Changes in Endotoxin-Binding Proteins during Major Elective Surgery: Important Role for Soluble CD14 in Regulation of Biological Activity of Systemic Endotoxin

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Assessment of circulating endotoxin during the perioperative period, which is only demonstrated by the Limulus amebocyte lysate (LAL) test, may be modulated by several endotoxin-binding proteins. Endotoxin-neutralizing capacity (ENC) and the plasma levels of soluble CD14 (sCD14), lipopolysaccharide-binding protein, and bactericidal/permeability-increasing protein (BPI) were determined in 40 patients 6 h prior to skin incision for major abdominal surgery. The bioactivity of plasma endotoxin was tested by the polymyxin B-inhibited stimulatory activity of the plasma samples on healthy monocytes as measured by the release of tumor necrosis factor alpha. Plasma endotoxin levels in almost all patients increased from 0.05 ± 0.01 to 0.23 ± 0.03 experimental units (EU) per ml (P < 0.001); more specifically, 17 of 40 samples showed endotoxin levels of greater than 0.2 EU per ml and corresponding reductions in ENC. Soluble CD14 plasma levels were decreased from 5.6 ± 0.3 to 4.6 ± 0.3 μg per ml (P < 0.05). ENC was strongly correlated with the sCD14 plasma concentration throughout the period of observation. The addition of sCD14-neutralizing monoclonal anti-sCD14 antibodies reduced ENC both pre- and postoperatively. No correlation could be established between ENC and the plasma levels of BPI, high-density lipoproteins, or low-density lipoproteins determined by measuring the concentrations of apoprotein A and apoprotein B. Biologically active endotoxin was found in only 6 of 17 samples with endotoxin levels greater than 0.2 EU per ml in the LAL test. These samples could be characterized by their perioperative loss of at least 35% of their sCD14. No change in sCD14 was detected in the remaining 11 samples. The perioperative loss of ENC is partly caused by the loss of sCD14 resulting from its consumption by endotoxin reaching the bloodstream. This study demonstrated the role of sCD14 on the bioactivity of circulating endotoxin in a human model of endotoxemia after major abdominal surgery.

A number of cell types, including hepatocytes (15, 33), local macrophages (16, 26, 40), and granulocytes (35, 36), have cellular endotoxin-neutralizing activity mediated via well-characterized mechanisms of lipopolysaccharide (LPS) inactivation. In addition to the cellular endotoxin neutralization system, soluble endotoxin-binding and -neutralizing factors that reduce the harmful action of circulating endotoxin are also present in plasma. Early studies showed that plasma itself is a potent inhibitor of endotoxin-mediated phenomena such as pyrogenicity (41, 42). Later experiments showed that several plasma proteins may bind endotoxin either in a specific or unspecific manner, which was assumed to be associated with an alteration of aspects of endotoxin bioactivity (14, 31, 45). Most recently, the soluble form of the endotoxin receptor CD14 (sCD14) was demonstrated to mediate the LPS-neutralizing action of high-density lipoproteins (22, 23, 47). Plasma sCD14 levels are increased during septic diseases (7, 29, 30) as well as after multiple-trauma and burn injuries (28). Bactericidal/permeability-increasing protein (BPI), a neutrophil granule protein, diminishes the bioactivity of LPS in vitro (1, 24) and in vivo (13, 44) and has been shown to increase significantly during sepsis (8, 17). The LPS-binding protein (LBP) first catalytically transfers an LPS monomer to a binding site on sCD14 (20), and the resulting LPS-sCD14 complexes diffuse readily, breaking LPS into lipoprotein particles (47–49). LBP is a classical acute phase protein, which is strongly enhanced during acute inflammatory responses (17, 19).

