Endotoxin Binding to Platelets in Blood from Patients with a Sepsis Syndrome

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Endotoxin, the lipopolysaccharide cell wall constituent of Gram-negative bacteria, produces symptoms of the Gram-negative sepsis syndrome. By measuring endotoxin in blood from septic patients it may be possible to select a subpopulation of patients in which mortality can be prevented by treatment with anti-endotoxin antibodies. We evaluated the performance of an endotoxin-free blood-collection tube. Within-run and between-run CVs of our endotoxin assay were 4–18% and 8–20%, respectively. In endotoxin-positive samples (LPS ≥6 ng/L), the concentration of endotoxin in platelet-rich plasma was significantly higher (P < 0.001) than in platelet-poor plasma. Apparent binding of endotoxin to platelets ranged from 0% to 92%. The correlation between the apparent percentage binding of LPS to platelets and the platelet count in platelet-rich plasma is linear and positive, but LPS is not bound solely to platelets. We conclude that endotoxin must be measured in platelet-rich plasma.

Indexing Terms: infection, bacterial/lipopolysaccharides/platelet-rich plasma/platelet-poor plasma

The clinical features of Gram-negative sepsis include fever or hypothermia, chills, hypotension, confusion, oliguria, leukocytosis or leukopenia, thrombocytopenia, metabolic acidosis, and diffuse intravascular coagulation. Despite maximal supportive medical care and optimal, potent antimicrobial treatment, mortality in patients with this syndrome remains high (1). Evidence from studies in humans and animal models suggests that many of the clinical features of Gram-negative sepsis are due to Gram-negative bacteria or their glycolipid cell wall constituents (endotoxin or lipopolysaccharide, LPS) in the patient’s bloodstream (2–4). In the bloodstream, LPS is (among other plasma components) bound with a high affinity to an acute-phase protein called LPS-binding protein (LBP) (5), and this complex can activate monocytes and macrophages by binding to the CD14 receptor on their plasma membranes (6). Activated monocytes and many other cells can be triggered (during sepsis) to synthesize cytokines such as tumor necrosis factor α, interleukin-1, and interleukin-6; these, in turn, may induce the host systemic inflammatory response syndrome (SIRS) (7) and the clinical features mentioned above (8–13).

Among the new therapeutic interventions being tested in randomized (double-blind) placebo-controlled clinical trials is one in which the toxic moiety of LPS is blocked by (monoclonal) antibodies directed against the lipid A domain. Indeed, some authors report that immunotherapy of Gram-negative sepsis with antibodies to lipid A may reduce mortality in such patients (14, 15). However, this therapy is expensive, and not all patients with a sepsis syndrome are likely to benefit from the treatment; e.g., patients with a Gram-positive sepsis or those with SIRS induced by other mechanisms may experience serious toxic effects if treated with anti-lipid A antibodies (16). Therefore, it may be helpful to measure LPS or Gram-negative bacteria before such immunotherapy is started. Testing for LPS may be preferred because the results are more quickly available than results obtained by culture techniques (2 h vs 24–48 h). Furthermore, modern quantitative chromogenic endotoxin tests based on the use of Limulus amebocyte lysate (LAL) are more sensitive than the older semiquantitative gel-clot assays.

Routine testing for endotoxin in human blood is subject to several pitfalls. For example, concentrations of LPS in blood are very low, although LPS is abundant in the environment; this makes contamination a serious problem during blood sampling, sample preparation, and testing. In addition, commercially available blood-collection tubes may contain endotoxin (17). Recently, the performance of an endotoxin-free blood collection tube (KabiTube ET; now named Endo tube ET) was evaluated (18). The tube contains endotoxin-free heparin and a silicone gel for separating cells from plasma during centrifugation at 3000g. This centrifugation produces platelet-poor plasma (PPP). The evaluators stated (18) that after centrifugation at 3000g, tubes could immediately be frozen and then be thawed and opened just before testing, thus minimizing sample handling and possible sources of contamination. However, some reports show that endotoxin from several bacterial sources binds to human platelets (19), and that endotoxin concentrations in PPP are much lower than in platelet-rich plasma (PRP) (20); others have found no difference between the amounts of LPS in PRP and PPP (18, 21, 22). Now, given the general acceptance that endotoxin elicits the cytokine response leading to SIRS, it will be important in evaluating new therapeutic drugs in clinical trials to establish uniform criteria for selecting patients and to develop validated laboratory tests that can be used to support uniform patient selection worldwide. We have therefore reevaluated the endotoxin-free blood-collection tube, especially with regard to the binding of

