (10). Based on the mAb concentrations achieved in serum ($\sim 5 \times 10^{-8}$ M) and the affinity of the mAb (6×10^{-12} M) (10), the expected ratio of bound over free IL-6 is about 5,000. In view of the

observed maximal levels of circulating total IL-6, <0.5 pg/ml of IL-6 was free in the animals treated with the anti-IL-6 mAb.

IL-6 may have opposite effects on TNF production and TNF activity. IL-6 suppresses endotoxin-induced TNF production by mononuclear cells in vitro, and in intact mice in vivo (8, 9). On the other hand, IL-6 can upregulate TNF receptors on several cell types, and potentiate the cytotoxic effect of TNF on U937 cells (22, 23). The extracellular domains of both known types of TNF receptors also exist in soluble forms in the extracellular milieu (24). These sTNFRs types I and II compete with the cell-bound TNF receptors for the binding of free TNF, and are considered important regulators of the bioavailability of this cytokine. The serum levels of sTNFRs are elevated in clinical and experimental sepsis, in which they may serve as a self-defense mechanism against excessive TNF activity (15, 16, 25). Our results demonstrate that endogenous IL-6 does not affect TNF production, or the shedding of TNF receptors, after a single injection of a low dose of endotoxin. The same is true for endotoxin-induced IL-8 production, to which, considering the unchanged IL-8 response in the presence of anti-IL-6, IL-6 does not seem to contribute.

Endotoxin administration was associated with a pronounced neutrophilic leukocytosis. Although IL-6 is capable of inducing neutrophilia in several species (7, 26), treatment with anti-IL-6 mAb did not influence this response, suggesting that other factors such as endotoxin itself, TNF, and/or IL-8 are responsible (27, 28). Neutrophil activation is thought to contribute to tissue damage in sepsis. Indeed, markers of neutrophil degranulation are elevated in the plasma of patients with sepsis, and correlate with mortality rates (18). Elastase is a highly potent proteinase derived from azurophilic granules of neutrophils, that circulates in complex with its inhibitor α_1-antitrypsin. In accordance with previous reports (11, 29), endotoxin provoked a transient rise in the plasma concentrations of elastase-α_1-antitrypsin complexes. Injection of anti-IL-6 did not prevent this increase, suggesting that, although IL-6 may cause degranulation of neutrophils in vitro (30), the cytokine is not of great importance in this endotoxin-induced response in vivo. Likely, TNF is more significant for neutrophil degranulation in endotoxemia, because TNF potently elicits neutrophil degranulation in healthy humans (27), and inhibition of TNF synthesis after injection of endotoxin by pentoxifylline strongly reduced the increase in elastase-α_1-antitrypsin complexes in this chimpanzee model (11). In addition, IL-8 may play a role, since this cytokine is an important stimulator of neutrophil functions, especially after priming with TNF (31, 32).

Disseminated intravascular coagulation is a major complication of sepsis. Our previous studies in humans and chimpanzees have demonstrated that endotoxin provokes a sustained activation of the coagulation system via the extrinsic tissue factor–mediated pathway, preceded by a transient activation of the fibrinolytic system (33–35). In this study anti-IL-6 inhibited endotoxin-induced activation of the coagulation system. The mechanism by which the antibody achieved this effect remains to be established. Although anti-IL-6 did not affect the induction of tissue factor on mononuclear cells stimulated by endotoxin in vitro, this does not absolutely rule out that IL-6 has an effect on tissue factor expression by monocytes in vivo, or a stimulatory role of IL-6 on tissue factor expression by endothelial cells. Indeed, baboons infused with IL-6 showed enhanced expression of tissue factor by circulating monocytes (Taylor, Fletcher B., personal communication), whereas very recently, our group found rises in the plasma concentrations of F1+2 and TAT complexes after infusion of IL-6 into cancer patients (Stouthard, Jacqueline M., et al., manuscript in preparation). Furthermore, in sepsis, IL-6 may contribute to coagulation activation indirectly by induction of C-reactive protein, which in turn is able to stimulate tissue factor production (36). Of interest, reciprocal interactions may exist between IL-6 and the extrinsic pathway of the coagulation system, since treatment of septic baboons with tissue factor pathway inhibitor (TFPI) has shown to reduce not only the coagulative response, but also IL-6 plasma concentrations (37). Therefore, another explanation for the inhibitory effect of anti-IL-6 treatment on endotoxin-induced coagulation activation might be that anti-IL-6 induces alterations in natural anticoagulant functions such as TFPI, but possibly also other coagulation protease inhibitors such as protein C.

Administration of anti-IL-6 did not affect the early fibrinolytic activation after injection of endotoxin, confirming our previous finding that the stimulation of fibrinolysis and coagulation proceeds independently in low grade endotoxemia (34, 35). Intervention with an anti-TNF mAb in this model has implicated TNF as an important intermediate factor in endotoxin-induced activation of the fibrinolytic system (38).

In conclusion, our study indicates that IL-6 produced in response to a bolus dose of endotoxin, has no major feedback effect on the ongoing activation of the cytokine network, and that it is not involved in the ensuing neutrophilia and neutrophil degranulation. In this model of low grade endotoxemia, IL-6 appears to be a pivotal factor, most likely in an indirect way, for coagulation activation. This may explain why treatment with anti-IL-6 mAb has been found to provide significant protection against mortality in the generalized Shwartzman reaction, and some protection in lethal endotoxemia in mice (5, 6). Further studies are needed to assess the role of IL-6 after more severe bacterial challenges.

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