Lymph of Patients with a Systemic Inflammatory Response Syndrome Inhibits Lipopolysaccharide-Induced Cytokine Production

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In patients with systemic inflammatory response syndrome (SIRS), tolerance of peripheral blood mononuclear cells to a second challenge with lipopolysaccharide (LPS) has been described. Thoracic duct lymph transports LPS and represents the extravascular, interstitial fluid compartment of the body. The aim of this study was to determine the capacity of lymph to influence LPS-induced cytokine production in vitro. Thoracic duct lymph was obtained from patients with SIRS and without SIRS (controls). The effect of lymph and simultaneously collected plasma on LPS-induced cytokine production by normal peripheral blood mononuclear cells was assessed. Both lymph and plasma of patients with SIRS reduced LPS-induced tumor necrosis factor-α and interleukin-6 production (P < .01); lymph of controls also inhibited cytokine production (P < .01), although to a lesser extent. This study suggests that LPS tolerance may occur both in the intra- and extravascular compartments.

Lipopolysaccharide (LPS) tolerance is characterized by down-regulation of the production of proinflammatory cytokines (tumor necrosis factor-α [TNF-α], interleukin [IL]-6) but with an enhanced synthesis of IL-1 receptor antagonist upon ex vivo restimulation of mononuclear cells with LPS [1, 2]. This phenomenon has been found in patients with systemic inflammatory response syndrome (SIRS) and in healthy humans injected with low-dose LPS [1, 2]. Although the precise mechanisms of LPS tolerance are unclear, it is caused at least in part by soluble mediators in the circulation, since serum of septic patients and endotoxemic volunteers partly reproduced the LPS-tolerant state in normal whole blood [2, 3]. Besides plasma, lymph may be an important body compartment in LPS transport, as LPS is present in thoracic duct lymph from patients with SIRS [4]. Moreover, thoracic duct lymph represents the extravascular, interstitial body compartment [5] and therefore may reflect processes at tissue level. The effect of lymph on LPS-induced cytokine production is unknown.

Several circulating factors known to modulate the toxicity of LPS might be involved in LPS tolerance. These are substances that either facilitate LPS-induced activation of cells, such as LPS-binding protein (LBP) [6], or neutralize LPS, such as bactericidal/permeability-increasing protein (BPI) [7]. In addition, the antiinflammatory cytokine IL-10 is considered to antagonize LPS-induced toxicity by deactivation of monocytes [8]. Increased levels of these LPS-modulating substances have been detected in plasma of patients with sepsis [9, 10].

In this study, we sought to determine the capacity of lymph to influence LPS-induced cytokine production. We therefore obtained thoracic duct lymph of patients with and without SIRS and assessed the effect of lymph and simultaneously collected plasma on cytokine production by normal peripheral blood mononuclear cells (PBMC) stimulated with LPS. Further, to obtain insight into the potential role of LBP, BPI, and IL-10, the concentrations of these substances were measured in lymph and plasma.

Patients and Methods

Plasma and thoracic duct lymph collection. Peripheral blood plasma and thoracic duct lymph were obtained from 8 patients with SIRS (6 men, 2 women, age [mean ± SE] 62 ± 5 years) and from 7 patients without SIRS (4 men, 3 women, age 64 ± 2 years). Patients with SIRS, admitted to the intensive care unit of the Academic Medical Center, Amsterdam, fulfilled the SIRS criteria [11] and had organ failure of at least two organ(s) (systems). In these patients, the thoracic duct was cannulated with a 14-gauge double-lumen catheter (Ohmeda, Swindon, UK) in the neck [4]. Lymph was obtained directly after the catheter was inserted into the thoracic duct.

In patients without SIRS, undergoing transthoracic resection including the thoracic duct for carcinoma of the esophagus or gastroesophageal junction, the thoracic duct was cannulated with a 14-gauge single-lumen catheter (Ohmeda) approximately at the level of the fifth thoracic vertebra. From both patient groups, an arterial blood sample was drawn at the time of lymph sampling. Blood was collected in sterile 4.5-mL tubes containing 0.048 mL of EDTA-K3 (Vacutainer Systems; Becton-Dickinson, Rutherford, NJ). Lymph was collected in pyrogen-free plastic tubes (Sarstedt, Nümbrecht, Germany) containing pyrogen-free heparin (Tromboli- quine; Organon, Oss, The Netherlands; final concentration 50 IU/mL). Following centrifugation (1600 g, 20 min), lymph superna-
tant and plasma were aliquoted and stored at −80°C until further processing.