The endotoxin-neutralizing capacity (ENC) of plasma can be easily determined by a direct Limulus amebocyte lysate (LAL) test without heat inactivation of the inhibitors present in plasma (4). Our previous studies showed that ENC was decreased significantly during aseptic abdominal surgery, which is associated with impending complications due to infection (4). Elective aseptic abdominal surgery represents a human model characterized by a significant and reproducible endotoxemia and a well-defined acute phase reaction (5, 6, 12, 37, 46). Although there are some indications that circulating endotoxin has bioactivity during the postoperative (5, 32) and posttraumatic courses (25), its pathophysiological relevance is far from being generally accepted. The complex nature of cellular and soluble neutralizing mechanisms may account for the observation that high endotoxin levels are not invariably correlated with clinical signs. We propose that the endotoxin-binding proteins, and sCD14 in particular, determine the biological activity of translocated endotoxin during surgery.

In this study, we aimed to (i) evaluate the sCD14, LBP, BPI, and endotoxin plasma levels and the ENC of the plasma during major elective abdominal surgery, (ii) estimate the relationship of sCD14, LBP, and BPI on ENC, and (iii) estimate the bio-

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TABLE 1. Clinical characteristics of presurgical status in the patients subjects

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Valuea</th>
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<tbody>
<tr>
<td>Sex (male/female)</td>
<td>22/18</td>
</tr>
<tr>
<td>Age (average [yr])</td>
<td>61</td>
</tr>
<tr>
<td>(range [yr])</td>
<td>42–77</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>27.5 ± 0.3</td>
</tr>
</tbody>
</table>

Diagnoses

- Chronic pancreatitis: 9
- Pancreatic cancer: 19
- Gastric cancer: 5
- Colon cancer: 7
- APACHE II score: 5.0 ± 1.0
- Total protein (g/dl): 6.5 ± 0.1
- Albumin (g/dl): 4.1 ± 0.1
- Hemoglobin (g/dl): 13.2 ± 0.1
- Total cholesterol (mg/dl): 220.5 ± 10.2
- Choline esterase (U/liter): 4.382 ± 201
- CRP (mg/liter): 9.0 ± 1.1

a Data are presented as means ± SE.
b Body mass index = body weight/height² (kg/m²).
c APACHE, Acute Physiology and Chronic Health Evaluation.
d C-reactive protein.

determination of endotoxin content. Endotoxin plasma levels were determined by using a two-step, endpoint micromethod as described previously in detail (4). The unknown samples were pretreated by heat inactivation for 10 min at 75°C and were incubated with the lysate (Charles River Endosafe, Sulzfeld, Germany) for 30 min at 37°C. After adding 5 mmol/l chromogenic substrate (Pefachrome from LPS, Sintall-Oberzell, Germany), samples were further incubated for 3 min at 37°C. The reaction was stopped, and the endotoxin content was quantified according to a simultaneously established standard curve in pyrogen-free plasma.

Estimation of ENC. The ENC of plasma, expressed as endotoxin recovery, was measured by using the LAL test and has been recently described in detail (4). The method principally relies on the determination of the recovery of exogenously added endotoxin to plasma samples. In contrast to the estimation of endogenous endotoxin, inactivation of the plasma samples was omitted. Ten microliters of a standard endotoxin of Salmonella abortus subsp. equi (1,000 EU/ml) (NPS; Pyrosquant Co., Moerfelden-Walldorf, Germany) were added to 90 µl of plasma. After incubation at 24°C for 30 min, the sample was diluted with 900 µl of pyrogen-free water (final concentration, 10 EU/ml). The endotoxin recovery was determined as described above except that the standard curve was established in pyrogen-free water. Intra- and interassay variation coefficients amounted to 6.5 and 7.2%, respectively, as ascertained in 30 single determinations.

In experiments designed to determine the role of sCD14, the plasma samples were preincubated with 10 µg of the monoclonal anti-CD14 antibody MEM-18 per ml (kindly provided by V. Horesi, Institute of Organic Chemistry and Biochemistry, Prague, Czech Republic) for 20 min at 24°C before LPS was added. Endogenous endotoxin levels of all samples were subtracted from the recovery data.

