Endotoxin Induces Downregulation of Tumor Necrosis Factor Receptors on Circulating Monocytes and Granulocytes in Humans

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Leukocytes rapidly lose their surface receptors for tumor necrosis factor (TNF) after exposure to various stimuli in vitro. To assess the effect of endotoxin on cellular TNF receptors in humans in vivo, binding of biotinylated TNF to circulating monocytes and granulocytes was determined by fluorescence-activated cell sorter analysis in six healthy subjects after intravenous injection of endotoxin (lot EC-5, 20 U/kg). Endotoxin administration was associated with a transient decrease in monocyte TNF receptors, reaching a nadir after 2 hours ($P < .0001$), and a more sustained decrease in granulocyte TNF receptors ($P < .001$). Although the decrease in cellular TNF receptors coincided with increases in soluble TNF receptors types I and II, no correlations were observed between trough monocyte or granulocyte TNF receptors and peak plasma concentrations of soluble TNF receptors. Stimulation of human whole blood with endotoxin resulted in reduced expression of both type I and type II TNF receptors on monocytes and granulocytes. Endotoxin induces downmodulation of monocyte and granulocyte TNF surface receptors in humans in vivo, which may represent a mechanism to reduce excessive activity of TNF during systemic infection.

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ENDOTOXIN IS a major component of the outer membrane of Gram-negative bacteria that is considered to be responsible for the toxic sequelae of Gram-negative infection. Intravenous administration of low-dose endotoxin to healthy humans is a widely adopted model to study the human response to acute infection. Injection of endotoxin causes the systemic release of proinflammatory cytokines, of which tumor necrosis factor ($\alpha$ (TNF)) is the first to appear in the circulation. This early TNF release initiates a number of inflammatory responses, including secretion of other cytokines and neutrophil activation. After more severe bacterial challenges, such as infusion of a lethal dose of live Escherichia coli into baboons, excessive TNF release causes lethality, as demonstrated by the ability of anti-TNF antibodies to prevent death.

Recent investigations have established that the host response to infection also comprises several endogenous mechanisms to counteract the potentially toxic action of TNF. TNF exerts its biologic effects by binding to specific cellular receptors, of which two distinct types with molecular weights of 55 kD (type I, CD120a) and 75 kD (type II, CD120b) have been identified. In vitro investigations have revealed that leukocytes rapidly lose their capacity to bind TNF after exposure to various agents. Endotoxin induces a rapid loss of monocyte TNF receptors, while TNF receptors on isolated granulocytes remain virtually unaltered after incubation with endotoxin. However, at present, studies of the in vivo effects of endotoxin on leukocyte TNF receptors are limited. One study reported decreased binding of TNF by granulocytes 3 hours after administration of endotoxin to normal humans. In the present study, we sought to determine the kinetics of changes in TNF receptors on circulating monocytes and granulocytes during human endotoxemia.

MATERIALS AND METHODS

In vivo study. Six male subjects, aged 29 ± 2 (mean ± SE) years, were admitted to the Adult Clinical Research Center of the New York Hospital-Cornell University Medical Center (New York, NY) after documentation of good health by history, physical examination, and hematologic and biochemical screening. The study was approved by the Institutional Review Board, and written informed consent was obtained from all subjects before enrollment in the study. All volunteers were studied in postabsorptive state. On the morning after admission, a radial arterial catheter was placed to continuously monitor heart rate and blood pressure (Datascope model 2000A; Datascope Corp, Paramus, NJ) and for blood sampling. A rectal probe was inserted to allow continuous measurement of core temperature. At 9:00 AM, the volunteers received a bolus intravenous injection of endotoxin (National Reference Endotoxin, E coli 0113; lot EC-5; provided by Dr H.D. Hochstein, Bureau of Biologies, Food and Drug Administration, Bethesda, MD) at a dose of 2 ng/kg body weight. Blood was obtained directly before the injection of endotoxin and 0.5, 1, 1.5, 2, 3, 4, 5, and 6 hours thereafter. In three volunteers, blood for flow cytometry was also obtained 24 hours after endotoxin administration. All blood samples (except samples for flow cytometry) were centrifuged at 4°C for 20 minutes at 1,600g and stored at −70°C until assayed.

