Tumor necrosis factor (TNF) has been implicated as a proximal mediator of the septic syndrome. To evaluate the possible role of TNF in leukocyte activation in septicemia, we performed a cross-over saline-controlled study in six healthy men who were intravenously injected with recombinant human TNF (50 pg/m³), and analyzed changes in circulating white blood cells and parameters for neutrophil and monocyte activation. TNF elicited a very rapid neutropenia, reaching a nadir after 15 minutes, followed by a neutrophilia. Lymphocytes showed a sustained decrease, whereas monocytes declined transiently. TNF injection was also associated with neutrophil activation, as reflected by a mean fivefold increase in the plasma concentrations of elastase-α1-antitrypsin complexes and a mean sevenfold increase in plasma lactoferrin levels. Serum neopterin, a marker of monocyte activation, was significantly increased 24 hours after the administration of TNF. These changes occurred in the absence of detectable complement activation, as indicated by unchanged C3a-desarg plasma values. Serum interleukin-6 showed a nearly 40-fold increase after TNF injection, whereas interleukin-1 remained undetectable throughout. We conclude that the systemic release of TNF, triggered early after invasive infection, may be involved in the alterations in circulating leukocyte numbers and in the activation of leukocytes, during the development of the septic syndrome.

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MATERIALS AND METHODS

The study was approved by the institutional research and ethics committees of the Academic Medical Center, University of Amsterdam, and written informed consent was obtained from all volunteers. All subjects were admitted to the Metabolic Research Ward and confined to bed during the 12-hour study period.

Study design. The present study was performed simultaneously with investigations on the coagulative and endocrine effects of TNF, results of which have been published previously. The outcomes of clinical parameters, ie, increases in body temperature, occurrence of chills, and the absence of hemodynamic changes were also reported in detail.

Six healthy male volunteers (age 27 to 33 years) participated in the study. Medical history, physical examination, and routine laboratory investigation were completely normal in all subjects. They did not use medication and had no febrile disease in the month before the study.

Each study period started at 7:30 AM. The volunteers fasted overnight until the end of the study. Each subject was studied twice with an interval of at least 3 weeks. On one occasion a bolus investigation, we aimed to assess the role of circulating TNF in these inflammatory reactions. For this purpose we studied six healthy men in a saline-controlled cross-over fashion after a bolus intravenous injection of recombinant human TNF.

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Intravenous injection of recombinant human TNF of 50 μg/ml dissolved in 10 ml of isotonic saline was administered; on the other occasion an equivalent volume of isotonic saline was administered. The order in which recombinant TNF and isotonic saline was given was determined by balanced assignment.

Venous blood samples were obtained by separate venipunctures, with the use of 19-gauge butterfly needles, directly before the injection of recombinant TNF or isotonic saline and 15, 30, and 45 minutes and 1, 2, 3, 4, 5, 6, 8, 10, and 12 hours thereafter. Additional samples were obtained 24 hours postinjection. Blood for the measurement of serum TNF concentrations was obtained immediately before the injection of recombinant TNF or saline and 5, 10, 15, 30, 60, 120, 180, and 240 minutes thereafter.

Recombinant human TNF was kindly provided by Boehringer Ingelheim (Ingelheim am Rhein, Germany). It was more than 99% pure as determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis analysis and contained less than 10 ng of endotoxin per milligram of protein as tested by the limulus amoebocyte lysate assay.

**Assays.** Leukocyte counts were determined in blood anticoagulated with K2-EDTA with the use of a flow cytometer (Technicon H1 system; Technicon Instruments, Tarrytown, NY). Leukocyte count differentials were determined both by flow cytometry and hand counting of peripheral blood smears, which showed identical results. Blood for the determination of elastase-α1-antitrypsin complexes, lactoferrin, and C3a-desarg was collected in siliconized Vacutainer tubes (Becton Dickinson, Plymouth, UK) to which EDTA (10 mmol/L) and Polybrene (Aldrich, Milwaukee, WI) (0.05%, wt/vol) were added to prevent any in vitro complex formation. Blood samples were centrifuged at 4°C for 20 minutes at 1,600g. The plasma concentrations of elastase-α1-antitrypsin complexes and lactoferrin were measured with a radioimmunoassay (RIA) that has been described in detail.1 Briefly, sepharose beads, to which polyclonal antibodies against human elastase or an MoAb against lactoferrin were coupled, were incubated with the samples to be tested. Elastase-α1-antitrypsin or lactoferrin bound to the beads was quantitated by incubation with 125I-MoAb against complexed α1-antitrypsin (RIA for elastase-α1-antitrypsin) or polyclonal 125I-1-antilactoferrin (RIA for lactoferrin). The plasma levels of elastase-α1-antitrypsin complexes and lactoferrin are expressed as nanograms per milliliter using preformed complexes and purified lactoferrin as standards, respectively. Complement activation was assessed by measuring the plasma concentrations of C3a-desarg by an RIA as reported previously.14 To prevent interference with native C3 in the assay, samples were incubated with polyethylene glycol before being tested. The results are expressed as nanomoles per liter.