**PBMC isolation.** Blood was obtained aseptically from 12 healthy male volunteers (age 33 ± 2 years). Blood from each volunteer was transferred immediately into pyrogen-free tubes that contained pyrogen-free heparin. Blood was diluted 1:1 in Hank's Buffered Salt Solution (HBSS; BioWhittaker, Verviers, Belgium) and subsequently PBMC of each volunteer were isolated by centrifugation over a density gradient (Lymphopaque Ficoll Paque; Pharmacia, Woerden, The Netherlands) at room temperature for 15 min at 600 g. Cells in the interphase were collected, washed twice and thereafter brought to a concentration of 1 × 10⁶ PBMC/mL in HBSS containing 10% sterile nonacute human serum (Central Laboratory of The Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam) [12].

**Experimental design.** Lymph of patients with SIRS was pooled, as was lymph of the patients without SIRS; equal amounts of lymph from each patient were used. The lymph pools were brought to final concentrations of 10% (vol%) in RPMI 1640 (BioWhittaker). Then, 0% lymph (RPMI 1640 only), 10% SIRS lymph, or 10% non-SIRS lymph was preincubated in the absence of LPS or in the presence of 1 or 10 ng/mL LPS (final concentrations) (Escherichia coli O111:B4; Sigma, St. Louis; 1 ng ≈ 12 endotoxin units) for 24 h in a CO₂ incubator at 37°C. Thereafter, PBMC (final concentration 0.5 × 10⁶/mL) of each healthy volunteer were incubated with the different lymph-LPS suspensions for 4 and 24 h in a CO₂ incubator at 37°C for measurement of TNF-α and IL-6, respectively. These durations of incubation were chosen after preliminary experiments had established that the concentrations of TNF-α and IL-6 peaked at these time points (data not shown).

After centrifugation at 2000 g for 30 min at 4°C, supernatants were aliquoted and stored at −80°C until assays were performed. An identical protocol was used to determine LPS-neutralizing capacities of 10% plasma of patients with SIRS, 10% plasma of patients without SIRS, 10% plasma of 10 healthy volunteers (10 women, age 29 ± 2 years), TNF-α (Medgenix, Fleurus, Belgium), IL-6 (PharMingen, San Diego), and IL-10 (PharMingen) were determined by ELISA according to the instructions of the manufacturer. LBP and BPI concentrations were determined using specific ELISAs as described [9, 13]. The lower levels of detection were 7 pg/mL (TNF-α), 14 pg/mL (IL-6), 8 pg/mL (IL-10), 100 pg/mL (LBP), and 200 pg/mL (BPI).

**Statistical analysis.** All values are expressed as mean ± SE. Data were compared by paired and unpaired Wilcoxon tests as appropriate. P < .05 was considered significant.

**Results**

**Incubation without LPS.** Incubation of normal PBMC without LPS only resulted in detectable levels of TNF-α and IL-6 in the presence of lymph or plasma of patients with SIRS. In these samples, TNF-α levels were 39 ± 15 pg/mL (incubation with lymph) and 10 ± 7 pg/mL (incubation with plasma); IL-6 concentrations were 1774 ± 88 and 502 ± 43 pg/mL, respectively. Therefore, the ex vivo production of TNF-α and IL-6 was calculated as the difference between cytokine concentrations found after incubation with LPS and those found after incubation without LPS.

**Effect of lymph.** Lymph of patients with SIRS and controls (patients without SIRS) reduced TNF-α and IL-6 production after stimulation with 1 ng/mL LPS (P < .01 vs. LPS only; figure 1). At 10 ng/mL LPS, IL-6 production was significantly inhibited only by SIRS lymph (P < .01 vs. LPS only; figure 1). SIRS lymph was more potent than lymph of controls in inhibiting TNF-α production elicited by 1 ng/mL LPS (P < .05) and in inhibiting both TNF-α and IL-6 production elicited by 10 ng/mL LPS (P < .05; figure 1).

**Comparison of lymph and plasma.** Plasma obtained from healthy volunteers inhibited TNF-α and IL-6 production (P < .05 vs. LPS only, except for IL-6 release at 10 ng/mL LPS; figure 1), confirming a previous report [2]. The extent of inhibition by healthy plasma was less (P < .05) compared with the inhibition by SIRS plasma and non-SIRS plasma (P < .01 vs. LPS only; figure 1). TNF-α and IL-6 release induced by 10 ng/mL LPS was inhibited more by SIRS plasma than by non-SIRS plasma (P < .05; figure 1). SIRS plasma was more potent in reducing cytokine production than SIRS lymph (P < .03), as was non-SIRS plasma compared with non-SIRS lymph (P < .03; figure 1).

**LBP, BPI, and IL-10.** Lymph of patients with SIRS contained higher concentrations of LBP and IL-10 than lymph of patients without SIRS (P < .03), while BPI levels were below the detection limit in lymph of both SIRS and non-SIRS patients (table 1). Plasma of patients with SIRS contained higher concentrations of LBP and BPI than plasma of patients without SIRS (P < .02; table 1), while IL-10 concentrations were not different between the 2 groups. LBP levels were higher in SIRS plasma than in SIRS lymph (P < .03). IL-10 concentrations were higher in SIRS lymph than in SIRS plasma (P < .02).