Whole blood stimulation. Endotoxin used for whole blood stimulation (from E coli serotype 0127:B8) was purchased from Sigma Chemical Co (St Louis, MO). Dilutions were made in sterile isotonic saline (Abbott Labs, North Chicago, IL). Blood was collected aseptically from five different healthy laboratory workers, and aliquots of 0.9 mL of whole blood were added to sterile polypropylene tubes (Becton Dickinson & Co, Rutherford, NJ). After addition of endotoxin (100 ng/mL; final concentration, 1 ng/mL or 1 μg/mL) or an equivalent volume of normal saline, tubes were incubated for various time periods (see Results) at 37°C. Thereafter, blood samples were processed for flow cytometry as described below.
Flow cytometry and assays. Leukocyte counts were determined by flow cytometric light scatter analysis. Saturation binding of TNF by monocytes and granulocytes was determined by fluorescence-activated cell sorter (FACS) analysis as described previously. Briefly, erythrocytes in 400 µL of blood were lysed with bicarbonate-buffered (pH 7.2) 0.826% ammonium chloride solution. Leukocytes were recovered by centrifugation and washed with ice-cold phosphate-buffered saline (PBS) containing 0.1% sodium azide (cPBS-A). Specific staining was with biotinylated human TNF (final concentration, 1 µg/mL), while nonspecific staining was with biotinylated TNF plus a 100-fold excess of unlabeled human TNF. Recombinant human TNF (provided by Dr Tadahiko Kohno, Amgen, Burlingame, CA) was biotinylated using sulfo-succinimidyl 6-(biotinamido)hexanoate (Immunopure, Rockford, IL) exactly according to the instructions of the manufacturer. All FACS experiments were performed using the same batch of biotinylated TNF. After incubation on ice for 15 minutes, cells were washed with cPBS-A and stained with 0.5 µg/mL phycoerythrin-conjugated streptavidin for 15 minutes on ice. Leukocytes were then washed with cPBS-A and resuspended for flow cytometric analysis. In a number of experiments (see Results), specific antibodies directed against either the type I TNF receptor (htr-20) or the type II TNF receptor (utr-4), donated by Dr Manfred Brockhaus (F. Hoffmann-La Roche, Basel, Switzerland), were used to assess the surface expression of these distinct TNF receptor species. In these experiments, erythrocytes in 200-µL aliquots of blood were lysed, and leukocytes were incubated with either htr-20, utr-4, or mouse IgGl (MOPC-21, Sigma; all 50 µL of a 20 µg/mL solution) for 45 minutes on ice. After washing with cPBS-A, leukocytes were then stained with an F(ab')2 fragment of phycoerythrin-conjugated sheep-antimouse antibody (Sigma) for 30 minutes on ice. Thereafter, leukocytes were washed with cPBS-A and resuspended for flow cytometric analysis. The flow cytometer photomultiplier gain was standardized using phycoerythrin-conjugated beads (Calibrite; Becton Dickinson Immunocytometry Systems, San Jose, CA). Mean channel fluorescence (MCF) at greater than 570 nm of forward and side angle scatter-gated monocytes and granulocytes was assessed. Data are presented as the difference (linear units) between MCF intensities of specifically and nonspecifically stained cells. In the experiments with biotinylated TNF, addition of a 100-fold excess of unlabeled TNF reduced the fluorescence signal to background (ie, to the signal obtained without adding any TNF, either biotinylated or unlabeled). Spiking human whole blood with 500 pg/mL of unlabeled recombinant TNF did not interfere with the fluorescence signal generated by biotinylated TNF and streptavidin-phycoerythrin (data not shown). This concentration of TNF was chosen because such TNF levels were found in volunteers injected with endotoxin and after stimulation of whole blood with 1 µg/mL endotoxin for 30 minutes, at which time point the binding of biotinylated TNF to monocytes and granulocytes (see Results) was maximally reduced. The plasma concentrations of TNF (CLB, Amsterdam, The Netherlands) and soluble TNF receptors (reagents provided by Dr W.A. Burman, University of Limburg, Maastricht, The Netherlands) were measured in heparinized plasma using specific and sensitive enzyme-linked immunosorbent assays (ELISAs). Statistical analysis. All values are given as means ± SEM. Data were analyzed by analysis of variance and Newman Keul's test. Correlations were calculated using the Pearson test. A P value less than .05 was considered to represent a statistically significant difference.

RESULTS

Monocyte and granulocyte TNF receptors. Injection of endotoxin induced a decrease in peripheral blood monocyte counts, reaching a nadir after 2 hours (from 0.47 ± 0.06 × 10⁷/L at baseline to 0.06 ± 0.02 × 10⁷/L; P < .01). Granulocyte numbers showed an initial decrease (from 3.55 ± 0.80 × 10⁹/L to 1.78 ± 0.31 × 10⁹/L after 1 hour), followed by an increase from 2 hours and onward (5 hours: 12.22 ± 1.70 × 10⁹/L; P < .0001).

