Brief Definitive Report

Regulation of Interleukin 10 Release by Tumor Necrosis Factor in Humans and Chimpanzees

By Tom van der Poll, Jaap Jansen, Marcel Levi, Hugo ten Cate, Jan Wouter ten Cate, and Sander J. H. van Deventer

From the Center of Hemostasis, Thrombosis, Atherosclerosis, and Inflammation Research, Academic Medical Center, University of Amsterdam, 1105 AZ Amsterdam, the Netherlands

Summary

Interleukin 10 (IL-10) has been shown to inhibit endotoxin-induced tumor necrosis factor (TNF) production. To assess the role of TNF in the induction of IL-10 in endotoxemia, four healthy men were studied after a bolus intravenous injection of recombinant human TNF (50 $\mu g/m^2$). In addition, 13 healthy chimpanzees were investigated after a bolus intravenous injection of *Escherichia coli* endotoxin (4 ng/kg), 6 animals received endotoxin only, 4 animals received a simultaneous intravenous injection of a monoclonal anti-TNF antibody, whereas 3 chimpanzees were treated with an anti-TNF $F(ab')_2$ fragment 30 min after the administration of endotoxin. TNF induced a modest rise in IL-10 concentrations peaking after 45 min (47 ± 32 pg/ml; $p < 0.05$). IL-10 peaked 2 h after injection of endotoxin (202 ± 61 pg/ml; $p < 0.005$). In both anti-TNF–treated groups, the early endotoxin-induced TNF activity was completely neutralized. Simultaneous anti-TNF treatment attenuated endotoxin-induced IL-10 release (73 ± 13 pg/ml; $p < 0.01$ versus endotoxin alone), whereas postponed anti-TNF treatment did not significantly affect this response ($p = 0.21$). These results indicate that TNF, in part, mediates the induction of IL-10 in endotoxemia, resulting in an autoregulatory feedback loop.

TNF is considered to occupy a central role in the pathogenesis of sepsis (1). In experimental sepsis, TNF is the first cytokine appearing in the circulation, and neutralization of this early TNF activity markedly reduces mortality associated with the intravenous administration of living bacteria (1, 2). In accordance, high levels of TNF have been found in the circulation of patients with sepsis (3, 4), and administration of the recombinant cytokine to animals or humans causes pathological changes also observed in clinical sepsis (5, 6).

IL-10 is a recently discovered cytokine that can be produced by a variety of cell types, including T cells, B cells, monocytes, and macrophages (7). Incubation of monocytes or human whole blood with endotoxin results in the production of TNF, followed by that of IL-10 (8, 9). In patients with sepsis, the serum concentrations of IL-10 are elevated (9). Several lines of evidence indicate that this induced IL-10 is part of an endogenous protective mechanism that involves inhibition of TNF production. First, IL-10 inhibits endotoxin-induced TNF release by mononuclear cells in vitro and in mice in vivo (8, 10–12). Second, neutralization of endogenous IL-10 potentiates endotoxin-induced TNF production by mononuclear cells (8, 9), and in intact mice (13, 14). Finally, administration of IL-10 to mice reduces endotoxin-induced lethality (11, 12), whereas passive immunization against IL-10 enhances lethality associated with the administration of endotoxin (13, 14).

It is interesting to note that a recent study has implicated TNF as a regulator of IL-10 expression by mononuclear cells in vitro, suggestive of the existence of an autoregulatory feedback mechanism (15). Therefore, in the present study we sought to determine whether TNF is involved in the appearance of endotoxin-induced IL-10 in vivo.

