Downregulation of Prolinflammatory Cytokine Release in Whole Blood From Septic Patients

By Wolfgang Ertel, Jean-Pierre Kremer, John Kenney, Ursula Stockholzer, Doraid Jarrar, Otmar Trenz, and Friedrich Wilhelm Schildberg

Using animal models or healthy volunteers, injection of lipopolysaccharide (LPS) or bacteria causes activation of macrophages with excessive synthesis and secretion of proinflammatory cytokines. Although these models mimic the effects of LPS in the host, they may represent more of an experimental expression of endotoxemia than natural infection itself. Therefore, as an ex vivo model of sepsis, whole blood from 15 patients with severe sepsis and 20 control patients without infection was stimulated with LPS to study the kinetics of mRNA expression and release of proinflammatory cytokines, tumor necrosis factor (TNF-α), interleukin (IL)-1β, and IL-6. Stimulation of whole blood with 1 μg/mL LPS resulted in a maximum increase of cytokine secretion in the control group, while a marked (P < .01) depression of TNF-α, IL-1β, and IL-6 release was observed in the septic group, which persisted up to 10 days after study enrollment.

**PROINFLAMMATORY cytokines** such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) have been implicated as principal mediators of cytokine production. The kinetics of proinflammatory cytokines in bacteria and endotoxemia are further support the pivotal role of these proinflammatory cytokines in bacteria and sepsis.

Experimental models fail to precisely reflect the pathophysiologic alterations that occur during natural infection, such as repeated contact of macrophages with endotoxin, as well as the downregulatory mechanisms of cytokine synthesis. Finally, because of the short half-life of TNF-α, IL-1β, and IL-6 with a rapid disappearance, measurements of cytokine blood levels in septic patients do not allow precise studies of cytokine cytokine synthesis and secretion in vivo. This may explain the discrepancies of previous reports describing the incidence and kinetics of cytokine blood levels in patients with severe sepsis.

Little is known about the capacity of peripheral blood mononuclear cells (PBMCs) to release proinflammatory cytokines under septic conditions, though PBMCs have a rapid and intense contact to bacteria and/or endotoxin. Therefore, it was the objective of this study to investigate the influence of natural infection on the capacity of PBMCs in whole blood to synthesize and secrete proinflammatory cytokines. To reduce the confounding factors associated with isolation of monocytes, such as adherence induced expression of cell-surface TNF or TNF-mRNA, LPS-stimulated whole blood as an ex vivo model of sepsis was used in this study. Although this ex vivo system cannot accurately depict inflammatory processes in the whole body, it provides an window to observe kinetics of cytokine mRNA expression, protein synthesis, and release by PBMCs after stimulation with LPS and may have considerable relevance with respect to local compartmentalized cytokine production.

While IL-1β mRNA expression was similar in peripheral blood mononuclear cells (PBMCs) harvested from LPS-stimulated whole blood in septic and control patients, the half-life and consequently the expression of TNF-α and IL-6 mRNA were strongly reduced in the septic group. These data indicate a downregulatory mechanism of cytokine release in whole blood from patients with severe sepsis that occurs on different levels. Although excessive secretion of proinflammatory cytokines has been considered deleterious for the host, the reduced capacity of PBMCs in whole blood from septic patients to synthesize and secrete proinflammatory cytokines to an inflammatory stimulus may result in immunodeficiency, because these cytokines in low concentrations are involved in the upregulation of essential cellular and humoral immune functions.

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**MATERIALS AND METHODS**

**Patient selection.** Patients eligible for this study were those with sepsis syndrome or septic shock. Sepsis syndrome was defined by fever or hypothermia (temperature >38.3°C or <35.6°C), tachycardia (>90 beats per minute in the absence of beta-blockade), and tachypnea (respiratory rate >20 breaths per minute or the requirement of mechanical ventilation) and by clinical signs of altered organ perfusion resulting in mental disorientation, oliguria, or elevated lactate levels. Septic shock was defined by clinical diagnosis of sepsis syndrome plus hypotension (systolic blood pressure below 90 mm Hg or a 40 mm Hg decrease below baseline systolic blood pressure) or the use ofvasopressor drugs to maintain blood pressure.

