Detection of Circulating Lipopolysaccharide-Bound Monocytes in Children with Gram-Negative Sepsis

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The possibility that gram-negative sepsis can be diagnosed by detection of lipopolysaccharide (LPS) bound on the surface of monocytes in the circulation of patients with gram-negative sepsis was investigated. Peripheral monocytes were analyzed by flow cytometer and an anti-LPS monoclonal antibody in 3 groups: children with gram-negative sepsis, children with gram-positive sepsis, and healthy children. LPS-bound monocytes were found in all patients with gram-negative sepsis but not in children with gram-positive sepsis or in healthy children. Therefore, the flow cytometry method developed for this study may be a novel method for diagnosing gram-negative sepsis.

Sepsis remains a leading cause of death, and gram-negative sepsis is also frequently associated with serious sequelae such as multiple organ failure [1]. Endotoxin (lipopolysaccharide [LPS]) has been suggested as a pivotal factor in such septic syndromes as shock and multiple organ failure. LPS binds to monocytes via CD14 receptor and toll-like receptor [2–4] and also stimulates the synthesis of such monokines as tumor necrosis factor (TNF)–α and interleukin-1 and -6, which contribute to the pathogenesis of septic syndrome [2]. The clinical diagnosis of gram-negative sepsis and endotoxemia is based on a positive blood culture and the detection of endotoxin in the plasma. However, identification of the causative bacterial species requires a blood culture that takes at least overnight, and the results are usually negative after the administration of antibiotics. Clinically important endotoxia may occur when endotoxin is present but below detectable levels. Unfortunately, no quick and accurate test to diagnose gram-negative sepsis and endotoxemia has been developed.

In the present study, we investigated whether we could diagnose gram-negative sepsis by detecting LPS-bound monocytes in the circulation. We previously detected LPS-bound neutrophils in gram-negative sepsis by indirect immunofluorescence staining with mouse anti-LPS monoclonal antibody (MAb) (first antibody) and the phycoerythrin (PE)–conjugated antibody (second antibody) [5]. The same immunofluorescence analysis also recognized LPS, which binds to the surface of neutrophils in vitro via the CD14 receptor, because the anti-CD14 blocking MAb eliminated the LPS binding to the neutrophils [6]. In the present study, we modified a method of direct immunofluorescence staining using anti-LPS MAb conjugated with fluorescein isothiocyanate (FITC) and investigated whether LPS-bound monocytes are found in the circulation of patients with gram-negative sepsis.

Materials and Methods

MAb and reagents. The murine IgM MAb directed against the lipid A component of bacterial endotoxin E5 was provided by Xoma (Berkeley, CA) [7]. This MAb can bind to LPS from a wide range of gram-negative bacteria [8]. The E5 was labeled with FITC with a conjugation kit (FluoroTag; Sigma Chemical, St. Louis). In addition, we prepared PE-conjugated anti-CD14 MAb, clone TuÈk4 (mouse IgG2a; Dako, Glostrup, Denmark), and the anti-CD14 blocking MAb, clone MEM-18 (mouse IgG1; Monosan, Uden, The Netherlands). Isotype-matched control MAbs (IgG2a and IgM) were purchased from Dako. LPS derived from Escherichia coli serotype F-583 and PMA were purchased from Sigma. TNF-α and IFN-γ were purchased from R&D Systems (Minneapolis). The endotoxin content of the reagents, PBS, and medium was <3 pg/mL as determined by limulus amebocyte lysate (LAL) assay (Endospecy test; Seikagaku, Tokyo).

In vitro assay of LPS binding to monocytes. Heparinized peripheral blood was obtained from healthy adult volunteers, and mononuclear cells were immediately isolated by density gradient centrifugation using Mono-Poly resolving medium (ICN Biochemicals, Costa Mesa, CA). After 2 washes with RPMI 1640, the cells (10⁶/mL) were suspended in RPMI 1640 plus 10% human serum and incubated for 1 h at 37°C in the presence or absence of LPS (100 ng/mL), PMA (10 nM), or TNF-α (10 ng/mL) plus IFN-γ (10 ng/mL). To determine whether LPS binds to monocytes via
CD14, anti-CD14 blocking MAb (MEM-18, 10 μg/mL) was added to the cells in the culture medium just before exposure to LPS.

**Patients and sample preparations.** We studied 5 healthy children (ages, 4–20 months; median, 12), 5 children with gram-negative sepsis (1 with *E. coli*, 4 with *Haemophilus influenzae*, ages 1 month–3 years; median, 11 months), and 4 children with gram-positive sepsis (*Streptococcus pneumoniae*; ages, 9 months–3 years; median, 19 months). Blood samples were obtained from all patients in the acute phase during the febrile period. After peripheral blood mononuclear cells were immediately isolated, they were suspended in PBS containing 1% bovine serum albumin. The plasma level of endotoxin was estimated by LAL assay (Endospecy test).

