Cytokines and chemokines in systemic and urinary tract infection by Escherichia coli
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Chapter 4

Sequential measurements of CC chemokines in urosepsis and experimental endotoxemia.

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Abstract

Background: Chemokines are a superfamily of small chemotactic proteins. While increased levels of interleukin-8 have been measured in serum and urine during urinary tract infection, little is known about other chemokines in this condition. Methods: Monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, MIP-1β and interferon-γ inducible protein (IP)-10 were measured in 30 patients with culture-proven urosepsis during a three-day follow-up, and in 11 healthy humans after intravenous injection of endotoxin (4ng/kg). Results: Urine and serum levels of MCP-1, MIP-1β and IP-10, but not of MIP-1α, were elevated in patients on admission, and decreased after initiation of antibiotic treatment. Endotoxin administration to healthy subjects induced increases in plasma and urine concentrations of all four chemokines. Conclusion: These data indicate that clinical and experimental gram-negative infection in humans is associated with enhanced production of chemokines that mainly act on mononuclear cells and that these chemokines are at least in part locally produced.
Introduction

Chemokines play an important role in adhesion of inflammatory cells to the endothelium and their migration to the site of infection [1]. They may further contribute to the inflammatory response by activation of leukocytes through induction of oxygen burst and degranulation [2]. Depending on their structure, chemokines can be divided into distinct families. Monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α and MIP-1β belong to the CC chemokine family and act primarily on monocytes and macrophages. Interferon γ inducible Protein (IP) -10 is a member of CXC chemokine family that acts on activated T cells and monocytes [1]. Increased serum levels of MCP-1 and MIP-1α have been found in patients with sepsis [3, 4].

The urinary tract is a common source of gram-negative sepsis [5], with Escherichia coli being the most frequently isolated pathogen [6]. Urosepsis is usually diagnosed on the basis of a positive urine culture and clinical findings associated with sepsis, including chills, fever and hypotension. While increased urinary and serum interleukin (IL)-6 and IL-8 levels have been widely reported in urinary tract infection [7-13], little is known about the production of chemokines other than IL-8 in this condition.

Although some studies have demonstrated elevated CC chemokine concentrations in the circulation of patients with sepsis [3,4], knowledge of local production of these mediators is limited. Patients with urosepsis, who by definition have a documented infectious source in their urinary tract, potentially provide the opportunity to obtain insight in the extent of chemokine production at the site of the infection, namely by measurements in urine. Therefore, in the present study we sequentially measured serum and urine concentrations of MCP-1, MIP-1α, MIP-1β and IP-10 in patients with urosepsis during a three-day follow up period. In addition, we also measured chemokine serum and urine concentrations in healthy humans intravenously injected with endotoxin (i.e. without a local inflammatory stimulus in the urinary tract).
Materials and Methods

Patients with urosepsis and controls. Thirty patients (mean age ± SD: 67 ± 17 years) with culture proven gram-negative urosepsis were studied. The diagnosis of urosepsis was based on the presence of a urine culture positive for a gram-negative micro-organism with pyuria (leukocytes > 10/hpf with few epithelial cells); and metabolic or hematologic signs of systemic infection including two of the following six: tachycardia (>90 beats/min), hypotension (systolic blood pressure < 90 mm Hg), hypoxemia (pO₂ ≤ 75 mm Hg), leukocytosis (>10,000/mm³), abnormal prothrombin time or activated partial thromboplastin time or thrombocytopenia (<100,000/mm³), or acute mental status change. Exclusion criteria were antibiotic use in the previous 72 hours, a very poor clinical condition, severe renal insufficiency (estimated creatinin clearance < 30 mL/min) or pregnancy. Further details of the study have been published elsewhere [7]. Patients were treated with intravenous imipenem 500 mg or intravenous ceftazidime 1000 mg every 8 hours for the first 72 hours. Since the type of antibiotic regimen (imipenem vs. ceftazidime) did not significantly influence chemokine levels, data from the two treatment groups were combined. Clinical data were collected and the APACHE II score was assessed before and during the therapy. Blood and urine were collected before the start of treatment (0 hours) and at 4, 24, 48 and 72 hours thereafter. The samples were centrifuged at 1500 g for 20 min. Supernatants were collected and stored at -20 °C until assays were performed. Serum and urine were also collected from 20 healthy individuals, all of whom had sterile urine.

