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Effect of a Recombinant Dimeric Tumor Necrosis Factor Receptor on Inflammatory Responses to Intravenous Endotoxin in Normal Humans

By Tom van der Poll, Susette M. Coyle, Marcel Levi, Patty M. Jansen, Mieke Dentener, Karen Barbosa, Wim A. Buurman, C. Erik Hack, Jan W. ten Cate, Jan M. Agosti, and Stephen F. Lowry

To determine the role of tumor necrosis factor (TNF) in lipopolysaccharide (LPS)-induced inflammation, 12 healthy subjects received an intravenous injection with LPS (2 ng/kg) preceded by infusion of either a recombinant human dimeric TNF receptor type II-IgG fusion protein (TNFR:Fc; 6 mg/m²; n = 6) or vehicle (n = 6) from 30 minutes to directly before LPS injection. LPS elicited a transient increase in plasma TNF activity, peaking after 1.5 hours (219 ± 42 pg/mL; P < .05). Infusion of TNFR:Fc completely neutralized endogenous TNF activity. LPS administration was associated with an early activation of fibrinolysis (plasma concentrations of tissue-type plasminogen activator, plasminogen activator activity, and plasmin-α2-antiplasmin complexes), followed by inhibition (plasma plasminogen activator inhibitor type 1), changes that were completely prevented by TNFR:Fc. By contrast, TNFR:Fc did not influence LPS-induced activation of coagulation (plasma levels of prothrombin fragment F1 + 2 and thrombin-antithrombin III complexes). TNFR:Fc strongly inhibited endothelial cell activation (plasma levels of soluble E-selectin), modestly reduced neutrophil responses (neutrophilic in-dotoxemia in normal humans. A remarkable finding of that study was, that, although both TNFR:Fc doses provided a large excess of TNF neutralizing capacity, the lower dose exerted the most potent antiinflammatory effects. In the present study, we sought to determine the antiinflammatory effects of an even lower dose of TNFR:Fc (6 mg/m²) on activation of coagulation, endothelial cells, and leukocytes during endotoxemia in normal humans.

MATERIALS AND METHODS

Study design and subjects. The present study was performed simultaneously with an investigation on the effects of TNFR:Fc on endotoxin-induced changes in the surface expression of TNF and interleukin-1 (IL-1) receptors, the results of which are reported elsewhere. Eighteen healthy male subjects, 28 ± 2 (mean ± SE) years of age, 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. Twelve subjects received an intravenous injection with lipopolysaccharide (LPS; National Reference Endotoxin, Escherichia coli 0113 [lot EC-5]; generously provided by Dr H.D. Hochstein, The Bureau of Biologies, Food and Drug Administration, Bethesda, MD) at a dose of 2 ng/kg body weight, at 9:00 am. These 12 subjects were randomized to also receive 30 minutes of intravenous infusion of TNFR:Fc; Immunex Corp, Seattle, WA) at a dose of 6 mg/m² (n = 6) or vehicle (n = 6). The remaining 6 subjects received 30 minutes of intravenous infusion of TNFR:Fc (6 mg/m²) only (ie, without LPS). TNFR:Fc was manufactured by fusing two identical

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extracellular portions of the type II TNF receptor with the Fc domain of IgG1. The resulting dimeric TNF receptor binds TNF with a 50-fold greater affinity ($K_d = 10^{13}$ mol/L$^{-1}$) than monomeric receptor.

TNFR:Fc was reconstituted with 1 mL of sterile water for injection and diluted in 100 mL of isotonic saline. TNFR:Fc (6 mg/m$^2$) was infused for 30 minutes. Data are the mean ± SE of 6 normal subjects obtained before the start of the infusion (Pre) and at various time points after the end of the infusion.