Fifteen patients who fulfilled these criteria were enrolled in this study. Of these, 8 patients met the criteria for sepsis syndrome and 7 for septic shock. Seven patients (47%) died during the observation period of 10 days because of sepsis induced multiple-organ failure. The source of infection included pneumonia (n = 7), peritonitis (n = 5), pleura empyema (n = 2), and osteomyelitis (n = 1). The isolated microorganisms were gram-negative bacilli in 6 cases, gram-
positive bacteria in 3 cases, mixed populations in 5 cases, and fungi in 1 case.

Control patients (n = 20) who were admitted to our hospital for hernia repair or cholecystectomy were comparable to the septic group with regard to age and sex.

Collection of blood. Blood from septic patients was collected on day of enrollment (D 0) and on day (D) 1, 2, 3, 5, 7, and 10 thereafter. Blood from control patients was obtained once preoperatively to exclude any influence of stress, anaesthesia, and surgical trauma.

Blood was drawn into heparinized syringes (20 U heparin/mL; heparin was tested for endotoxin: <5 pg endotoxin/mL heparin). Aliquots of 5 mL blood were placed in sterile polypropylene tubes (Falcon; Becton Dickinson, Lincoln Park, NJ). One sample was processed immediately as described below to serve as the baseline value. For each blood sample, total and differential white blood cell counts were obtained using a Coulter Counter (Coulter Corp, Hialeah, FL). The other blood samples were inoculated with 1 pg/mL LPS-concentrations of 1 pg/mL induces a 202% increase in oxygen levels within 1 hour incubation period. Oxygen levels increased by 202% without LPS were handled similarly. At 0, 0.5, 1, 2, 4, and 8 hours thereafter, density gradient centrifugation for separation of PBMCs and plasma was carried out at 4°C to minimize degradation of RNA during the 30-minute isolation procedure of PBMCs. The plots were hybridized with 32P-labeled probes as described above, and subjected to quantitative autoradiography by means of the FUJI digital imaging system (exposition on FUJI imaging plates and subsequent evaluation with a Fujix BAS 1000 Bio Imaging Analyzer: FUJI, Duesseldorf, Germany).

Transcript stability of TNF-α and IL-6 mRNA. To determine stability of cytokine mRNA, PBMCs from whole blood were isolated after a 24-hour incubation period with LPS (1 ng/mL) followed by inhibition of transcription using actinomycin D (5 μg/mL; Sigma Chemical Co, St Louis, MO). At different time points (0, 0.5, 1, 2, 4, and 8 hours) thereafter, density gradient centrifugation for separation of PBMCs and plasma was carried out at 4°C to minimize degradation of RNA during the 30-minute isolation procedure of PBMCs. The plots were hybridized with 32P-labeled probes as described above, and subjected to quantitative autoradiography by means of the FUJI digital imaging system (exposition on FUJI imaging plates and subsequent evaluation with a Fujix BAS 1000 Bio Imaging Analyzer: FUJI, Duesseldorf, Germany).

Statistics. Results are presented as mean ± SEM. Data were analyzed by the unpaired Wilcoxon rank sum test with Bonferroni correction for multiple comparisons. Differences were considered significant with P values less than 0.05.

RESULTS AND DISCUSSION

The kinetics of proinflammatory cytokine release into whole blood from septic patients were studied over a 24-hour incubation period after stimulation with a maximum dose of LPS (1 μg/mL) and a physiologically relevant LPS concentration (1 ng/mL). Although in control patients TNF-α, IL-1β, and IL-6 were not detectable at 0 hour of incubation, cytokine levels were slightly elevated in whole blood from septic patients (TNF-α: 1.3 ± 0.7 U/mL; range, 0 to 9 U/mL; IL-1β: 22 ± 16 pg/mL; range, 0 to 250 pg/mL; IL-6: 197 ± 84 U/mL; range, 0 to 1347 U/mL) at time point 0 hour (Table 1). Control studies without LPS did not show a spontaneous release of cytokines into whole blood in any of the two groups during the 24-hour incubation period (data not shown).