Experimental endotoxemia. In addition to the patients with urosepsis, 11 healthy subjects (mean age ± SE: 24 ± 1 years) were studied after intravenous administration of endotoxin (lipopolysaccharide, LPS). The subjects did not smoke, use any medication or have a febrile illness in the month preceding the study. They were admitted to the clinical research unit at the Academic Medical Center after their medical history, physical examination, hematological and biochemical tests, chest X-ray and ECG had proved normal. Endotoxin (LPS standard lot G from E.coli; the United States Pharmacopeia Convention Inc., Rockville, MD) was given over one minute in an ante-cubital vein, at a dose of 4 ng/kg of body weight. Blood was collected by venipunctures directly before LPS administration and
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0.5, 1.0, 1.5, 2.0, 3.4, 5.6, 8.8 and 12 hours thereafter. EDTA plasma was obtained by centrifuging at 1500 g for 20 min. All the urine produced by the subjects was collected before, and 3 and 6 hours after LPS administration.

Assays. Chemokine concentrations were measured by ELISA. MCP-1 was measured using purified monoclonal mouse anti-human MCP-1 (2 μg/ml; PharMingen, San Diego, CA) as coating antibody, biotinylated rabbit anti-human MCP-1 (1 μg/ml; PharMingen) as detecting antibody and human recombinant MCP-1 (PharMingen) as standard. For determination of MIP-1α and MIP-1β levels, purified monoclonal mouse anti-human MIP-1α and anti-human MIP-1β as coating antibodies were used (4 μg/ml; R&D Systems, Abingdon, United Kingdom), biotinylated affinity purified goat IgG anti-human MIP-1α and MIP-1β as detecting antibodies (20 ng/ml; R&D Systems) and recombinant human MIP-1α and MIP-1β as standards (R&D Systems). For determination of IP-10, purified monoclonal mouse anti-human IP-10 (4 μg/ml; R&D Systems) was used as coating antibody, biotinylated goat anti-human IP-10 (50 ng/ml; R&D Systems) as detecting antibody and recombinant human IP-10 (R&D Systems) as standard. Detection limits of the assays in the diluted serum and urine samples were: 16.4 pg/ml (MCP-1; 1:2), 78.0 pg/ml (MIP-1α and MIP-1β; both 1:5) and 120 pg/ml (IP-10; 1:2).

Statistical analysis. Data are given as median and range unless stated otherwise. Log transformed concentrations of chemokines were analyzed in time by one-way analysis of variance (ANOVA), followed by Dunnett’s test in both uroseptic patients and healthy subjects given endotoxin. Comparisons of levels in patients on admission (0 hours) and in healthy controls, and of concentrations in patients with positive and negative blood cultures, were done by Mann-Whitney U test. Correlations were calculated using Pearson correlation coefficient. α for all tests was set at .05.

When cytokine levels in urine were normalized for urinary creatinin concentrations, analysis of differences between patients and controls, and effect of treatment yielded similar results (data not shown).

The study was approved by the institutional scientific and ethics committees. Written informed consent was obtained from all patients and healthy subjects.
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**Results**

*Healthy subjects.* MCP-1 was detectable in serum (0.09 (0.02-0.67)) ng/ml and urine (0.13 (0.01-0.81)) ng/ml from all 20 healthy individuals. MIP-1α, MIP-1β and IP-10 were not detectable in any of the normal serum or urine samples.

*Patients with urosepsis.* Patient characteristics and cultured microorganisms have been reported previously [7]. *Escherichia coli* was cultured from the urine of 28 patients (93 per cent). 10 patients had blood cultures positive for a microorganism that was also cultured from urine (9 *Escherichia coli*, 1 *Pseudomonas aeruginosa*). On admission, the median APACHE II score was 11 (range 2 - 20). All patients fully recovered after treatment. As shown in Figure 1, both serum and urine levels of MCP-1 were higher in patients with urosepsis on admission (0.59 (0.19-12.00) and 1.07 (<0.02-10.55) ng/ml respectively) than in healthy controls (both P < .001). Both serum and urine MCP-1 concentrations decreased after initiation of the antibiotic therapy (serum: P < .01; urine: P < .001). Furthermore, serum and urine MCP-1 levels were higher in patients with positive blood cultures than in patients with negative blood cultures (P < .01 and P < .05 respectively) (Table). MIP-1α was detectable in serum and