### Table 1. Effects of TNFR:Fc in Normal Subjects

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>TAT Complexes (ng/mL)</th>
<th>PAP Complexes (nmol/L)</th>
<th>Soluble E-Selectin (ng/mL)</th>
<th>Secretory PLA2 (ng/mL)</th>
<th>Neutrophils ($\times 10^9$/L)</th>
<th>Elastase-1-Antitrypsin Complexes (ng/mL)</th>
<th>IL-6 (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre</td>
<td>6.4 ± 0.9</td>
<td>4.1 ± 0.3</td>
<td>13.3 ± 3.1</td>
<td>2.38 ± 0.54</td>
<td>1.91 ± 0.65</td>
<td>&lt;40</td>
<td>&lt;4</td>
</tr>
<tr>
<td>2</td>
<td>5.9 ± 0.8</td>
<td>4.6 ± 0.9</td>
<td>13.8 ± 2.8</td>
<td>1.97 ± 0.45</td>
<td>2.14 ± 0.39</td>
<td>&lt;40</td>
<td>&lt;4</td>
</tr>
<tr>
<td>4</td>
<td>6.4 ± 1.9</td>
<td>5.6 ± 1.4</td>
<td>13.2 ± 2.5</td>
<td>1.62 ± 0.39</td>
<td>3.25 ± 0.62</td>
<td>&lt;40</td>
<td>&lt;4</td>
</tr>
<tr>
<td>6</td>
<td>7.3 ± 2.4</td>
<td>5.5 ± 0.6</td>
<td>13.5 ± 2.7</td>
<td>1.73 ± 0.33</td>
<td>3.31 ± 0.46</td>
<td>&lt;40</td>
<td>&lt;4</td>
</tr>
<tr>
<td>12</td>
<td>2.4 ± 0.2</td>
<td>6.8 ± 0.9</td>
<td>13.7 ± 2.8</td>
<td>1.60 ± 0.32</td>
<td>2.78 ± 0.24</td>
<td>&lt;40</td>
<td>&lt;4</td>
</tr>
<tr>
<td>24</td>
<td>2.0 ± 0.2</td>
<td>4.4 ± 0.7</td>
<td>12.6 ± 2.5</td>
<td>1.83 ± 0.27</td>
<td>2.16 ± 0.30</td>
<td>&lt;40</td>
<td>&lt;4</td>
</tr>
</tbody>
</table>

In addition, no significant changes were registered in the inflammatory responses under investigation (Table 1).

#### Plasma TNF activity and clinical responses

Details of the in vivo neutralizing capacity of TNFR:Fc at the dose administered are reported elsewhere. Infusion of TNFR:Fc effectively neutralized LPS-induced TNF activity. In subjects injected with LPS only, plasma TNF activity peaked after 1.5 hours ($219 ± 42$ pg/mL; $P < .05$ v time), whereas in subjects infused with LPS and TNFR:Fc, TNF activity remained undetectable ($P < .05$ v LPS only). Furthermore, the addition of 10 ng/mL recombinant TNF to plasma samples obtained from TNFR:Fc-treated subjects at 1.5 hours or 24 hours after injection of LPS did not result in detectable TNF activity, indicating that the amount of TNFR:Fc administered offered an excess of TNF neutralizing capacity. LPS induced a febrile response, which was significantly blunted by TNFR:Fc: peak temperatures were 38.2°C ± 0.1°C (LPS only) and 37.7°C ± 0.1°C (LPS with TNFR:Fc; $P < .05$). TNFR:Fc did not significantly influence flu-like symptoms induced by LPS, including chills, headache, and nausea.

#### Coagulation and fibrinolysis

LPS injection was associated with a potent activation of the coagulation system, as reflected by increases in the plasma concentrations of F1 + 2 prothrombin fragment and TAT complexes, peaking after 4 hours (7.21 ± 2.24 nmol/L) and 5 hours (77.8 ± 26.1 ng/mL), respectively (both $P < .05$ v time; Fig 1). This LPS-induced coagulant response was not significantly influenced by treatment with TNFR:Fc. Peak levels of F1 + 2 and TAT complexes in TNFR:Fc-infused subjects were 6.77 ± 1.58 nmol/L ($P = .99$ v LPS only) and 52.8 ± 18.2 ng/mL ($P = .87$), respectively (Fig 1).

Changes in the fibrinolytic system induced by LPS were characterized by an initial activation, as reflected by transient increases in the plasma concentrations of tPA, PA activity, and PAP complexes, followed by inhibition, as indicated by a more delayed increase in the plasma levels of PAI-1 (all $P < .05$ v time; Fig 2). Peak concentrations of indexes of activation of fibrinolysis were measured after 1.5 to 2 hours (tPA, 28.5 ± 2.4 ng/mL; PA activity, +227% ± 39%; PAP complexes, 34.7 ± 3.9 nmol/L), and peak levels of PAI-1 were measured after 4 hours (179.2 ± 11.1 ng/mL). Infusion of TNFR:Fc completely prevented LPS-induced fibrinolytic changes. Thus, none of the parameters of fibrinolysis increased significantly from baseline (all $P < .05$ v LPS only; Fig 2).