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4 Nonstandard abbreviations: LPS, lipopolysaccharide; LBP, lipopolysaccharide-binding protein; LAL, Limulus amebocyte lysate; PRP, platelet-rich plasma; PPP, platelet-poor plasma; SIRS, systemic inflammatory response syndrome; BPI, bactericidal permeability-increasing factor; and HDL, high-density lipoprotein.

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endotoxin to platelets in blood obtained from patients with sepsis.

Materials and Methods

Patient Selection and Sample Preparation

Patients admitted to the intensive care unit of our hospital and showing signs of a sepsis syndrome according to generally accepted criteria were selected for study (23). The procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 1983. Blood was sampled in duplicate in Endo tube ET (Chromogenix, Mölndal, Sweden) at the time the sepsis criteria were met and, later, every 6 h during a 72-h follow-up period (for further details about this tube, see ref. 18). Immediately after it was withdrawn, the blood was transported to the laboratory. One sample was centrifuged at 4°C for 15 min at 3000g to obtain PPP. The other sample was centrifuged at 180g for 15 min at 4°C (PRP). After centrifugation the complete plasma layer was separated and then mixed, and platelets were counted in an aliquot of each sample (Sysmex E5000; Toa Medical, Kobe, Japan). A second aliquot was stored at −78°C in sterile polypropylene tubes (Greiner, Frickenhausen, Germany) until measurement.

Measurement of Endotoxin

Endotoxin in plasma was measured with a quantitative chromogenic endpoint assay (Coastest Endotoxin; Chromogenix). In short, a sample was diluted 10-fold with sterile endotoxin-free water (LAL water; Chromogenix) and heated at 75°C for 15 min. After the sample was cooled, 40 μL was pipetted into wells of a sterile flat-bottom microplate (Greiner). The plates were incubated at 37°C in a micro-Hywell incubator (Chromogenix); after 5 min, 40 μL of LAL reagent was added. Activation of LAL took exactly 25 min at 37°C, after which we added 80 μL of prewarmed (37°C) chromogenic substrate (S2423; 1 mmol/L in Tris buffer, pH 9.0). Exactly 5 min later, substrate conversion was stopped by adding 80 μL of 200 mL/L acetic acid. Absorbances were measured at 405 nm in a microplate reader (ICN Biomedicals, Costa Mesa, CA). Samples were measured in duplicate.

To a second aliquot of each sample we added endotoxin standard (Escherichia coli O111:B4; 50 ng/L) before assaying (in duplicate). Blank absorbances were measured for each sample (40 μL of sample, 120 μL of water, and 80 μL of acetic acid reagent). Recovery of added endotoxin standard within the 75–125% range was considered normal.

Sample handling was performed aseptically as possible. On the basis of clinical studies by others using the same endotoxin assay, we considered the reference range to be 0–5 ng/L of PRP (24, 25).

Calibration

Calibration was performed by using 10-fold-diluted plasma (obtained in an Endo tube ET from the blood of normal healthy volunteers) to which we added five different concentrations of endotoxin standard [E. coli O111:B4; 0–100 ng/L; 1 ng corresponds to 12 endotoxin units (EU) calibrated against US Food and Drug Administration standard EC-5 lot F]. Calibration curves should be linear (r ≥ 0.980 by linear regression) for calibration to be valid.

Binding of Endotoxin to Platelets

Binding of endotoxin to platelets was estimated as:

Apparent binding (%) = [(endotoxin PRP − endotoxin PPP)/endotoxin PRP] × 100%

Validity of Endotoxin Assay

Stability of endotoxin in human whole blood. Three different quantities of endotoxin standard (E. coli O111: B4) dissolved in endotoxin-free physiological saline solution were added to blood from healthy volunteers, collected into Endo tubes ET. After the addition of saline and thorough mixing, the samples were either centrifuged directly for preparation of PRP or were incubated for 1 and 2 h at three different temperatures (0, 22, and 37°C). After incubation, the incubated samples were also centrifuged to prepare PRP. The PRP samples were stored at −78°C until measurement of endotoxin.