![MCP-1 in serum](image)

![MCP-1 in urine](image)

**Figure 1.** Serum (left panel) and urine (right panel) levels of MCP-1 in healthy subjects and patients with urosepsis on admission and 4, 24, 48 and 72 hours after initiation of antibiotic therapy. Horizontal lines represent median. Patients had higher serum and urine levels than controls (both P < .001). Asterisks represent significant change in concentrations when compared with levels on admission. Dotted line represents detection limit of the assay.
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urine from only three patients (nonsignificant versus healthy controls; data not shown). Serum and urine levels of MIP-1β were higher in patients (0.14 (<0.08-13.37) and <0.08 (<0.08-0.77) ng/ml respectively) than in healthy individuals (both P < .001) (Figure 2). MIP-1β decreased during therapy in serum and urine, but only the decrease in urine reached statistical significance (P < .001). MIP-1β levels were higher in serum, but not in urine, from patients with positive blood cultures than from patients whose blood cultures were negative (P = .002) (Table). Although IP-10 was significantly elevated in serum (0.23 (<0.12-4.73) ng/ml) (P < .001) and urine (<0.12 (<0.12-1.21) ng/ml) (P = .002) from patients compared to controls (Figure 3), IP-10 was not detectable in urine from 18 of 30 patients (60 per cent). While IP-10 levels in serum did not change during follow-up, urine levels decreased significantly (P < .001).

Both MCP-1 and MIP-1β showed a significant correlation between the serum and urine levels measured on admission (Pearson coefficient = 0.42, P <.05; Pearson coefficient = 0.39, P <.05 respectively). Of all four chemokines measured in serum and urine, only the serum and urine levels of MCP-1 measured on admission showed a significant correlation with the APACHE II score (Pearson coefficient = 0.41, P < .05; Pearson coefficient = 0.39, P < .05 respectively).

Table. Concentrations of MCP-1, MIP-1β and IP-10 in serum and urine of patients with urosepsis. Comparison of patients with positive and negative blood cultures.

<table>
<thead>
<tr>
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<th>Positive blood culture</th>
<th>Negative blood culture</th>
<th>Difference between the two groups</th>
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<tbody>
<tr>
<td></td>
<td>N = 10</td>
<td>N = 20</td>
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<tr>
<td>MCP-1 (ng/ml)</td>
<td>Serum 2.60 (0.40-12.00)</td>
<td>0.51 (0.19-4.15)</td>
<td>P &lt; .01</td>
</tr>
<tr>
<td></td>
<td>Urine 1.37 (0.33-10.55)</td>
<td>0.97 (0.02-5.05)</td>
<td>P &lt; .05</td>
</tr>
<tr>
<td>MIP-1β (ng/ml)</td>
<td>Serum 0.38 (0.12-13.37)</td>
<td>0.11 (&lt;0.08-6.27)</td>
<td>P &lt; .005</td>
</tr>
<tr>
<td></td>
<td>Urine 0.08 (&lt;0.08-0.67)</td>
<td>0.09 (&lt;0.08-0.77)</td>
<td>N.S.</td>
</tr>
<tr>
<td>IP-10 (ng/ml)</td>
<td>Serum 0.39 (&lt;0.12-4.73)</td>
<td>&lt;0.12 (&lt;0.12-2.40)</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>Urine &lt;0.12 (&lt;0.12-1.21)</td>
<td>&lt;0.12 (&lt;0.12-0.67)</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

Data represent median (range) values.
N.S. = nonsignificant
Fig 2. Serum (upper panel) and urine (lower panel) levels of MIP-1β in healthy subjects and patients with urosepsis on admission and 4, 24, 48 and 72 hours after initiation of antibiotic therapy. Horizontal lines represent median. Patients had higher serum and urine levels than the controls (both P < .001). Asterisks represent significant change in concentrations when compared with levels on admission. Dotted line represents detection limit of the assay.