#### Soluble E-selectin and secretory PLA2

Injection of LPS elicited a marked increase in the plasma concentrations of
s soluble E-selectin, peaking after 12 hours (81.2 ± 9.4 ng/mL; P < .05 v time). Infusion with TNFR:Fc almost completely prevented this increase, with peak soluble E-selectin levels being detected of only 26.4 ± 3.4 ng/mL (P < .05 v LPS only; Fig 3). sPLA2 concentrations reached a plateau between 8 and 12 hours after LPS administration (8 hours, 37.1 ± 10.0 ng/mL; P < .05 v time), a response that was not significantly influenced by treatment with TNFR:Fc (Fig 4).

**Neutrophils.** LPS administration resulted in an increase in neutrophil counts, peaking after 6 hours (7.2 ± 0.7 × 10⁹/L; P < .05 v time; Fig 5). LPS also elicited degranulation of neutrophilic granulocytes, as reflected by increases in the plasma concentrations of elastase-α₁-antitrypsin complexes and lactoferrin, peaking after 4 hours (132.2 ± 24.0 and 467.9 ± 50.4 ng/mL, respectively; both P < .05 v time; Fig 5). Treatment with TNFR:Fc attenuated these LPS-induced responses. Neutrophil counts increased to only 5.1 ± 1.0 × 10⁹/L (P = .06 v LPS only), and the increases in elastase-α₁-antitrypsin complexes and lactoferrin were reduced to 90.3 ± 8.4 and 240.4 ± 30.9 ng/mL, respectively (both P < .05 v LPS only; Fig 5).

**Cytokines.** LPS induced transient increases in the plasma concentrations of IL-6, IL-8, and IL-10 (all P < .05 v time;
Fig 3. Mean (± SE) plasma concentrations of soluble E-selectin after intravenous injection of LPS (lot EC-5; 2 ng/kg) at t = 0 hours in subjects receiving either (□) TNFR:Fc (6 mg/m²; n = 6) starting at t = −0.5 hours or (●) vehicle (n = 6). P value indicates the difference between treatment groups.

Fig 4. Mean (± SE) plasma concentrations of sPLA₂ after intravenous injection of LPS (lot EC-5; 2 ng/kg) at t = 0 hours in subjects receiving either (□) TNFR:Fc (6 mg/m²; n = 6) starting at t = −0.5 hours or (●) vehicle (n = 6). P value indicates the difference between treatment groups.

Fig 5. Mean (± SE) neutrophil counts and plasma concentrations of elastase-α₁-antitrypsin complexes and lactoferrin after intravenous injection of LPS (lot EC-5; 2 ng/kg) at t = 0 hours in subjects receiving either (□) TNFR:Fc (6 mg/m²; n = 6) starting at t = −0.5 hours or (●) vehicle (n = 6). P value indicates the difference between treatment groups.

DISCUSSION

Sepsis is associated with excessive activation of a number of host mediator systems, including the cytokine network, the hemostatic mechanism, and leukocytes, each of which can contribute to the development of tissue injury. TNF is the first cytokine detectable in the circulation during models of systemic infection. Numerous studies have documented that neutralization of this early TNF activity inhibits the induction of the cytokine network and has a strong protective effect against lethality associated with intravenous bacterial
EFFECTS OF DIMERIC TNF RECEPTOR DURING ENDOTOXEMIA

In a recent study, TNFR:Fc administered at higher doses (10 and 60 mg/m²) exerted a paradoxical effect on cytokine and stress hormone release during human endotoxemia, ie, as the dose of TNFR:Fc increased, the degree of inhibition decreased. Because the lower TNFR:Fc dose used in the earlier volunteer study still provided a large excess of TNF neutralizing capacity, we chose to administer TNFR:Fc at 6 mg/m². Furthermore, in light of these findings, we infused 6 healthy men with TNFR:Fc only (ie, without LPS) to exclude an inflammatory effect of TNFR:Fc per se, which could not be detected. The absence of changes in standard laboratory measurements, such as coagulation parameters, hematology, and chemistry, was previously reported after administration of TNFR:Fc to normal subjects in doses up to 60 mg/m². Similar to our study, TNF activity remained neutralized up to 24 hours after LPS injection, thereby ruling out the occurrence of delayed and prolonged release of TNF activity from TNF-TNFR:Fc complexes, as has been reported in a mouse model of lethal gram-negative sepsis.