Determination of albumin, apo A, and apo B plasma levels. The plasma levels of albumin, apoprotein A (apo A), and apoprotein B (apo B) were determined by nephelometry (20 analyzer, Behring Co., Liederbach, Germany).

ELISA assays. Plasma TNF-α was quantified with a commercially available enzyme-linked immunosorbent assay (Immunotech, Hamburg, Germany). Soluble CD14 was measured by using a sandwich ELISA with two monoclonal antibodies against CD14 (IBL, Hamburg, Germany). Plasma samples which were diluted 1:200 with phosphate-buffered saline (PBS) were assayed in ELISA following manufacturer’s instructions. Plasma BPI and LBP levels were determined by using a sandwich ELISA as reported elsewhere in detail (9). In short, microwell plates were coated with human-BPI-specific monoclonal antibody 4E3 or with polyclonal anti-human LBP immunoglobulin G (IgG). Washing and dilution buffers for BPI and LBP determination contained 80 mM and 40 mM MgCl₂, respectively. Mg²⁺ ions were added to prevent the influence of LPS on BPI or LBP measurement. Human recombinant BPI or recombinant LBP (provided by M. Marra, InCyte, Palo Alto, Calif.) was used for the standard curve.

![Fig. 1](http://cvi.asm.org/...onOctober%2028,%202017%20by%20guest)
Statistical evaluations of continuous data were performed by one-way analyses of variance and unpaired Student’s t tests for intergroup differences. Differences were considered significant at \( P < 0.05 \). A decrease of plasma LBP levels (5.7 ± 0.8 to 4.0 ± 0.5 \( \mu \)g/ml; \( P < 0.05 \)) was observed (Fig. 2B). Plasma BPI levels did not change during the observation period (Fig. 2C). The plasma levels of albumin, apo A, and apo B were all significantly decreased (Table 2).

Relationship between ENC and endotoxin-binding proteins in plasma. A significant correlation was found between the sCD14 plasma level and the postoperative recovery of endotoxin (Fig. 3B) \(( r = -0.66; \ P < 0.0001)\). Plasma LBP values showed a negative correlation with endotoxin recovery after surgical stress (Fig. 4B) \(( r = -0.60; \ P < 0.0001)\). Plasma BPI levels did not correlate with endotoxin (data not shown). There was no correlation between albumin, apo A, and apo B plasma levels and the recovery of sCD14, LBP, BPI, or endotoxin.

Figure 5 represents endotoxin recovery in the presence or absence of MEM18. The addition of MEM18 significantly increased endotoxin recovery \(( P < 0.01)\); change in endotoxin recovery, 0.05 ± 0.01 EU/ml in the preoperative plasma samples. A large, but not significant, increase \(( P = 0.06)\) in endotoxin recovery compared to the MEM18 negative assay was detected for postoperative samples (change in endotoxin recovery, 0.12 ± 0.04 EU/ml).

Correlation of plasma sCD14 with the biological activity of postoperative plasma. In LAL tests, 17 of 40 (43%) postsurgical plasma samples showed endotoxin levels which were all above 0.20 EU/ml. These 17 samples were used to stimulate healthy monocytes in order to evaluate bioactivity as determined by TNF-\( \alpha \) release (Fig. 6). Preoperative plasma did not significantly stimulate the monocyte culture (data not shown). The activity of 6 of the 17 postoperative plasma samples could be blocked by the addition of 5 \( \mu \)g of polymyxin B per ml \(( P < 0.01)\) (Fig. 6A), indicating that biologically active endotoxin

![FIG. 2.](http://cvi.asm.org/download/)

TABLE 2. Plasma levels of albumin, apo A, and apo B after surgery

<table>
<thead>
<tr>
<th>Protein</th>
<th>Avg value for 40 samples</th>
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<tr>
<td></td>
<td>Preoperative</td>
</tr>
<tr>
<td>Albumin (g/liter)</td>
<td>39.2 ± 1.4</td>
</tr>
<tr>
<td>Apo A (g/liter)</td>
<td>1.4 ± 0.0</td>
</tr>
<tr>
<td>Apo B (g/liter)</td>
<td>1.0 ± 0.1</td>
</tr>
</tbody>
</table>

\( ^{a} \) Data are presented as means ± SEs.
\( ^{b} \) Each of these values showed significant difference from preoperative values \(( P < 0.001)\) by Student’s \( t \) test.