Fig 3. Serum (upper panel) and urine (lower panel) levels of IP-10 in healthy subjects and patients with urosepsis on admission and 4, 24, 48 and 72 hours after initiation of antibiotic therapy. Horizontal lines represent median. Patients had higher serum and urine levels than the controls (serum P < .001, urine P = .002). Asterisks represent significant change in concentrations when compared with levels on admission. Dotted line represents detection limit of the assay.

Endotoxemia in healthy humans. Intravenous injection of E. coli LPS was associated with transient increases in the plasma and urine concentrations of all four chemokines measured. LPS induced a rise in plasma MCP-1 levels starting at 1.5 hours and peaking at 4 hours (mean ±
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MCP-1 concentrations remained significantly elevated until 12 hours after LPS administration. Urine MCP-1 started to increase within 3 hours after LPS administration and continued throughout the 6 hours follow up (mean ± SE: 2.60 ± 0.71 ng/ml; P < .001). Plasma levels of MIP-1α increased, starting 1.0 hour after LPS injection and reached peak values at 1.5 hours (mean ± SE: 2.44 ± 0.46 ng/ml; P = .001) (Figure 4). After 4 hours they were no longer significantly elevated. Urine MIP-1α increased, albeit not significantly reaching its peak values between 3 and 6 hours after LPS (mean ± SE: 0.02 ± 0.0001 ng/ml; N.S.). Plasma levels of MIP-1β increased from 1.0

Fig 4. Mean ± SE plasma and urine concentrations of MCP-1, MIP-1α, MIP-1β and IP-10 in healthy subjects after administration of endotoxin. Serum concentrations were measured prior to endotoxin injection (t0) and during a follow up of 12 hours. Urine concentrations were measured prior to endotoxin injection (t0) and in all urine excreted within 3 hours after infusion of endotoxin (t0-3) and between 3 and 6 hours after endotoxin administration (t3-6). Asterisks indicate significant difference from the levels at t=0.
hour onwards, reaching peak concentrations at 2 hours (mean ± SE: 10.08 ± 0.46 ng/ml; P < .001) (Figure 4). It stayed significantly elevated until 12 hours after LPS administration. Its urine levels increased within 3 hours after LPS administration and continued to do so throughout the 6-hour follow-up (mean ± SE: 0.11 ± 0.03 ng/ml; P < .001). Plasma IP-10 increased from 3 hours onwards after LPS administration, peaking at 5 hours (mean ± SE: 13.11± 1.22 ng/ml; P < .001) (Figure 4) and stayed elevated until 12 hours after LPS administration. Urine IP-10 increased only between 3 and 6 hours after LPS administration (mean ± SE: 1.32 ± 0.34 ng/ml; P < .001)

No leukocytes could be found in the urine from any of the subjects at any time point.

**Discussion**

Chemokines are 8-10 kDa proteins that selectively target and activate different leukocyte subpopulations. They can be produced by a variety of immune and nonimmune cells in response to many different stimuli [1]. In the present study, we measured MCP-1, MIP-1α, MIP-1β and IP-10 in serum and urine from patients with culture-proven urosepsis and in plasma and urine from healthy subjects after administration of LPS derived from *E. coli*, the micro-organism that was responsible for 28 of the 30 cases of urosepsis studied. We found that MCP-1, MIP-1β and IP-10, but not MIP-1α, were elevated in serum and urine from patients, and that intravenous injection of LPS induced transient increases in the plasma and urine concentrations of all four chemokines.