Materials and Methods

Study Design. Four healthy men (27–33-yr-old) received a bolus intravenous injection of human recombinant TNF (50 $\mu g/m^2$; Boehringer Ingelheim, Ingelheim am Rhein, Germany). Details of the experimental procedures and outcomes of clinical parameters have been published previously (6). In addition, 13 healthy adult chimpanzees, recruited from the primate colony at the Laboratory for Experimental Medicine and Surgery in Primates, New York University School of Medicine (Tuxedo, NY) were studied after a bolus intravenous injection of *Escherichia coli* endotoxin (4 ng/kg; lot EC-5, kindly provided by Dr. H. D. Hochstein, Food and Drug Administration, Bethesda, MD). The experimental procedures have been reported in detail previously (16, 17). Briefly, the chimpanzees were sedated with ketamin chloride and intubated. General anesthesia was maintained with nitrous oxide and halothane until the end of the study. Vital functions were registered during the

entire experiment. The dose of endotoxin chosen does not induce serious clinical effects, and all animals fully recovered from the experiment (16, 17). Six animals received only the bolus injection of purified endotoxin. In four animals the injection of endotoxin was immediately followed by the administration of anti-TNF mAb (provided by Bayer, Wuppertal, Germany) given as a bolus injection of 15 mg/kg body weight (16). In three chimpanzees, the administration of endotoxin was followed after 30 min by the administration of an anti-TNF $F(ab')_2$ fragment (MAK 195F; provided by Knoll, Ludwigshafen, Germany) given as a bolus injection of 0.1 mg/kg body weight. MAK 195F is derived from a murine TNF neutralizing mAb (IgG_3) produced with standard methods using hybridoma cell cultures (18). A $F(ab')_2$ fragment was made by removing the Fc portion of the antibody by enzymatic cleavage with pepsin. Both the anti-TNF mAb and the anti-TNF $F(ab')_2$ fragment potently neutralize human and chimpanzee TNF, and have no cross-reactivity with a series of other cytokines, including lymphotoxin (16, 18).

Sampling and Assays. From the human volunteers, blood was drawn directly before the administration of recombinant TNF and at 0.25, 0.5, 0.75, 1, 2, 3, 4, and 5 h thereafter. From the chimpanzees, blood was obtained for measurement of IL-10 directly before the injection of endotoxin and at 1, 2, 3, 4, and 5 h thereafter. IL-10 was measured by ELISA. Microtiter plates were coated overnight at 4°C with anti-IL-10 mAb JES3-9D7 (Pharmingen, San Diego, CA; 1 μg/ml in 0.1 M bicarbonate buffer, pH 9.6). After washing and blocking the plates with 1% BSA in PBS, standards (recombinant human IL-10; Medgenix, Fleurus, Belgium) and samples, diluted at least 1:1 in assay buffer (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, the Netherlands), were incubated for 1 h at room temperature. After washing, biotinylated anti-IL-10 mAb JES3-12G8 (Pharmingen), 1 μg/ml in assay buffer, containing 2.5% normal rat serum (GIBCO, Life Technologies, Paisley, UK), was added and incubated for 1 h at room temperature. Thereafter, the wells were washed again and horseradish peroxidase conjugated to streptavidin (poly-HRP-streptavidin; Central Laboratory of the Netherlands Red Cross Blood Transfusion Service) was added (final concentration 1:10,000 in assay buffer) and allowed to incubate for 1 h at room temperature. After washing, ortho-phenylenediamine-dihydrochloride substrate was added and incubated for 30 min. The reaction was stopped with H_2SO_4 and read at 490 nm. The detection limit of the assay was 5 pg/ml. In the chimpanzees, serum TNF was measured both with an immunological assay (IRMA; Medgenix) that detects total TNF irrespective of its binding to soluble receptors (19), and with a biological assay using WEHI-164 mouse fibrosarcoma cells (20). Cytotoxicity was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) cytotoxicity assay. Concentrations of TNF activity are given in picograms per milliliter using recombinant human TNF (Cetus, Emeryville, CA) as the standard. All samples were stored at −70°C until assays were performed.

Statistical Analysis. All values are given as means ± SEM. Differences in results within and between groups were analyzed by analysis of variance. Correlations were calculated with the Pearson test. $p < .05$ was considered to represent a significant difference.

Results

Injection of TNF into Humans. At baseline, IL-10 was not detectable in any of the human volunteers. TNF induced a transient rise in circulating IL-10 concentrations (Fig. 1). IL-10 peaked after 45 min (47 ± 32 pg/ml; $p < 0.05$ versus baseline).