Samples diluted in the above buffer (1:2 for BPI, 1:2,000 for LBP) were assayed. Biotinylated polyclonal rabbit anti-human BPI IgG and biotinylated rabbit anti-human LBP IgG were used as secondary antibodies, followed by visualization using peroxidase-conjugated streptavidin (Dakopatts, Glostrup, Denmark). The levels of detection of both assays were 200 pg/ml.

Isolation of monocytes. Peripheral blood mononuclear cells (PBMCs) were obtained from 10 healthy male donors. Venous blood mixed with 10 IU of sodium heparin was separated in a 50-ml polystyrene tube with a porous filter disk (LuecoSep; Greiner, Frickenhausen, Germany) using Ficoll-Paque solution (Seromed, Berlin, Germany) at 450 g for 20 min. PBMCs were washed three times with PBS and then finally suspended at 2 × 10^7 PBMCs per ml of RPMI 1640 (GIBCO, Eggenstein, Germany) containing 10% autologous plasma.

Plasma samples were incubated for 20 min with or without 5 \( \mu \)g of Escherichia coli O55:B5 (BioWhittaker, Walkersville, Md.) per ml in monocytic cultures. In this experiment, we assessed that polymyxin B abolishes the bioactivity of 50 pg of LPS in pre- and postsurgical plasma samples was tested by incubating the plasma, diluted to 50% with RPMI 1640, with monocytes of healthy volunteers in the presence or absence of polymyxin B (Pfizer, Karlsruhe, Germany) using Ficoll-Paque solution (Seromed, Berlin, Germany) at 450 g for 20 min. PBMCs were washed three times with PBS and then finally suspended at 2 × 10^7 PBMCs per ml of RPMI 1640 (GIBCO, Eggenstein, Germany) containing 10% autologous plasma. Monocytes were separated from lymphocytes by adherence (12 h at 37°C) to 12-well polystyrene plates (Greiner). After removal of nonadherent cells, monocytes were extensively washed with PBS containing 5% autologous plasma to remove residual nonadherent cells.

Assessment of biological activity of LPS in surgical plasma samples. The bioactivity of LPS in pre- and postsurgical plasma samples was tested by incubating the plasma, diluted to 50% with RPMI 1640, with monocytes of healthy volunteers in the presence or absence of polymyxin B (Pfizer, Karlsruhe, Germany). Plasma samples were incubated for 20 min with or without 5 \( \mu \)g of polymyxin B per ml at room temperature, and subsequently 4 × 10^5 monocytes were added. The cells were cultured at 37°C in a 5% CO2 atmosphere for 5 h. After incubation, the supernatant was collected and frozen at -70°C. In control experiments, we assessed that polymyxin B abolishes the bioactivity of 50 pg of Escherichia coli O55:B5 (BioWhittaker, Walkersville, Md.) per ml in monocytic cultures. In this experiment, a mixture containing 5% autologous plasma to remove residual nonadherent cells.

Statistical analysis. Data were expressed as means ± standard errors (SEs). Statistical evaluations of continuous data were performed by one-way analyses of variance and unpaired \( t \) tests for intergroup differences. Differences were considered significant at \( P < 0.05 \).