**Discussion**

The present findings demonstrate that lymph of patients with SIRS can partially reproduce LPS tolerance, possibly indicating that this phenomenon also occurs in the extravascular compartment.

In patients with SIRS, the body may combat LPS toxicity by reducing the capacity of mononuclear cells to produce proinflammatory cytokines upon restimulation with LPS. It has been shown that soluble mediators are involved, since serum of septic patients and endotoxemic volunteers partially reproduced the LPS-tolerant state in normal whole blood [2, 3]. It has not previously been studied whether, in the interstitial fluid, an LPS-tolerant state is present, presumably reflecting processes at tissue-level. Therefore, we aimed to investigate the capacity of thoracic duct lymph from patients with SIRS to influence LPS-induced cytokine production, since thoracic duct lymph has been shown to represent the interstitial fluid compartment [5].

Patients without SIRS, undergoing a transthoracic resection of the esophagus for a carcinoma of the esophagus, were used as controls. It is possible that these patients have characteristics that differ from healthy individuals. However, we assume that
Figure 1. Mean (± SE) tumor necrosis factor-α (TNF-α) and interleukin (IL)-6 concentrations: 10% lymph and plasma of patients with SIRS, 10% lymph and plasma of patients without SIRS, and 10% plasma of healthy volunteers were preincubated with lipopolysaccharide (LPS) for 24 h. Then peripheral blood mononuclear cells of 12 healthy volunteers were incubated with different lymph-LPS or plasma-LPS suspensions for 4 h (TNF-α measurements) and 24 h (IL-6 measurements). For results of statistical testing, see text.

Table 1. Concentrations (mean ± SE) of lipopolysaccharide-binding protein (LBP), bactericidal/permeability-increasing protein (BPI), and interleukin-10 (IL-10) in lymph and plasma of patients with and without SIRS.

<table>
<thead>
<tr>
<th>Patients with SIRS (n = 8)</th>
<th>Patients without SIRS (n = 7)</th>
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<tbody>
<tr>
<td>Lymph</td>
<td>Plasma</td>
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<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>LBP (μg/mL)</td>
<td>BPI (pg/mL)</td>
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<tr>
<td>35 ± 10*</td>
<td>&lt;200</td>
</tr>
<tr>
<td>48 ± 9†</td>
<td>924 ± 198†</td>
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<tr>
<td>Lymph</td>
<td>Plasma</td>
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<tr>
<td>11 ± 6</td>
<td>8 ± 2</td>
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<tr>
<td>371 ± 109</td>
<td>261 ± 110</td>
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</table>

* P < .03 vs. lymph of patients without SIRS.
† P < .02 vs. plasma of patients without SIRS.
‡ P < .02 vs. plasma of patients with SIRS.

Indeed, lymph of patients with SIRS inhibited LPS-induced TNF-α and IL-6 production by normal PBMC. This was also found for plasma of patients with SIRS, as described previously [3]. Lymph and plasma of patients without SIRS, undergoing major surgery, also inhibited the LPS-induced proinflammatory cytokine release, although to a lesser extent. These findings are in line with a recent report indicating that major surgery itself can induce an LPS-tolerant state [14].

To obtain insight into the possible roles of LBP, BPI, and IL-10, circulating factors known to modulate LPS toxicity, concentrations of these substances were measured in lymph and plasma of patients with and without SIRS. The increased lymph concentration of IL-10 and the increased plasma concentration of BPI in patients with SIRS may explain why SIRS

these patients closely approximate the condition of healthy volunteers, the ideal control group.
lymph and SIRS plasma inhibited cytokine release more strongly than did lymph and plasma of patients without SIRS, respectively.

Many other substances may bind LPS (e.g., antibodies, complement, albumin) or deactivate mononuclear cells. For example, lipoproteins are known to bind and neutralize LPS [12, 15]. Concentrations of apolipoproteins A-1 and B were significantly lower in lymph and plasma of patients with SIRS compared with concentrations in lymph and plasma of patients without SIRS (data not shown). Lipoprotein concentrations can therefore not explain the more potent inhibition of proinflammatory cytokine production by SIRS lymph or SIRS plasma compared with non-SIRS lymph or non-SIRS plasma, respectively. The present investigation does not elucidate which other mediators might be involved. It thus seems that alterations in LBP concentrations do not contribute significantly to the development of LPS tolerance.

LPS tolerance is associated with a reduced capacity of mononuclear cells to produce cytokines upon stimulation with LPS. Here we show that lymph obtained from patients with SIRS can reproduce an LPS-tolerant state when added to cultures of normal PBMC. These data suggest that soluble mediators present in lymph (and plasma) are responsible, at least in part, for the phenomenon of LPS tolerance.

References