**Staining procedure and flow cytometric analysis.** Cells (5 x 10⁷/mL) were incubated with FITC-conjugated E5 or the non-specific mouse IgM FITC for 30 min at 4°C. After being washed, the cells were exposed to PE-conjugated anti-CD14 MAb (TuÈk4) or the nonspecific mouse IgG2a PE (negative control MAb) for 30 min at 4°C. All samples were analyzed by flow cytometer (FACS-Calibur; Becton Dickinson, San Jose, CA). After setting the gates around the monocyte population, the data were obtained by the CellQuest software package (Becton Dickinson). The results are shown as single-color histograms with anti-LPS(E5)-FITC and as 2-color diagrams of monocytes stained with anti-LPS(E5)-FITC and anti-CD14-PE. The cursor setting was based on the pattern of isotype negative control MAb. The percentage of double-positive cells, the E5 mean channel fluorescence (MCF), and CD14 MCF were calculated for each sample.

**Results**

After culture of the mononuclear cells isolated from healthy donors in the presence and absence of LPS in vitro, the cells were stained with anti-LPS(E5)-FITC. The monocytes treated with LPS showed an increased number of LPS-positive cells when compared with monocytes treated with medium only, PMA, and cytokines (TNF-α plus IFN-γ; figure 1A). These results provide evidence supporting the validity of the assay for LPS-bound monocytes. Furthermore, the addition of CD14-blocking MAb (MEM-18) abolished the induction of positive staining with anti-LPS MAb, indicating that LPS binds to the surface of the monocytes via CD14 receptors (figure 1B).

After the mononuclear cells were isolated from healthy children and children with sepsis, the cells were immediately stained with anti-LPS(E5)-FITC and anti-CD14-PE (figure 2). Although no increase in the number of LPS-positive cells was found in the healthy children or in those with gram-positive sepsis according to the histograms, an increase in the number of LPS-positive cells was observed in the children with gram-negative sepsis. In 2-color diagrams of healthy children and patients with gram-positive sepsis, most cells were LPS negative and CD14 positive: There was no dramatic increase in the number of LPS-positive and CD14-positive cells. On the other hand, in children with gram-negative sepsis, the percentage of double-positive (LPS-positive, CD14-positive) cells increased concomitantly with the decreased fluorescence intensity of CD14 expression on the monocytes. Plasma endotoxin was detectable in 2 of 5 children with gram-negative sepsis by the LAL assay (6 and 10 pg/mL).

**Discussion**

In the present study, direct immunofluorescence staining with fluorescence-conjugated anti-LPS MAb (E5) demonstrated LPS-bound monocytes in all patients with gram-negative sepsis but not in the patients with gram-positive sepsis or in healthy children. The presence of circulating LPS-bound monocytes implies that the patients had bacteremia due to gram-negative species and that LPS derived from the outer membranes binds to the surface of monocytes. On the other hand, detectable endotoxin in plasma was found in only 2 of 5 patients with gram-negative sepsis. The significance of the plasma endotoxin concentration remains controversial; different findings have been reported regarding the plasma endotoxin measurements. Guidet et al. [9] reported that the plasma endotoxin concentration could not predict either gram-negative infection or bac-
Figure 2. Lipopolysaccharide (LPS) binding and CD14 expression on circulating monocytes in vivo. Mononuclear cells from healthy children and from patients with gram-negative and gram-positive sepsis were stained with anti-LPS(E5)-fluorescein isothiocyanate (FITC) and anti-CD14-phycocerythrin (PE). In histograms, thin and bold lines indicate cells staining for nonspecific mouse IgM-FITC and anti-LPS(E5)-FITC, respectively. Numbers in quadrants of each plot are % of positive cells. Boxes show mean ± SE of % of double-positive cells, mean channel fluorescence (MCF) of monoclonal antibody (MAb) E5, and MCF of CD14 from entire population in each group.

teremia. Therefore, the flow cytometric analysis developed in the present study may be a more sensitive test for detecting gram-negative sepsis and endotoxemia than the LAL assay. Since the plasma endotoxin level may be influenced by the amount of LPS that binds to the circulating phagocytes (monocytes and neutrophils), further investigations are called for.

We reported elsewhere that LPS binds to the surface of the neutrophils in patients with gram-positive sepsis with use of mouse anti-LPS MAb (E5, first MAb) and anti-mouse MAb-PE (second MAb) [5]. This method of indirect immunofluorescence staining failed to detect any LPS bound on the surface of monocytes [5] because all monocytes were LPS positive (even in the healthy controls). In the present study, direct immunofluorescence staining with anti-LPS(E5)-FITC eliminated false-positive results and we also could differentiate LPS-positive from LPS-negative monocytes.

CD14 is a receptor for LPS, which lacks a transmembrane and cytoplasmic domain and has an abundant expression on the surface of monocytes in comparison with neutrophils [10, 11]. The CD14 molecules are shed from the surface of monocytes in response to LPS in vitro [11, 12]. Clinical observations have shown the CD14 expression on circulating monocytes is down-regulated in the acute phase of gram-negative sepsis [13, 14], while the plasma soluble CD14 levels increase in sepsis, especially in gram-negative sepsis [15]. Our dot plot analysis revealed that CD14 expression decreased more in gram-negative sepsis than in gram-positive sepsis, suggesting that LPS derived from gram-negative bacteria induces the shedding of CD14 molecules from the surface of monocytes. Thus, the 2-color immunofluorescence analysis with anti-LPS(E5) and anti-CD14 MAbs is considered to be useful for detection of LPS-bound monocytes associated with the decreased CD14 expression as seen in gram-negative sepsis. We believe this new method can assist physicians with more accurate diagnosis of gram-negative sepsis and endotoxemia.

References