Serum for the determination of neopterin, TNF, IL-6, and IL-1 was prepared by centrifugation of clotted blood for 20 minutes at 1,600g (room temperature). The serum concentrations of neopterin were measured with an RIA (IMMUTest Neopterin; Henning, Berlin, Germany) and expressed as nanomoles per liter. The serum levels of TNF were determined by IRMA (Medgenix, Fleurus, Belgium) as described previously.15 Polypropylene tubes were coated with a combination of MoAbs to recombinant TNF that recognize distinct epitopes of TNF. The tubes were incubated overnight with a mixture of the sample to be tested and anti-TNF antibody labeled with 125I. After decantation, the bound fraction was counted in a gamma counter, and the level of TNF was expressed in picograms per milliliter in relation to a standard binding curve for recombinant TNF. The serum concentrations of IL-6 were measured using the B9 assay as described previously.15 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. Serum IL-6 levels are expressed as units per milliliter, where 1 U/ml is the concentration that leads to half-maximal proliferation. One unit equals about 1 pg of IL-6. The identity of IL-6 in positive sera was confirmed with a neutralizing antiserum to recombinant IL-6. The limit of detection of the B9 assay is 7 U/ml. Samples with values below the limit of detection were assigned a value of 7 U/ml. The serum concentrations of IL-1 were measured using the D10 assay as reported previously.20 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-1a. The limit of detection of the D10 assay is 30 pg/ml.

**Statistical analysis.** Values are given as means ± SEM. Differences in results between the TNF and saline experiments were tested by analysis of variance and Newman-Keul’s test for multiple comparison, as indicated. A P value <.05 was considered to represent a significant difference.

**RESULTS**

**Leukocyte counts.** Baseline leukocyte counts were similar and within normal limits in both study periods (Fig 1). TNF injection was associated with a biphasic change in total leukocyte counts, which largely reflected the change in the number of circulating neutrophils (both P < .0001 by analysis of variance). Marked initial decreases in total leukocytes and neutrophils were observed, reaching a nadir after 15 minutes. Total leukocytes dropped from 5.2 ± 0.4 × 10⁹/L at baseline to 1.2 ± 0.1 × 10⁹/L; neutrophils from 2.6 ± 0.3 × 10⁹/L to 0.2 ± 0.04 × 10⁹/L. Early blood sampling in three subjects showed that the decrease in circulating neutrophils was already apparent as early as 5 minutes after TNF injection. The initial neutropenia was followed by a neutrophilia becoming significant after 1 hour. From this timepoint and onward band cells appeared in the circulation, with a maximum of 18% to 26% of circulating white blood cells after 2 to 4 hours. Total leukocyte and neutrophil counts peaked after 6 hours (12.9 ± 1.3 × 10⁹/L and 11.9 ± 1.3 × 10⁹/L, respectively) and were still significantly elevated after 12 hours. Total leukocyte and neutrophil counts had returned to control values in samples obtained after 24 hours (data not shown).

TNF administration also elicited a rapid and sustained lymphopenia (P < .0001 by analysis of variance), which reached a nadir after 5 hours (from 2.0 ± 0.2 × 10⁹/L at baseline to 0.3 ± 0.1 × 10⁹/L). Lymphocytes remained decreased until the end of the 12-hour study period; 24 hours after TNF or saline injection lymphocyte counts were similar (data not shown). TNF induced a transient monopenia lasting 2 hours postinjection (P < .005 by analysis of variance). Minimal monocyte counts were observed after 15 minutes, when monocytes had almost completely disappeared from the peripheral blood (from 0.3 ± 0.1 × 10⁹/L at baseline to 0.02 ± 0.01 × 10⁹/L).