Chemokines are divided into families based on the relative position of their cysteine residues [1]. MCP-1, MIP-1α and MIP-1β are CC chemokines, referring to the fact that their first two cysteine residues are adjacent to each other. In CXC chemokines, such as IP-10, the first two cysteine residues are separated by a single amino acid. Unlike most CXC chemokines, IP-10 does not act on neutrophils due to the absence of the sequence glutamic acid – leucine – arginine preceding the CXC sequence [1]. Hence, all chemokines measured in the present study exert their main effects on monocytes and lymphocytes.
Our finding of elevated serum concentrations of these chemokines in patients with urosepsis extends earlier studies in more heterogeneous patient populations with clinically defined sepsis syndrome. In such patients MCP-1 was found elevated in 57 per cent [4] and MIP-1α in 45 per cent on admission [3]. In our patients, all with documented gram-negative infection originating from the urinary tract, MCP-1 was consistently elevated throughout a 3-day follow-up in all patients in serum and in the vast majority of patients in urine. Presumably, the discrepancy with the earlier report is at least in part due to 20-fold lower sensitivity of the MCP-1 ELISA used in that study [4]. The fact that in our investigation MIP-1α only was detectable in a small subset of patients, is likely related to differences in the severity of the septic insult. Indeed, while our patients all recovered, in the previous study the mortality rate was 43 per cent [3]. In addition, it is of interest that the increase in plasma MIP-1α after injection of LPS in healthy subjects lasted for only a brief period compared to the rises in the plasma levels of MCP-1, MIP-1β and IP-10.

Transient release of MCP-1 into the circulation has been reported previously in healthy humans injected with low dose of LPS [14] and in baboons after infusion of a sublethal dose of E. coli [15]. Interestingly, MCP-1 release was sustained in baboons with lethal E. coli sepsis, suggesting that the duration of MCP-1 production during sepsis is influenced by the severity of the infection [15]. In murine endotoxemia, MCP-1 has been identified as an anti-inflammatory mediator, i.e. passive immunization against MCP-1 enhanced LPS-induced lethality while treatment with recombinant MCP-1 exerted a protective effect [16].

As in human endotoxemia, intraperitoneal administration of LPS to mice induced a transient rise in serum MIP-1α levels [17]. Neutralization of endogenously produced MIP-1α reduced early mortality and attenuated lung neutrophil and mononuclear phagocyte recruitment and pulmonary injury. Together these data suggest that MIP-1α has a proinflammatory role during endotoxic shock [17].

Normal subjects had low levels of MCP-1 in their serum in spite of the fact that MCP-1 is known to bind to the Duffy receptor on erythrocytes [18]. We recently found that not only free MCP-1, but also erythrocyte-bound MCP-
1 increases after a systemic challenge with endotoxin in healthy humans (manuscript in preparation), suggesting that Duffy receptors on normal erythrocytes are not saturated. Possibly, an equilibrium exists between MCP-1 bound to the Duffy receptor and free MCP-1, both in physiological and pathological conditions.

Urine levels of chemokines measured in patients with renal disease may reflect their clearance from blood, local production in the urinary tract or both. Indeed, mesangial, epithelial, endothelial and interstitial cells within the kidney are among the many different cell types that can produce chemokines [19]. In addition, in an isolated perfused kidney model, perfusion with LPS caused expression of MCP-1 especially in peritubular capillary endothelial cells [20]. It should be noted that the chemokines measured in this study attract mainly monocytes and lymphocytes whereas neutrophils are the predominant cells found in urine from patients with urinary tract infection. Previous studies have documented elevated MCP-1 concentrations in urine of patients with lupus nephritis, a disease in which the recruitment of monocytes plays a role in the development of renal inflammation [21, 22]. Evidence exists that IL-8 which can be found in high concentrations in urine of patients with urinary tract infection [7, 8, 12], is an important factor for the migration of neutrophils into the urinary system [8, 13, 23]. Taken together, it is likely that the chemokines measured in urine in the present study serve a function unrelated to leukocyte trafficking in the urinary tract during urosepsis.

In the present study, we observed that urine levels of MCP-1 and MIP-1β were higher in patients with urosepsis than from subjects who were intravenously challenged with LPS. This suggests that these chemokines measured in urine from patients with urosepsis may in part be locally produced and in part come from the circulation. However, it should be noted that the patients with urosepsis experienced a release of LPS throughout a longer period of time while the healthy subjects received just one, relatively small dose of LPS.

Chemokines have recently been recognized as a large family of low molecular weight chemotactic cytokines that play an important role in the pathogenesis of a number of inflammatory and infectious diseases. We demonstrate here that MCP-1, MIP-1β and IP-10, chemokines that act on
mononuclear cells, are elevated during urosepsis and experimental endotoxemia. Future studies are warranted to determine the exact function of individual chemokines during gram-negative infection.

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