In line with previous reports, injection of LPS was associated with an early activation of the fibrinolytic system mediated by the release of tPA, followed in time by an abrupt inhibition of fibrinolytic activity by the appearance of PAI-1. Only later, the common pathway of the coagulation system became activated. Administration of TNF to humans or baboons causes similar changes in the fibrinolytic and coagulation systems as LPS. TNF has a net procoagulant effect on endothelial cells by enhancing the synthesis and surface expression of tissue factor, the essential cofactor of the extrinsic pathway of the coagulation system; by downregulating thrombomodulin and protein S secretion; and by inhibiting the fibrinolytic response via inducing the synthesis and secretion of PAI-1. Although these data strongly suggest that TNF is an important mediator of LPS-induced stimulation of coagulation and fibrinolysis, TNFR:Fc only inhibited fibrinolytic changes. These results are in accordance with studies in endotoxemic and bacteremic primes, in which infusion of a neutralizing anti-TNF MoAb did not influence activation of the coagulation system. Thus, TNF is crucial for LPS-induced stimulation of fibrinolysis, but does not contribute to LPS-induced coagulation activation. The latter response has been found to be mediated by tissue factor, possibly in conjunction with IL-6. Hence, inhibition of TNF during sepsis may have a net procoagulant effect by

Fig 6. Mean (± SE) plasma concentrations of IL-6, IL-8, and IL-10 after intravenous injection of LPS (lot EC-5; 2 ng/kg) at t = 0 hours in subjects receiving eitherTNFR:Fc (6 mg/m²; n = 6) starting at t = −0.5 hours or vehicle (n = 6). P value indicates the difference between treatment groups. NS, nonsignificant.

Table 2. Administration of TNFR:Fc Does Not Significantly Influence Plasma Concentrations of LBP During Endotoxemia

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>LPS Only</th>
<th>LPS + TNFR:Fc</th>
</tr>
</thead>
<tbody>
<tr>
<td>−0.5</td>
<td>18.9 ± 1.8</td>
<td>20.8 ± 2.5</td>
</tr>
<tr>
<td>0</td>
<td>17.5 ± 2.0</td>
<td>18.9 ± 3.0</td>
</tr>
<tr>
<td>8</td>
<td>58.0 ± 8.7</td>
<td>43.8 ± 4.3</td>
</tr>
<tr>
<td>12</td>
<td>66.7 ± 6.9</td>
<td>54.9 ± 6.1</td>
</tr>
<tr>
<td>24</td>
<td>58.9 ± 8.2</td>
<td>52.4 ± 6.8</td>
</tr>
</tbody>
</table>

Mean ± SE plasma concentrations of LBP (in micrograms per milliliter) after intravenous injection of LPS (lot EC-5; 2 ng/kg) at t = 0 hours in subjects receiving either TNFR:Fc (6 mg/m²; n = 6) starting at t = −0.5 hours or vehicle (n = 6).
selective inhibition of fibrinolysis without a corresponding effect on the coagulation system. Furthermore, this study shows that also in humans activation of coagulation and fibrinolysis in response to intravenous LPS are independent phenomena, as previously shown in chimpanzees in which inhibition of coagulation with an antithrombin factor MoAb did not affect fibrinolytic changes.

TNFR:Fc infusion not only prevented tPA release, but also strongly attenuated the increase in the plasma levels of soluble E-selectin, another molecule that is shed by the vascular endothelium upon activation. These data therefore suggest that TNF plays a significant role in endothelial cell activation during endotoxemia. By contrast, the release of sPLA\(_2\) was not influenced by TNFR:Fc. sPLA\(_2\) is a central mediator of inflammation controlling the synthesis of eicosanoids and platelet-activating factor that can be produced by a variety of cell types, including endothelial cells and macrophages. The plasma concentrations of sPLA\(_2\) have been found elevated in sepsis, in which it may contribute to tissue injury. We previously showed that injection of TNF into baboons results in an increase in plasma sPLA\(_2\) levels. TNF may elicit sPLA\(_2\) release directly by an effect on endothelial cells. In baboons infected with live E coli, the plasma concentration of sPLA\(_2\) was not increased by TNFR:Fc. sPLA\(_2\) is a central mediator of inflammation, controlling the synthesis of eicosanoids and platelet-activating factor that can be produced by a variety of cell types, including endothelial cells and macrophages.