**Neutrophil activation.** Activation of neutrophils was assessed by measurements of the plasma concentrations of elastase-α1-antitrypsin complexes and lactoferrin. Preinjection values were similar in both study periods (Fig 2). TNF elicited sharp increases in the plasma levels of elastase-α1-antitrypsin complexes and lactoferrin, both becoming significant after 30 minutes and peaking after 3 hours. Elastase-
At baseline, neopterin levels were not different in both study periods (6.0 ± 1.3 nmol/L before TNF; 5.3 ± 0.4 nmol/L before saline), and they remained unchanged until 8 hours after the initial injections. Twelve hours after TNF injection a modest nonsignificant increase in serum neopterin was found compared with the control period (7.8 ± 1.4 nmol/L vs 5.8 ± 1.2 nmol/L), which became significant 24 hours postinjection (8.7 ± 0.8 nmol/L vs 4.5 ± 0.8 nmol/L).

**Complement activation.** Activation of the complement system was monitored by measuring the plasma concentrations of C3a-desarg. As compared with saline, TNF did not affect C3a-desarg levels throughout the entire observation period (data not shown).

**Cytokines.** The serum levels of TNF have been reported previously. Briefly, TNF was not detectable in serum obtained at baseline or during the saline control period (Fig 3). After injection of recombinant TNF, the highest serum level was measured after 5 minutes (4,261 ± 785 pg/mL). Thereafter, serum TNF concentrations decreased rapidly.

α1-antitrypsin increased from 43 ± 6 ng/mL at baseline to 223 ± 28 ng/mL (P < .0001 by analysis of variance); lactoferrin from 212 ± 41 ng/mL to 1526 ± 227 ng/mL (P < .0001 by analysis of variance). From 3 hours and onward gradual decreases in elastase-α1-antitrypsin and lactoferrin were observed, whereby the former remained elevated until the end of our 12-hour observation period.

**Monocyte activation.** The serum concentrations of neopterin were determined as a measure of monocyte activation.
circles); IL-6 remained below the limit of detection (7 U/mL) in the saline control period. After injection of recombinant TNF, TNF was not detectable in serum (Fig 3). In addition, mean (+SEM) serum levels of TNF are shown (solid triangles) after injection of recombinant TNF. TNF was not detectable during the control period.

Serum IL-6 concentrations were below the limit of detection (7 U/mL) in the saline control period. TNF induced an increase of serum IL-6 concentrations that became significant after 30 minutes (Fig 3). Serum IL-6 peaked after 2 hours (265 ± 56 U/mL; \( P < .0001 \) by analysis of variance).

IL-1 activity was not detectable in serum during either study period (data not shown).

DISCUSSION

TNF is a pleiotropic protein that occupies a pivotal early role in the pathogenesis of the septic syndrome. In the course of septicemia, initiation of the cytokine network is followed by activation of other mediator systems, which is considered to importantly contribute to the development of the septic syndrome. Among these mediator systems, leukocytes are of particular interest. The objective of the present study was to evaluate the effects of circulating TNF on human leukocytes, because in rats TNF-induced neutrophilia is associated with decreased neutrophil counts in the bone marrow. Additionally, in cancer patients, labeled autologous leukocytes reinfused 15 minutes before TNF infusion rapidly disappeared from the circulation and did not return when leukocytosis developed. TNF injection also provoked a sustained lymphopenia, which may have been caused by enhanced lymphocyte adhesion to vascular endothelium secondary to expression of intercellular adhesion molecule 1 (ICAM-1) and vascular adhesion molecule 1 (VCAM-1) on the surface of endothelial cells by a direct action of TNF. Although IL-1 remained undetectable in the circulation after TNF injection, confirming previous findings in cancer patients infused with TNF in higher doses, cell-bound IL-1 may have been involved as a secondary mediator in the TNF-induced changes in circulating white blood cells as it shares many effects with TNF on the interaction between leukocytes and endothelium. IL-6 does not affect the adhesive capacity of endothelial cells, but has been shown to induce neutrophilia and a mild lymphopenia in rats in vivo. Since, in the present study, serum IL-6 showed a nearly 40-fold increase after TNF injection, this cytokine may have contributed to altered neutrophil and lymphocyte counts observed several hours after the administration of TNF.