Endotoxin administration was associated with a marked decrease in TNF binding by both circulating monocytes and granulocytes (Fig 1). Monocyte TNF receptors reached a nadir 2 hours after endotoxin injection in all volunteers (P < .0001), at which time the TNF-binding capacity of monocytes had declined to 14.6% ± 4.8% of baseline capacity. Granulocyte TNF receptors demonstrated a more sustained decrease (P < .001), with minimal TNF binding to granulocytes being detected between 2 and 5 hours after administration of endotoxin (42.0% ± 4.9% of baseline values). Blood was also obtained from three volunteers 24 hours after endotoxin administration. At that time, monocyte TNF receptors were increased when compared with baseline in all three subjects (to 157%, 123%, and 130% of their baseline values, respectively), while TNF binding by granulocytes was changed in a variable direction (89%, 100%, and 117% of baseline values, respectively). A significant positive correlation existed between trough monocyte TNF receptors and trough granulocyte TNF receptors (r = .86, P = .01).

TNF and soluble TNF receptor levels. Endotoxin in-
caused a virtually complete disappearance of TNF binding to monocytes within 30 minutes; at which time, both the type I and the type II TNF receptors were downmodulated (Fig 5). The decrease in TNF binding to granulocytes after incubation with endotoxin at 1 ng/mL was more delayed, becoming apparent only after 2 hours (Fig 4). Endotoxin at 1 µg/mL resulted in a more rapid decrease, within 15 minutes, in TNF binding to granulocytes (Fig 4), which was caused by a downmodulation of both TNF receptor species (Fig 6). Hence, in human whole blood, the endotoxin-induced reduction in TNF binding to monocytes and granulo-

duced a monophasic increase in the plasma concentrations of TNF, peaking after 1.5 hours (592 ± 136 pg/mL, P < .0001; Fig 2A). Additionally, the plasma levels of soluble TNF receptor types I and II increased after endotoxin administration (for both, P < .0001; Fig 2B). Both soluble TNF receptor species reached plateaus between 1.5 and 3 hours (1.5 hours: type I, 2.12 ± 0.43 ng/mL; 2 hours: type II, 11.29 ± 1.46 ng/mL). Significant inverse correlations existed between peak TNF concentrations, and trough monocyte TNF receptors (r = -.80, P < .05) and trough granulocyte TNF receptors (r = -.76, P < .05). In contrast, no correlations were found between trough monocyte or granulocyte TNF receptors, and peak levels of either soluble TNF receptor types.

Whole blood stimulation. To assess whether endotoxin reduced TNF binding by monocytes and granulocytes through downmodulation of both TNF receptor species, human whole blood obtained from five additional volunteers was stimulated with endotoxin at 37°C for up to 2 hours. Figure 3 shows TNF and soluble TNF receptor levels in supernatant plasma after incubation of whole blood with either saline or endotoxin (1 ng/mL, 1 µg/mL). Incubation of whole blood in the absence of endotoxin resulted in an increase in TNF binding to monocytes and granulocytes (Fig 4). In contrast, incubation of whole blood with endotoxin led to a decrease in TNF binding by both monocytes and granulocytes (Fig 4). Monocytes were more sensitive than granulocytes. Endotoxin at a concentration of 1 ng/mL

Fig 2. Mean (±SE) plasma concentrations of TNF (A) and soluble TNF receptors type I (B) and type II (C) after intravenous injection of endotoxin (lot EC-5, 2 ng/kg) into six healthy subjects. *P < .05 versus baseline by analysis of variance and Newman Keul's test.

Fig 3. Mean (±SE) plasma concentrations of TNF (A) and soluble TNF receptors type I (B) and type II (C) after incubation of whole blood in the absence (□) or presence of endotoxin (1 ng/mL, △, 1 µg/mL, ○) for the time periods indicated.
TNF RECEPTORS DURING ENDOTOXEMIA

A

![Graph A](image1)

B

![Graph B](image2)

**Fig 4.** Decrease in TNF binding by monocytes (A) and granulocytes (B) after incubation of whole blood in the absence (□) or presence of endotoxin (1 ng/mL, ○; 1 μg/mL, □) for the time periods indicated. The means ± SE of five independent experiments are shown. Results (difference between specific and nonspecific MCF) are expressed as percentage change from baseline.

...cytes was associated with a decreased surface expression of both type I and type II TNF receptors.

**DISCUSSION**

Regulation of the availability of cellular TNF receptors to induce signal transduction may represent a mechanism to protect the host against excessive activity of TNF. Incubation of leukocytes with several infectious and immunologic agents in vitro leads to a reduction in the expression of surface TNF receptors.\(^{12-19}\) The present results establish that after administration of endotoxin to healthy humans, circulating monocytes and granulocytes express fewer TNF receptors, as determined by their capacity to bind biotinylated TNF. TNF binding by monocytes was decreased transiently, whereas TNF binding by granulocytes was still reduced 6 hours after endotoxin administration. This downmodulation of cellular TNF receptors may result in a diminished responsiveness of host cells to the actions of TNF produced during endotoxemia.