The kinetics of LPS-induced cytokine release in whole blood from control patients without infection are in line with previous reports by DeForge et al.31 (Table 1). However, the release of TNF-α, IL-1β, and IL-6 into LPS-stimulated whole blood obtained from patients with severe sepsis was significantly decreased compared with the control group. The
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Table 1. Kinetics of Proinflammatory Cytokine Release in Septic Patients

<table>
<thead>
<tr>
<th>Time (d)</th>
<th>Control</th>
<th>Sepsis</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0 ± 0</td>
<td>1.3 ± 0.7</td>
</tr>
<tr>
<td>1</td>
<td>98.1 ± 21.2</td>
<td>35.6 ± 11.3</td>
</tr>
<tr>
<td>2</td>
<td>246.5 ± 30.6</td>
<td>80.5 ± 24.5*</td>
</tr>
<tr>
<td>4</td>
<td>538.4 ± 69.0</td>
<td>101.0 ± 34.5*</td>
</tr>
<tr>
<td>8</td>
<td>535.9 ± 75.0</td>
<td>71.2 ± 23.1*</td>
</tr>
<tr>
<td>24</td>
<td>170.9 ± 31.6</td>
<td>1.0 ± 0.6*</td>
</tr>
</tbody>
</table>

Kinetics of TNF-α (U/mL per 1 × 10⁶ monocytes), IL-1β (ng/mL per 1 × 10⁶ monocytes), and IL-6 (U/mL per 1 × 10⁶ monocytes) release into whole blood obtained from septic patients (n = 15) on day of study enrollment and control patients (n = 20) after stimulation with LPS (1 μg/mL) for 0, 1, 2, 4, 8, and 24 hours.

* P < .05 sepsis versus control.
† P < .01 sepsis versus control.

Table 2. Cytokine Release in Septic Patients on Consecutive Days

<table>
<thead>
<tr>
<th>Time (d)</th>
<th>Control (n = 20)</th>
<th>Sepsis (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>535.9 ± 75.0</td>
<td>71.2 ± 23.1*</td>
</tr>
<tr>
<td>1</td>
<td>81.4 ± 24.3*</td>
<td>39.8 ± 8.6*</td>
</tr>
<tr>
<td>2</td>
<td>98.1 ± 42.4*</td>
<td>5.6 ± 1.7*</td>
</tr>
<tr>
<td>4</td>
<td>128.5 ± 39.0*</td>
<td>8.8 ± 2.4*</td>
</tr>
<tr>
<td>8</td>
<td>109.4 ± 37.2*</td>
<td>11.2 ± 2.6*</td>
</tr>
<tr>
<td>24</td>
<td>239.3 ± 108.9*</td>
<td>11.4 ± 0.7*</td>
</tr>
</tbody>
</table>

Release of TNF-α (U/mL per 1 × 10⁶ monocytes), IL-1β (ng/mL per 1 × 10⁶ monocytes), and IL-6 (U/mL per 1 × 10⁶ monocytes) into whole blood from septic patients (n = 15) after stimulation with LPS (1 μg/mL) for 8 hours. Blood from septic patients was obtained on consecutive days 0, 1, 2, 3, 5, 7, and 10 after diagnosis of sepsis, and compared with control patients (n = 20).

* P < .01 sepsis versus control.
† P < .05 sepsis versus control.

The marked reduction of cytokine release in the septic group persisted during the whole observation period of 10 days (Table 2). These data indicate that the reduced responsiveness of PBMCs to adequately synthesize and secrete proinflammatory cytokines to an endotoxin challenge represents a long-lasting phenomenon throughout manifest sepsis. Moreover, the observed significant inhibition of proinflammatory cytokine release into whole blood from septic patients was consistent, whether cytokine levels were normalized to 1 × 10⁶ leukocytes/mL, 1 × 10⁶ PBMCs/mL, or 1 × 10⁶ MO/mL. However, cytokine levels were normalized to 1 × 10⁶ MO/mL, because monocytes are the primary source of proinflammatory cytokines after stimulation of whole blood with LPS, and lymphocytes, as well as neutrophils, only produce low amounts of proinflammatory cytokines. Furthermore, this excludes the possibility that the reduction of cytokine release in the septic group was due to altered percentages of MO in the differential white blood cell counts when compared with the control group.