RESULTS

Endotoxin plasma levels and plasma endotoxin recovery. We previously demonstrated significant endotoxiaemia only at the time point of 6 h after surgical stress in patients with multiple trauma (25). Therefore, the time point of 6 h after skin incision was selected for this study design. As shown in Fig. 1A, preoperative endotoxin plasma levels were 0.05 ± 0.01 EU/ml (normal; <0.07 EU/ml). Six hours after the skin incision, a significant increase to 0.23 ± 0.05 EU/ml was observed \(( P < 0.001)\). The plasma endotoxin levels did not increase in 4 of 40 patients (10%). The recovery of exogenously added endotoxin (10 EU/ml) to pre- and postoperative plasma samples is given in Fig. 1B. Endotoxin recovery increased from a mean of 0.06 ± 0.01 to 0.31 ± 0.03 EU/ml \(( P < 0.001)\) in each of the 40 patients. Endogenous endotoxin levels of all samples were subtracted from the recovery data.

Kinetics of sCD14, LBP, and BPI levels during elective abdominal surgery. The plasma concentration of sCD14 (Fig. 2A) decreased perioperatively from 5.6 ± 0.3 \( \mu \)g/ml to 4.6 ± 0.3 \( \mu \)g/ml \(( P < 0.05)\). A decrease of plasma LBP levels (5.7 ± 0.8 to 4.0 ± 0.5 \( \mu \)g/ml; \( P < 0.05 \)) was observed (Fig. 2B).
was responsible for this plasma activity. The observation that the activity of the other 11 plasma samples could not be blocked by polymyxin B (Fig. 6B) indicates that the endotoxin present in these samples is not biologically active but that other plasma components may be responsible for the observed monocyte activation. The sCD14 content of these 6 of the 17 samples decreased more than 65%, whereas the sCD14 levels in the remaining 11 samples did not change (Fig. 7A). The endotoxin recovery was also significantly higher in the six samples containing bioactive endotoxin than in the 11 other samples (P < 0.01) (Fig. 7B).

**DISCUSSION**

Although the presence of endotoxin in the systemic circulation has been extensively studied in a variety of clinical settings, there is no agreement on the occurrence of endotoxemia due to surgical stress. Many studies have reported significant endotoxemia in humans after trauma (25), major abdominal surgery (5), and cardiac surgery (32). However, other well-designed studies have failed to detect endotoxemia after trauma (27, 34) or hemorrhagic shock (11). These conflicting results were all obtained after making complex measurements of endotoxin content by the LAL test, and the debates over the development of gut-derived endotoxemia have generally been based on the LAL test. We, however, believe that it may be more relevant to focus on the biological activity of circulating endotoxin, which is related to clinical outcome. Recent major studies have corroborated the pathophysiological importance of gut-derived endotoxemia in experiments using antiendotoxin agents, such as polymyxin B (51), a cationic antibiotic that stoichiometrically neutralizes the lipid A moiety of endotoxin, or BPI (50), an endogenous endotoxin-neutralizing protein that reduces endotoxin translocation during experimental hemorrhage in rats and improves clinical outcome. In humans, the selective decontamination of the digestive tract has been demonstrated to reduce perioperative endotoxemia and release of interleukin 6 in cardiac patients (32). In the present study, we assessed the biological activity of the translocated endotoxin after major elective surgery.

**FIG. 3.** Correlation between ENC and sCD14. (A) Preoperative time point. (B) Postoperative time point. Plasma sCD14 levels are given on the x axis. Levels of endotoxin recovery are shown on the y axis.

**FIG. 4.** Correlation between ENC and LBP. (A) Preoperative time point. (B) Postoperative time point. Plasma LBP levels are demonstrated on the x axis. Levels of endotoxin recovery are depicted on the y axis.
Our results demonstrated the presence of significant endotoxemia during major abdominal surgery, which is related to a loss of plasma ENC, as described previously (4). Preoperative patient plasma neutralized almost all exogenously added endotoxin, whereas postoperative plasma did not have enough capacity to neutralize the same quantity of endotoxin, resulting in postoperative loss of ENC. This study demonstrated reduced sCD14 plasma levels in the very early postoperative stage, a finding similar to that of Kruger et al., who observed decreased sCD14 concentrations immediately after multiple trauma (28). Another new finding was a strong correlation between sCD14 levels and ENC as determined by the LAL test. High sCD14 levels were associated with high ENC. The addition of MEM18, a neutralizing anti-CD14 antibody (2), significantly diminished the ENC of preoperative plasma samples. The effect on postoperative plasma was less pronounced and not significant. One explanation for this observation may be the presence of endogenous endotoxin in postoperative plasma, as detected by the conventional LAL test after heat inactivation. Pretreatment by heating is known to destroy sCD14-endotoxin complexes, but ENC levels were determined without any inactivation step. Thus, endogenous endotoxin may block sCD14, resulting not only in loss of ENC but in a less pronounced effect of anti-CD14 antibodies. These antibodies can only bind to s/mCD14 before the addition of LPS (18). Preformed CD14-LPS complexes are no longer accessible to the neutralizing action of MEM18, and thus the ENC of postoperative plasma cannot be influenced by MEM18.