Both TNF receptor species may be processed to a soluble form, resulting in the soluble TNF receptor type I and type II, which can be found in the circulation of healthy humans and increase in a number of noninfectious and infectious diseases including sepsis.\(^{23-27}\) In addition, the plasma concentrations of soluble TNF receptors increase after intravenous administration of endotoxin to normal humans\(^{24-27,28}\) (and the present study). Because soluble TNF receptors retain their affinity for TNF but do not induce signal transduction, it has been proposed that they can function as natural inhibitors for TNF, especially when they are present in a large excess.

**Fig 5.** Both types of TNF receptors are downmodulated on monocytes after exposure of whole blood to endotoxin. Whole blood was incubated for 30 minutes with endotoxin (1 ng/mL), and leukocytes were stained with either biotinylated TNF (total TNF receptor expression; A), htr-20 (TNF receptor type I; B), or utr-4 (TNF receptor type II; C). Similar results were obtained in three independent experiments. Data are frequency histograms with cell number on the ordinate and fluorescence intensity (arbitrary units) on the abscissa. The histogram in the foreground is that of nonspecifically stained monocytes; the histogram in the background is that of specifically stained monocytes.
Fig 6. Both types of TNF receptors are downmodulated on granulocytes after exposure of whole blood to endotoxin. Whole blood was incubated for 30 minutes with endotoxin (1 µg/mL), and leukocytes were stained with either biotinylated TNF (total TNF receptor expression; A), htr-20 (TNF receptor type I; B), or utr-4 (TNF receptor type II; C). Similar results were obtained in three independent experiments. Data are expressed as in Fig 5. The histogram in the foreground is that of nonspecifically stained granulocytes; the histogram in the background is that of specifically stained granulocytes.

over the cytokine. In the current study, the increase in soluble TNF receptors coincided with the decrease in monocyte and granulocyte TNF receptors, suggesting that the observed reduction in monocyte and granulocyte TNF binding was related to shedding of surface TNF receptors by these cell types. However, considering the fact that no correlations existed between the extent of downmodulation of cellular TNF receptors and peak plasma levels of soluble TNF receptors, it seems likely that either soluble TNF receptors in plasma are, at least in part, derived from surface TNF receptors of cells that are not present in blood (eg, endothelial cells, tissue macrophages), or the downmodulation of peripheral blood monocyte and granulocyte TNF receptors is at least partly caused by internalization of their surface receptors, rather than by shedding. In accordance with the latter possibility is the in vitro finding that exposure of macrophages or monocytes to endotoxin results in a rapid and complete loss of cell surface TNF-binding sites, secondary to internalization of TNF receptors. Although endotoxin may also induce shedding of TNF receptors by monocytes in vitro, this effect is slow, reaching a plateau only after 2 days. As shown in Fig 3, exposure of whole blood to endotoxin results in a relatively fast increase in soluble TNF receptors. In all three subjects from whom blood was obtained 24 hours after endotoxin administration, monocyte TNF receptors were upregulated at that time when compared with baseline. This observation is in line with an earlier report showing enhanced expression of both type I and type II TNF receptors on isolated monocytes after stimulation with endotoxin for 24 hours.

In contrast to its effect on TNF receptor expression on isolated monocytes in vitro, endotoxin has been reported not to influence the TNF-binding capacity of isolated granulocytes in vitro. Using a different approach, ie, stimulating whole blood with endotoxin, and FACS analysis of unseparated leukocytes, we found a decrease in TNF binding by both monocytes and granulocytes. The reduction in TNF binding to granulocytes was especially evident when compared with the increased TNF binding to granulocytes after incubation of whole blood in the absence of endotoxin. The present data considered together with earlier studies with isolated granulocytes suggest that downmodulation of granulocyte TNF receptors observed after exposure to endotoxin is mediated indirectly, requiring the presence of other white blood cells besides neutrophils.

At the time the in vivo studies in healthy humans were conducted, antibodies directed against the type I or type II TNF receptor were not available to us. However, we consider it likely that both TNF receptor species were downregulated after injection of endotoxin into healthy humans, because downmodulation of the surface expression of both type I and type II TNF receptors occurs after stimulation of isolated monocytes with endotoxin, and stimulation of whole blood with endotoxin causes a reduction of type I and type II surface TNF receptors on monocytes and neutrophils (Figs 5 and 6).

TNF is produced early in the course of sepsis and endotoxemia. The present study demonstrates that the rise in circulating TNF levels after administration of endotoxin is associated with downmodulation of surface TNF receptors on monocytes and granulocytes, which likely represents an attempt of the host to reduce excessive activity of this potent cytokine. Although such responses may confer protection against mild challenges, it is clear that during overwhelming sepsis, they are insufficient to completely prevent detrimental effects of TNF.

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