It could be argued that the observed phenomenon may be related to the unphysiologically high LPS concentration of 1 μg/mL, because Wright et al. suggested an unspecific activation of MO with consecutive cytokine release in human whole blood using LPS concentrations greater than 10 ng/mL. Therefore, additional studies were carried out using an LPS concentration of 1 ng/mL, as this dose reflects a physiologically relevant concentration detected in plasma from septic patients. Despite using concentrations of 1 ng/mL, the release of TNF-α, IL-1β, and IL-6 remained markedly decreased in the septic group compared with the control group (Table 3).

To gain further insight into the mechanisms of reduced cytokine synthesis in LPS-stimulated whole blood from septic patients, Northern blotting was performed. Although IL-1β mRNA expression was similar in both groups, TNF-α and IL-6 mRNA expression were decreased in PBMCs from septic patients compared with control patients (Fig 1). These results were validated by the fact that the total amount of RNA per lane was similar for each sample and group, as indicated by comparable intensity of the 28S rRNA lanes of the two groups. To get information on regulatory mechanisms possibly involved in the suppression of TNF-α and IL-6 mRNA expression, stability of TNF-α and IL-6 mRNA was assessed. After an initial 2-hour induction period of TNF-α and IL-6 synthesis in whole blood cells by LPS, further transcription was blocked by addition of actinomycin D. As determined by digital densitometric analysis of mRNA
Table 3. Proinflammatory Cytokine Release Into Blood Stimulated With Low-Dose LPS

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Control</th>
<th>Sepsis</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF (U/mL)</td>
<td>0</td>
<td>37</td>
</tr>
<tr>
<td>IL-1β (ng/mL)</td>
<td>37</td>
<td>192</td>
</tr>
<tr>
<td>IL-6 (U/mL)</td>
<td>1,590</td>
<td>11,309</td>
</tr>
</tbody>
</table>

Kinetics of TNF-α (U/mL per 1 x 10^6 monocytes), IL-1β (ng/mL per 1 x 10^6 monocytes), and IL-6 (U/mL per 1 x 10^6 monocytes) release into whole blood from two patients with sepsis syndrome and two control patients after stimulation with LPS (1 ng/mL) for 0, 1, 2, and 4 hours.

levels at different time points after addition of actinomycin D, stability of both TNF-α and IL-6 mRNA was markedly reduced in septic patients when compared with a control patient without infection (Fig 2). Therefore, reduced levels of TNF-α and IL-6 transcripts detected in PBMCs from patients with severe sepsis is caused, at least in part, by a reduction of mRNA half-life. However, this does not exclude a further depression of transcriptional activity of the genes involved in reduced mRNA expression in patients with severe sepsis.

These results suggest that the defect of cytokine synthesis in whole blood from patients with severe sepsis occurs on different levels. Although we did not perform nuclear run-on studies to precisely document transcript regulation, TNF-α and IL-6 synthesis seems to be inhibited on a transcriptional level, because the half-life of TNF-α and IL-6 mRNA in PBMCs from septic patients is markedly reduced. In contrast, the significant inhibition of IL-1β secretion may be due to reduced synthesis of the IL-1β protein rather than defects in the transcription process, as the expression and the half-life (data not shown) of IL-1β mRNA were similar in both groups.