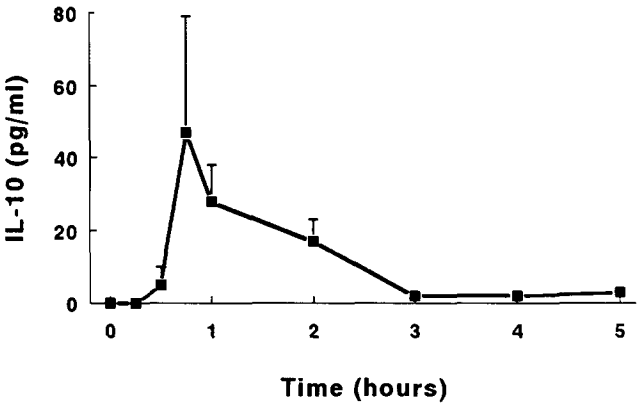

Figure 1. Mean (± SE) plasma concentrations of IL-10 after an intravenous bolus injection of recombinant human TNF (50 μg/m²) into four healthy humans. TNF induced a significant increase in IL-10 levels ($p < 0.001$ by analysis of variance).

Anti-TNF in Chimpanzee Endotoxemia. Administration of endotoxin to the chimpanzees resulted in a transient increase in serum TNF immunoreactivity and bioactivity, both reaching a summit after 1.5 h (192 ± 76 pg/ml and 150 ± 46 pg/ml, respectively; both $p < 0.005$ versus baseline). Endotoxin administration was also associated with a transient rise in IL-10 concentrations in the circulation (Fig. 2). Peak IL-10 levels were reached after 2 h (202 ± 61 pg/ml; $p < 0.005$ versus baseline) and showed a significant negative correlation with peak immunoreactive TNF concentrations ($r = -0.85$, $p < 0.05$; Fig. 3), supporting earlier studies demonstrating that endogenous IL-10 inhibits endotoxin-induced TNF release in mice (13, 14).

Both anti-TNF strategies used, i.e., simultaneous treatment with an anti-TNF mAb and treatment with an anti-TNF

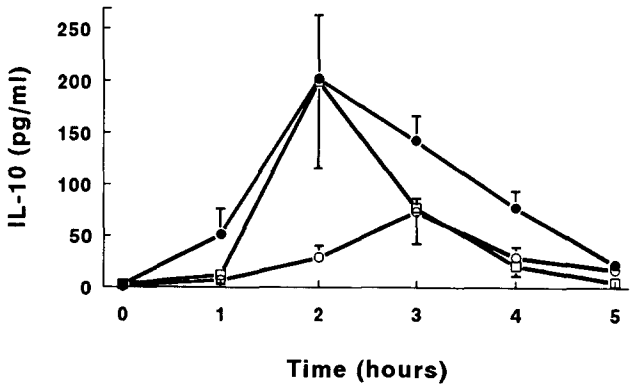

Figure 2. Mean (± SE) serum concentrations of IL-10 in chimpanzees intravenously injected with a bolus dose of *E. coli* endotoxin alone (4 ng/kg; ●; $n = 6$), with endotoxin and a simultaneous intravenous injection of an anti-TNF mAb (○; $n = 4$), or with endotoxin followed after 30 min by an intravenous injection of an anti-TNF $F(ab')_2$ fragment (□; $n = 3$). Simultaneous injection of anti-TNF significantly attenuated the endotoxin-rise in IL-10 concentrations ($p < 0.01$ versus endotoxin alone by analysis of variance), whereas postponed treatment with anti-TNF did not ($p = 0.21$).

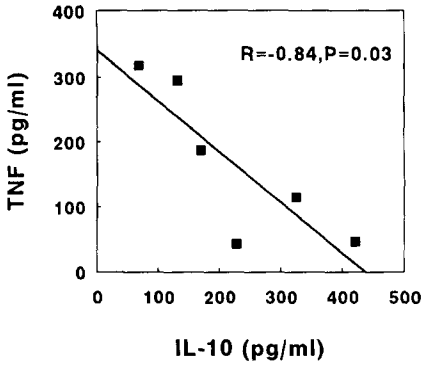

Figure 3. Correlation between peak IL-10 and peak TNF concentrations in six chimpanzees intravenously injected with *E. coli* endotoxin (4 ng/kg).