Assay precision. Pooled plasma was obtained from blood of healthy volunteers. Blood was collected into Endo tubes ET and PRP was prepared. Three different quantities of endotoxin standard dissolved in water were added to three aliquots of this plasma. Plasma dilution was kept constant for each aliquot by addition of water if necessary. After mixing, aliquots of all samples were stored at −78°C until measurement. Five aliquots of each sample were analyzed simultaneously and on five different days for calculation of within-run and between-run precision.

Results

Stability of endotoxin in whole blood of healthy persons was highest at 0°C and lowest at 37°C (Table 1), suggesting a temperature-dependent process by which LPS is cleared from the PRP. For example, after 2 h of

<table>
<thead>
<tr>
<th>Incubation temp. °C</th>
<th>LPS added, ng/L</th>
<th>0 h</th>
<th>1 h</th>
<th>2 h</th>
</tr>
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<tr>
<td>0</td>
<td>23</td>
<td>17 (2)</td>
<td>19 (2)</td>
<td>20 (4)</td>
</tr>
<tr>
<td>22</td>
<td>47</td>
<td>36 (2)</td>
<td>46 (7)</td>
<td>39 (5)</td>
</tr>
<tr>
<td>37</td>
<td>70</td>
<td>49 (3)</td>
<td>62 (8)</td>
<td>66 (7)</td>
</tr>
</tbody>
</table>

Table 1. Stability of endotoxin in human whole blood and recovery of LPS in PRP.

* LPS standard was added to normal human whole blood.

* Mean values (SEM) obtained from blood of four different blood donors.
incubation, recoveries of 70 ng/L added LPS were 94%, 73%, and 50% at 0, 22, and 37°C. Furthermore, recoveries after 1 and 2 h of incubation on melting ice seemed to be higher than those obtained immediately after addition of LPS to the blood.

Within-run precision (CV) ranged from 18.0% to 4.3% at LPS concentrations of 3.66 and 43.1 ng/L, respectively (Table 2). Between-run precision ranged from 20.0% to 8.4% at approximately the same LPS concentrations (Table 2). We obtained 150 blood samples from 12 patients with sepsis. In all 12 patients, endotoxin was ≥6 ng/L in PRP on at least one occasion (total, 74 samples). In 76 samples, PRP endotoxin was <6 ng/L. Most patients had a few positive endotoxin PRP samples in their follow-up period, with modestly increased endotoxin concentrations (e.g., LPS 6–20 ng/L). Five patients (nos. 1, 4, 7, 8, and 11) had endotoxin concentrations >20 ng/L (Table 3). In most PRP samples with endotoxin ≥6 ng/L, endotoxin appeared to be bound to platelets (range 0–92%, Table 3). Binding of endotoxin to platelets varied from patient to patient and among samples from a single patient. Two patients had platelet counts <100 × 10⁹/L throughout the follow-up period (nos. 6 and 10); they also had the lowest mean binding of endotoxin to platelets (16% and 14%, respectively; Table 3). Mean endotoxin concentrations in PRP were significantly higher (P < 0.001, Student's t-test) than in the corresponding samples of PPP (PRP 21.7 ng/L, SD 38.1, range 6–208 vs PPP 7.3 ng/L, SD 11.5, range 0–88).

For all 150 blood samples the recovery of added LPS was good: 55–60% (n = 1); 60–75% (n = 42); 75–125% (n = 103); and 125–135% (n = 4). PRP samples with LPS ≥6 ng/L were, depending on the platelet count, divided into groups according to 50 × 10⁹/L increments in platelet count. For each group the mean platelet count and mean apparent percentage binding of LPS to platelets were calculated. When, for all groups, we plotted the mean apparent percentage binding against the mean PRP platelet count, we found a positive (linear) correlation: y = 0.22x + 12 (r = 0.95, P < 0.002; Fig. 1). For patient no. 11, we plotted the time course for the measured LPS (PRP) and the apparent percentage binding of LPS to platelets (Fig. 2). The LPS curve showed two peaks, as did the apparent percentage binding curve.