Plasma LBP levels were positively correlated with the ENC and sCD14 values. LBP is thought to facilitate the formation of LPS-s/mCD14 complexes, and it enhances LPS-induced cell activation (21, 43, 52). LBP may also catalyze sCD14-high-density lipoprotein-dependent LPS neutralization (21, 49). Therefore, the positive correlation between LBP and ENC reflects the catalytic effect of LBP on sCD14-LPS binding and is compatible with these LPS-neutralizing mechanisms. However, a more detailed study is required to reveal the role of LBP itself in LPS neutralization.

Plasma BPI was not found to be correlated with ENC or LBP. BPI is a potent LPS-neutralizing factor produced by polymorphonuclear leukocytes (39) and has an antagonistic effect on LBP-related LPS-cell interaction (10, 24). However, BPI did not seem to contribute to ENC, at least during the perioperative period, and BPI release during that period seems to be modest. Therefore, significant secretion of BPI later in the postoperative period may play a role as an LPS-neutralizing mechanism.

The bioactivity of endotoxin in postsurgical plasma was determined by measuring TNF-α release from healthy monocytes.
after incubation with plasma samples in the presence and absence of polymyxin B. In a preliminary study, there was no procedural activation of monocytes following steps taken to purify them from whole blood. The presence of very low concentrations of endotoxin (0.05 ng/ml) caused a significant release of TNF-α by monocytes that could be completely abolished by the addition of polymyxin B. For the first time, the biological activity of circulating endotoxin was demonstrated in our study. Almost all of the 17 postoperative plasma samples with endotoxin contents above 0.20 EU/ml stimulated TNF-α secretion by healthy monocytes, whereas no stimulation was observed in the stimulation assay with preoperative plasma. The stimulatory activity of only 6 of the 17 postoperative plasma samples could be inhibited by the addition of polymyxin B, indicating that biologically active endotoxin was present in these six samples. In addition, these six samples had the lowest ENC and the most pronounced decrease in sCD14 compared with preoperative values. It should be noted that the absolute level of sCD14 was not found to be associated with endotoxin bioactivity, despite the relative loss of sCD14 during the perioperative course. These six patients, however, were not distinguished by blood loss, duration of operation, or volume of infusion. Based on these preliminary results obtained in 6 of the 17 patients, we hypothesized that endotoxin present in the blood after major surgical procedures may be bound to and purified from whole blood. The presence of even very low concentrations of endotoxin (0.05 ng/ml) caused a significant release of TNF-α by monocytes that could be completely abolished by the addition of polymyxin B. In a preliminary study, there was no activation of lipopolysaccharide binding protein during endotoxemia or sepsis. Arch. Surg. 129:220–226.

In conclusion, our findings suggest that sCD14 is an important modulator of plasma ENC in the perioperative course, regulating the endotoxin-dependent biological activity of plasma.

REFERENCES


FIG. 7. Loss of sCD14 and ENC in plasma from six patients with biologically active endotoxin and 11 patients without biologically active endotoxin. (A) The postoperative sCD14 plasma content is given on the y axis as a percentage (postoperative sCD14/preoperative sCD14 × 100). (B) ENC is shown on the y axis as the recovery of LPS exogenously added to the plasma samples.