$F(ab')_2$ fragment administered 30 min after the injection of endotoxin, completely prevented the appearance of TNF bioactivity in the circulation (both $p < 0.01$ versus endotoxin alone). In addition, in all anti-TNF–treated animals, immunoreactive TNF remained undetectable, indicating that the in vivo–administered antibodies bind to epitopes that overlap with those bound by the antibodies used in the immunoassay. In the chimpanzees that were injected simultaneously with endotoxin and anti-TNF, the increase in IL-10 was significantly attenuated ($p < 0.005$ versus endotoxin alone), and maximal IL-10 levels (73 ± 13 pg/ml) were reached 1 h later when compared with administration of endotoxin alone (Fig. 2). In contrast, in the animals in which anti-TNF treatment was postponed for 30 min after endotoxin injection, neither the peak IL-10 response, nor the kinetics of the IL-10 response was significantly altered. In this group, peak IL-10 concentrations were found after 2 h (199 ± 84 pg/ml; $p = 0.21$ versus endotoxin alone; Fig. 2).

Discussion

The capability of IL-10 to inhibit endotoxin-induced TNF production has been carefully documented (8, 10–12). Investigations with antibodies against IL-10 have revealed that IL-10 negatively controls TNF synthesis in endotoxemia (13, 14). The present study shows that TNF produced early after administration of endotoxin to chimpanzees contributes to the later appearance of IL-10. Hence, in endotoxemia, TNF is involved in the induction of a cytokine that provides a negative feedback to its own production.

Injection of recombinant TNF into normal humans resulted in a rapid release of IL-10 into the circulation. To our knowledge, only one study has reported the ability of TNF to stimulate IL-10 production to date. In that investigation, TNF was shown to be unique among a number of proinflammatory cytokines, including IL-1 and IL-6, in upregulating IL-10 expression in PBMC in vitro (15). TNF induced IL-10 mRNA in a dose-dependent manner, an effect that was detected after incubation with TNF for 24 h. Incubations for up to 8 h did not result in significant IL-10 mRNA induction, which contrasts with the present in vivo finding that demonstrates maximal IL-10 induction within 45 min after TNF administration.

After injection of endotoxin, peak IL-10 levels were reached 1 h later than after administration of TNF. These differential time frames suggested that TNF may be involved in endotoxin-induced IL-10 production. Indeed, simultaneous administration of anti-TNF importantly reduced the appearance of IL-10 in endotoxemia. It is notable that the capacity of TNF to induce IL-10 production may be enhanced in endotoxemia, since TNF has been shown to potentiate endotoxin-induced IL-10 synthesis by mononuclear cells in vitro (15). It is remarkable that delayed treatment with anti-TNF did not significantly affect IL-10 induction after endotoxin injection, in spite of the fact that it still completely prevented the appearance of TNF activity in the circulation. This suggests that within 30 min after injection of endotoxin, some TNF is induced at tissue level and/or in cell-associated form that contributes to the synthesis of IL-10, possibly synergizing with endotoxin (15) or yet unidentified factors. Indeed, studies in rodents have revealed that endotoxin induces a very rapid expression of the TNF gene in vivo, with TNF mRNA levels peaking in various organs as early as 15 min after the administration of endotoxin (21).

Enhanced production of IL-10 in endotoxemia and sepsis likely represents an attempt of the host to counteract excessive activity of proinflammatory cytokines. The proximal role of TNF in the induction of the proinflammatory cytokine cascade in gram-negative infection has been widely recognized (1, 2, 16). Our study demonstrates that TNF is capable of eliciting IL-10 release, and that endogenous TNF has an important role in IL-10 secretion in endotoxemia. These data suggest that in endotoxemia TNF contributes to the production of an important antiinflammatory cytokine that at least, in part, functions to inhibit ongoing TNF synthesis.

T. van der Poll is a fellow of the Netherlands Organization of Scientific Research; S. J. H. van Deventer is a fellow of the Royal Dutch Academy of Arts and Sciences.

Address correspondence to Dr. Tom van der Poll, Cornell University Medical College, Laboratory of Surgical Metabolism, Room LC-705, 1300 York Avenue, New York, NY 10021.

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