Discussion

The stability of LPS in normal human blood is adequate at 0°C during a 2-h period (Table 1). However, it is uncertain whether LPS-positive samples from patients with a sepsis syndrome show the same stability characteristics. In daily practice, it may be better to cool

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**Table 2. Precision of endotoxin assay.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within-run</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>3.56</td>
<td>0.64</td>
<td>18.0</td>
</tr>
<tr>
<td>II</td>
<td>13.7</td>
<td>1.24</td>
<td>9.1</td>
</tr>
<tr>
<td>III</td>
<td>43.1</td>
<td>1.84</td>
<td>4.3</td>
</tr>
<tr>
<td>Between-run</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>3.20</td>
<td>0.64</td>
<td>20.0</td>
</tr>
<tr>
<td>II</td>
<td>14.4</td>
<td>2.32</td>
<td>16.1</td>
</tr>
<tr>
<td>III</td>
<td>39.4</td>
<td>3.29</td>
<td>8.4</td>
</tr>
</tbody>
</table>

*Means of five experiments.

**Table 3. Binding of endotoxin to platelets in blood from patients with sepsis.**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Endotoxin PRP, ng/L</th>
<th>Apparent binding to platelets, %</th>
<th>Platelets PRP, 10⁹/L</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18 (6–40)</td>
<td>18 (25–86)</td>
<td>198 (157–213)</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>9 (7–13)</td>
<td>17 (0–71)</td>
<td>323 (107–440)</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>7 (6–7)</td>
<td>32 (0–57)</td>
<td>163 (86–228)</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>22 (8–37)</td>
<td>68 (25–89)</td>
<td>189 (181–196)</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>10 (6–15)</td>
<td>34 (17–58)</td>
<td>280 (267–300)</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>12 (7–20)</td>
<td>16 (0–47)</td>
<td>70 (50–89)</td>
<td>6</td>
</tr>
<tr>
<td>7</td>
<td>13 (7–23)</td>
<td>33 (0–70)</td>
<td>117 (95–184)</td>
<td>7</td>
</tr>
<tr>
<td>8</td>
<td>17 (7–33)</td>
<td>43 (0–91)</td>
<td>286 (171–553)</td>
<td>6</td>
</tr>
<tr>
<td>9</td>
<td>11 (6–16)</td>
<td>79 (67–88)</td>
<td>287 (245–338)</td>
<td>3</td>
</tr>
<tr>
<td>10</td>
<td>10 (6–13)</td>
<td>14 (0–36)</td>
<td>41 (34–56)</td>
<td>11</td>
</tr>
<tr>
<td>11</td>
<td>57 (7–208)</td>
<td>42 (14–92)</td>
<td>205 (107–362)</td>
<td>14</td>
</tr>
<tr>
<td>12</td>
<td>11 (6–15)</td>
<td>38 (14–87)</td>
<td>214 (165–296)</td>
<td>4</td>
</tr>
</tbody>
</table>

n, number of samples.
and centrifuge blood samples immediately after they are withdrawn. Further experiments are needed to clarify this issue. Calculated recoveries ranged from 40% to 45% (if one assumes a hematocrit of 0.40 and no binding of LPS to blood cells, Table 1); these results are similar to those of Sturk et al. (22). An interesting issue is that prolonged incubation of LPS standard in normal human blood at 0°C resulted in higher recoveries. This effect, which was also noticed by Harris et al. (21), was most pronounced at the highest concentration of LPS. It may be explained by a rapid binding of LPS to blood cells and a subsequent redistribution of LPS from blood cells to plasma components such as LBP, bactericidal permeability increasing factor (BPI), and high-density lipoprotein (HDL) after prolonged incubation (5, 6). Increasing the temperature results in a time-dependent loss of LPS from PRP, presumably by cellular uptake of LPS by blood cells other than platelets [e.g., erythrocytes, neutrophils, and monocytes (26, 27)]. Within-run CV ranged from 4% to 18% and between-run CV from 8% to 20% for high and low concentrations of LPS, respectively (Table 2). At very low concentrations of LPS (3 ng/L) precision was quite acceptable. Our precision data at higher LPS concentrations were better than, and comparable with, those obtained by other authors (21, 28, 29). LPS in PRP from 15 healthy individuals ranged from 0 to 3.2 ng/L [mean (SD) 1.9 (0.7) ng/L].