The results of the present study suggest that, during low-grade endotoxemia, TNF does not play an important role in this inflammatory reaction. Thus, the production of sPLA\(_2\) during endotoxemia seems to be regulated by different (TNF-independent) mechanisms and/or different cell types than the production of the more specific endothelial cell markers tPA and soluble E-selectin. It remains to be established which factors sustain sPLA\(_2\) production. IL-6, of which the release into the circulation was inhibited but not prevented by TNFR:Fc, is also able to induce sPLA\(_2\) release in vitro.

TNFR:Fc infusion abrogated LPS-induced neutrophil responses, which confirms and extends an earlier study in human volunteers. However, the reduced neutrophilia in subjects treated with TNFR:Fc contrasts with the previously reported enhancement of neutrophilia in endotoxemic subjects infused with higher doses of TNFR:Fc, suggesting that indeed lower doses of the compound exert more potent antiinflammatory effects. This supposition is further supported by our finding of slightly reduced LBP levels in TNFR:Fc-infused subjects, whereas in the earlier volunteer study the acute-phase protein response tended to be enhanced.

Recently, a dose-response relation between treatment with TNFR:Fc, administered at doses of 0.15, 0.45, or 1.5 mg/kg body weight, and mortality was found in a randomized, placebo-controlled trial with patients with septic shock. The explanation of this unexpected finding remains uncertain. Considering the present data and data from other laboratories, a toxic effect of TNFR:Fc is unlikely. Furthermore, it seems unlikely that TNFR:Fc functioned as an intravascular carrier of TNF prolonging the activity of the cytokine, because neither in this nor in the previous human study with TNFR:Fc could delayed appearance of TNF activity be detected. However, it is conceivable that some patients included in clinical sepsis trials do not benefit from complete neutralization of endogenous TNF activity. Indeed, animal data suggest that anti-TNF treatment may impair host defense during localized infections, such as peritonitis or pneumonia. Hence, at early stages of an infection, the local production of TNF likely is needed to combat invading microorganisms. Therefore, the failure of anti-TNF treatment (whether administered in the form of antibodies or TNF receptor fusion proteins) in patients with clinically defined sepsis may be related to the fact that the currently used inclusion criteria do not appropriately identify patients who may benefit from elimination of excessive TNF activity.

The results presented herein indicate that TNF cannot be considered the central mediator of LPS-induced systemic inflammatory responses in humans. Neutralization of TNF in this model was associated with a shift toward a more procoagulant state due to prevention of the fibrinolytic response while leaving coagulation activation unaltered. Furthermore, TNF is not involved in sPLA\(_2\) release and only modestly contributes to neutrophil responses during human endotoxemia. Although systemic administration TNF can reproduce the majority of inflammatory effects observed in models of systemic infection, it seems likely that TNF is not absolutely necessary for many of such responses during sepsis.

REFERENCES

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EFFECTS OF DIMERIC TNF RECEPTOR DURING ENDOTOXEMIA


Response to cyclosporin in pure red blood cell aplasia. A 54-year-old man has had pure red blood cell aplasia associated with B-cell chronic lymphocytic leukemia since 1994. At diagnosis, the Direct Antiglobulin Test was negative. In May 1996, while receiving prednisolone (70 mg/d), his reticulocyte count was $7.1 \times 10^9/L$ (reference range, 20 to $80 \times 10^9/L$); his hemoglobin level varied from 5.6 to 6.2 g/dL before red blood cell transfusions that he required every 1 to 2 weeks; his platelet count was $133 \times 10^9/$L; his leukocytes were $22.7 \times 10^9/$L, with a lymphocytosis of $15.9 \times 10^9/$L; and he had a normal granulocyte count of $5.6 \times 10^9/$L. The bone marrow showed severe erythroid hypoplasia, a reduced number of megakaryocytes, a normal proportion of granulocyte precursors, and marked lymphocytic infiltration (A). Two weeks after starting cyclosporin A at 250 mg twice daily, the drug concentration in whole blood was $155 \mu g/L$ and his reticulocyte count had increased to $145 \times 10^9/$L. By 4 weeks, the hemoglobin level had increased to 11.4 g/dL without red blood cell transfusion and the prednisolone decreased to 5 mg/d. Repeat marrow biopsy 3 weeks after initiating cyclosporin therapy showed an increased number of erythroid precursor cells (arrows in B). (Courtesy of I.E. Okpala, MRCPATH, FWACP, Brongla General Hospital, Aberystwyth, UK.)