The downregulatory mechanisms of decreased cytokine release in whole blood from septic patients remain unclear. There is evidence from in vitro and in vivo studies\(^{54,55}\) that following stimulation with endotoxin, regulation of IL-1 and TNF-α synthesis and release by PBMCs may be different, which is in line with our results. Potential mediators that are effective in suppressing IL-1 protein synthesis without influencing IL-1 mRNA accumulation are PGE\(_2\) and dexamethasone.\(^{52,54}\) Prostaglandin E\(_2\), as well as corticosteroids, are increased during endotoxemia.\(^{55,56}\) In addition, both anti-inflammatory substances inhibit mRNA expression of TNF-α.\(^{57,58}\) Moreover, antiinflammatory mediators (IL-4,\(^{49}\) IL-10,\(^{50}\) IL-13,\(^{51}\) or TGF-β1\(^{52}\)) may be involved in the suppression of proinflammatory cytokine release by PBMCs during sepsis. Measurements of IL-4 secretion into whole blood excluded an inhibitory influence of IL-4 on proinflammatory cytokine release, because stimulation of whole blood with LPS did not induce synthesis and secretion of IL-4 in any of the two groups (Ertel et al, manuscript in preparation). Though levels of soluble TNF-receptors are increased in blood obtained from patients with severe sepsis,\(^{53}\) they may not be responsible for the reduced release of TNF-α into whole blood from septic patients. Neither, are they effective intracellularly, nor, are they able to neutralize IL-1β or IL-6. However, the profile of decreased cytokine synthesis and release on different levels observed in whole blood from septic patients is similar to observations made when TGF-β1 was added to LPS-stimulated whole blood from control patients.\(^{54}\) This hypothesis is supported by the fact that sepsis results in a significant increase of TGF-β1 serum levels.\(^{55}\) It seems unlikely that the observed phenomena are due to decreased expression of the LPS binding receptor CD14, reduced opsonization of LPS, or inhibition of LPS binding to receptors on monocytes/macrophages, be-
cause IL-1β mRNA expression in PBMCs isolated from septic patients was similar to that found in control patients. This suggestion is supported by studies indicating that desensitization of macrophages is not caused by downregulation of LPS-binding sites.66

Our data suggest a reduced responsiveness of PBMCs to a maximum and a physiologically relevant endotoxin stimulus. This phenomenon, referred to as endotoxin tolerance,8 seems to be due to a reduced capacity of white blood cells to synthesize and/or secrete proinflammatory cytokines. Our data are in line with previous studies by Granowitz et al67 and Rodrick et al.68 These investigators described a suppression of proinflammatory cytokine release in PBMCs isolated from endotoxin-injected human volunteers. Granowitz et al67 hypothesized that the phenomenon of endotoxin tolerance may be due to a defect in transcriptional and/or translational regulation. Our data demonstrating the reduced responsiveness of blood monocytes from septic patients to an adequate endotoxin challenge do not only confirm the clinical relevance of their results in endotoxin-injected human volunteers, but also elucidate the reduction of mRNA half-life in PBMCs from septic patients as one of the underlying mechanisms.

Although cytokines released in high amounts cause tissue damage and multiple-organ failure,79,80 TNF-α, IL-1β, and IL-6 stimulate and regulate a wide array of immune functions,31-36 thus playing an important role in the host defense system. Moreover, previous investigations suggest that TNF-α mediated immune responses may be important in the host defense against parasitic infections64,65 and that injection of IL-1α protects animals from lethal infection by intracellular bacteria.56 In addition, recombinant TNF-α attenuated the lethal effect of gram-negative sepsis induced by cecal ligation and puncture (CLP),67 and studies by Cross et al68 described a protective effect of TNF-α in combination with IL-1α in a bacteremia mouse model. These reports were confirmed by studies from Alexander et al69 who demonstrated that hypotension after a lethal infusion of E. coli LPS was completely prevented and tissue injury substantially ameliorated by pretreatment with recombinant TNF-α. These results indicate that a controlled immune response in the host to endotoxin mediated by TNF-α and IL-1β may represent a potent protective mechanism against infection with invasive microorganisms causing septic shock in humans. In this light, the reduced capability of PBMCs in whole blood from septic patients to produce and release adequate amounts of proinflammatory cytokines after exposure to endotoxin may indicate the inability of septic patients to adequately respond to repeated or persisting invasion of microorganisms and to maintain an effective defense system.

It is our hypothesis that two essential mechanisms (systemic inflammation and an effectively functioning immune system) of the host to eliminate invading microorganisms are compromised during clinical sepsis in different ways dependent on the timepoint of macrophage activation. Although the downregulation of excessive proinflammatory cytokine synthesis and release in the early period after contact with microorganisms may be an evolutionary process to reduce the incidence of tissue necrosis and consequence multiple organ failure through overwhelming cytokinemia, an almost complete inhibition of proinflammatory cytokine synthesis and release in the late period may lead to immunodeficiency in patients with severe sepsis. Therefore, only the balanced synthesis and release of proinflammatory cytokines may be beneficial for the host.

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Downregulation of proinflammatory cytokine release in whole blood from septic patients

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