We found increased concentrations of LPS in all sepsis patients. The patient (no. 11) with positive blood cultures (Pseudomonas aeruginosa) had the highest concentration of LPS (Table 3). Blood cultures from all other patients were negative.

In two recent studies, the prevalence of endotoxemia in septic patients was 50% and 80% (24, 30). All patients in our study had increased concentrations of endotoxin. This may be explained by the small study population, and by our 72-h follow-up period. Regardless of the results of the blood culture studies, Casey et al. found increased LPS in patients with sepsis (31). Furthermore, all our patients were treated with antimicrobial agents, which may stimulate the release of endotoxin to the bloodstream (32). One patient (no. 3) had LPS concentrations just above our cutoff value of 6 ng/L. Given the precision of our assay at this LPS concentration (i.e., CV = 15%), we estimate the 95% confidence interval ranges from 4.2 to 7.8 ng/L. In 50 of 74 LPS-positive samples (Table 3), concentrations of LPS were greater in PRP than in PPP (P <0.001). In the other 24 samples, the LPS concentrations in PRP and PPP were similar.

The linear relation between the mean PRP platelet count and the mean apparent percentage binding of LPS to platelets is statistically highly significant, suggesting that the different concentrations of LPS in PRP and PPP are due to binding of LPS to platelets. Binding of LPS to platelets varied considerably within individual patients and between patients. Previous reports on the binding of LPS to platelets have been contradictory. Spielvogel proved that endotoxin binds to platelets by the lipid A part of LPS (26) and found evidence that lipid A-associated protein (a protein component of bacterial endotoxin) facilitated the attachment of endotoxin to the platelet membrane. Springer and Adye (33) demonstrated the presence of endotoxin-binding sites on membranes of human platelets. Das et al. (34) reported that LPS (E. coli strain O26:B6) added to human PRP could be recovered in the platelet fraction only at concentrations <5 μg/L; at 10 to 100 times greater concentrations they were able to demonstrate the presence of LPS in PPP. When LPS (E. coli O111:B4) was added to normal human blood, several authors found the same recoveries in PRP and PPP (18, 21, 22) or in whole blood, PRP, and PPP (19). In similar experiments in which we used an E. coli O111:B4 LPS standard preparation we also found no difference in LPS concentrations in PRP and PPP (data not shown). However, when LPS preparations from other bacterial sources were used (e.g., E. coli O26:B6; Salmonella minnesota 9700; S. enteritidis; or S. typhimurium), recoveries of LPS in PRP and PPP were significantly different (20). For S. enteritidis and S. typhimurium LPS the recoveries for whole blood and PRP were the same when a perchloric acid extraction step was used (19). In a population of surgical patients, Tachiyama et al. (20) found no differences in LPS concentrations in PRP and PPP, except in one patient who had an intraabdominal abscess with E. coli; however, whether or not these patients had a sepsis syndrome was not stated.

Our results indicate that significant binding of LPS to platelets occurs in blood from patients with a sepsis syndrome. We found a positive linear correlation between the apparent percentage binding of LPS to platelets and the PRP platelet count. The LPS measured in PRP was statistically higher than in PPP; this is also documented for LPS from bacteria other than E. coli in in vitro experiments of LPS recovery with normal human blood (20). Data from our patient with the most pronounced endotoxemia suggest that the concentration of LPS in PRP correlates positively with the apparent percentage binding of LPS to platelets (Fig. 2). However, LPS binds not only to platelets but also to erythrocytes, neutrophils, and monocytes (26, 35) and to several plasma components such as HDL, LBP, BPI, and naturally present anti-LPS antibodies (5, 6). Flegel et al. (27) showed that normal human blood has an LPS neutralizing capacity equivalent to an LPS concentration of 10 ng/L. LBP in normal human blood (0.5 μg/L) can increase rapidly by as much as 100-fold during a strong acute-phase reaction. However, in the event that large quantities of LPS are released from an infected organ, LPS-binding plasma components may become saturated, leading to an increase in LPS binding to platelets—up to 90%, as found in some of our patients. Birkenmaier et al. (36) found that CD14 expression on monocytes from patients with sepsis is lower than in healthy persons. Measurement of the LPS-binding capacities of HDL, LBP, BPI, and natural anti-LPS antibodies in normal persons and patients with a sepsis syndrome will be necessary to gain further insight into these phenomena.
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References