Activation of neutrophils likely plays a pivotal part in the development of organ damage in septicemia. The capability of neutrophils to disrupt the normal architecture of tissues is derived from the combined cooperative action of released chlorinated oxidants and proteinases. Elastase is a very potent protease, degrading almost all components of the extracellular matrix, as well as a variety of plasma proteins. Moreover, elastase is an important mediator of endothelial cell injury, resulting in vascular leakage. The host's primary defense against uncontrolled action of elastase is the protease inhibitor \( \alpha_1 \)-antitrypsin, which rapidly and irreversibly binds to elastase, forming elastase-\( \alpha_1 \)-antitrypsin complexes. Lactoferrin is a glycoprotein from neutrophil specific granules. Several proinflammatory functions have been postulated for lactoferrin, including antimicrobial activity and regulation of neutrophil adhesiveness. Neutrophils secrete elastase and lactoferrin on stimulation with various agonists in vitro. As neutrophils are the predominant source of circulating elastase, and the exclusive source of circulating lactoferrin, the plasma concentrations of these proteins are commonly used as a measure of neutrophil activation in vivo. In patients with sepsis, elevated plasma levels of elastase-\( \alpha_1 \)-antitrypsin complexes and lactoferrin have been consistently found. TNF induced marked increases in the plasma concentrations of both neutrophil markers, reaching significance after 30 minutes and peaking after 3 hours. TNF may have provoked this neutrophil degranulation directly, at later stages possibly in concert with IL-6. In addition, granulocyte-macrophage colony-stimulating factor (GM-CSF), an important stimulator of the proliferation and maturation of leukocytes, may have been involved because TNF induces the production of this protein by several cell types in vitro.

![Graph showing mean (+SEM) serum concentrations of IL-6 after intravenous bolus injection of recombinant human TNF (50 pg/mL; solid circles); IL-6 remained below the limit of detection (7 U/mL) in the saline control period.](https://example.com/graphics/graph.png)
We did not measure GM-CSF in serum because, to our knowledge, release of GM-CSF to the circulation has never been found in infectious or other diseases.

Intravenous endotoxin has also been shown to induce increases in the plasma levels of elastase-α1-antitrypsin and lactoferrin.15,20 However, in these experiments the increase in elastase-α1-antitrypsin occurred only after 2 to 3 hours,15 whereas lactoferrin started to increase after 90 minutes.15 The differential time courses of the increases in neutrophil markers after endotoxin and TNF injection indicate that degranulation of neutrophils in endotoxemia may proceed via endotoxin-induced TNF. These results, taken together with previous studies reporting that TNF stimulates respiratory burst activity of neutrophils in vitro26 and primes neutrophils for hypochlorous acid production in vivo,31 implicate this cytokine as an important factor in the activation of neutrophils in septicemia.

Neopterin is exclusively released by activated monocytes and macrophages. Patients with septic shock have very high levels of neopterin in the circulation, which is interpreted to reflect monocyte activation.38 Our study establishes that TNF may play a role in this process. TNF induced an increase in neopterin serum concentrations, becoming significant after 24 hours, which contrasted with the rapid activation of neutrophils. It is likely that TNF stimulated neopterin release indirectly, as it does not affect the production of neopterin by monocytes in vitro.38

Severe septicemia is commonly associated with activation of the complement system.24 Intravenous injection of TNF did not elicit complement activation, as indicated by unchanged plasma levels of C3a-desarg. This finding confirms our previous observation after injection of endotoxin into healthy subjects, that activation of granulocytes can occur in the absence of complement activation.42 It is unlikely that our inability to detect complement activation was due to inappropriate sensitivity of the assay for C3a-desarg. Using the same assay we could readily detect activation of the complement system in several disease states, including sepsis28 and after treatment with recombinant IL-2 in cancer patients.40 However, these patients were severely ill and showed signs of shock and multiple organ failure, which contrasts with the relatively mild clinical response to intravenous endotoxin and TNF in healthy subjects. Conceivably, complement activation only occurs in more severe clinical conditions. It remains to be established whether repeated and/or prolonged exposure to higher amounts of TNF does stimulate the complement cascade.

In conclusion, this study shows that a single intravenous injection of TNF induces marked alterations in the peripheral blood counts and functional properties of leukocytes. These data substantiate the proximal role of TNF in the initiation of leukocyte activation in septicemia.

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