Correlation of Antibiotic-Induced Endotoxin Release and Cytokine Production in Escherichia coli—Inoculated Mouse Whole Blood Ex Vivo

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Escherichia coli were incubated in mouse whole blood ex vivo supplemented with β-lactam antibiotics that possessed preferential affinities for penicillin-binding proteins (PBPs). After 4 h, viable bacteria were undetectable in the presence of any of the 3 antibiotics tested, whereas significant increases in colony-forming units were detected in samples not treated with antibiotics. Differential levels of endotoxin in platelet-rich plasma were detected using the limulus amebocyte lysate assay, according to differential antibiotic affinities for the various PBPs. Levels of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in antibiotic-treated cultures after 8 h of incubation correlated well with the levels of endotoxin at 4 h \( r = .96, P < .0001 \) for TNF-α; \( r = .91, P = .0002 \) for IL-6. These data indicate that differential affinities of β-lactam antibiotics for PBPs affect both endotoxin and cytokine responses ex vivo in mouse blood and correlate with in vivo protective efficacy of these antibiotics in gram-negative experimental models.

Over the past several decades, in vitro and in vivo data from many laboratories have provided compelling support for the concept that endotoxin derived from the gram-negative microbe may play a pivotal role in the pathogenesis of the sepsis syndrome [1, 2]. It has been increasingly appreciated that free, nonmicrobe-associated endotoxin is significantly more biologically active than is endotoxin associated with the outer cell membrane of gram-negative microbes. Thus, the concept that the release of endotoxin, both in rate and extent, might be an important variable has been subject to experimental investigation [3]. Antibiotics, particularly those that act as inhibitors of cell wall biosynthesis, have been studied as potential variables that may influence endotoxin release. There is now an abundance of experimental data from in vitro and in vivo animal models that different cell wall–active antibiotics differ in their ability to cause endotoxin release from gram-negative microbes [4–8].

Although analyses with experimental animal models (primarily mice and rats) have been used extensively to increase our understanding of the in vivo physiologic significance of this phenomenon, these species often must be treated to make them sensitive to endotoxin, infection, or both. Most mouse and rat strains are intrinsically relatively resistant to the pathophysiologic effect of endotoxin. Because one of the ultimate goals of these studies is to contribute to potential therapeutic intervention strategies for treatment of septic patients, the clinical relevance of the findings has been considered within the framework of differential antibiotic-induced endotoxin release during treatment of systemic gram-negative bacterial infection [9, 10]. However, to date, relatively few studies have directly addressed this question, at least in part because of the difficulty in designing well-controlled clinical studies [11]. The available clinical evidence is nevertheless not inconsistent with the conclusion that, all other things being equal, antibiotic-induced release of endotoxin may contribute to the pathophysiology of the sepsis syndrome [12, 13].

With regard to antibiotic-induced endotoxin release, much attention has been directed to cell wall–active β-lactam antibiotics [4]. Variance in affinities of the individual antibiotics for penicillin-binding proteins (PBPs) exists even within a given subclass, and PBPs affect bacterial growth, morphology, and lysis, resulting in different amounts of endotoxin being liberated from microbes. Imipenem is a subclass of carbapenem antibiotic that binds preferentially to PBP-2 of Escherichia coli. Ceftazidime, in contrast, is a cephalosporin that binds to PBP-3. Meropenem is also a carbapenem, and it binds preferentially to PBP-2 and PBP-3 [4, 14]. PBP-1, -2, and -3 inhibitors can induce bacterial lysis and spheroplast and filament formation, respectively [15]. It has been proposed that the propensities of these antibiotics to cause release of endotoxin depend on the cell mass related to microbial morphology.

We previously reported in vivo lethality data that strongly support the conclusion that antibiotic-induced endotoxin release may contribute to the pathogenesis of experimental sepsis [8]. In vivo, in vitro, and ex vivo experiments have examined
the differential effects of these antibiotics. The ex vivo experiments used human whole blood from both healthy people and septic patients [16, 17]. These data suggested potential clinical significance of antibiotic-induced endotoxin release. However, to fully extrapolate the data from these in vitro, ex vivo, and in vivo experimental models of sepsis into human sepsis or other animals on the basis of mechanism, it will be important to correlate data from experimental animal models with both in vitro and ex vivo experimental approaches.

Here we report the results of an ex vivo study in which we used whole blood from the same mouse strain that we used previously for in vivo experiments [8]. We also correlated our findings with in vitro and in vivo data.

Materials and Methods

Mice. We used 10- to 12-week-old female CF-1 outbred mice (Harlan Sprague-Dawley, Indianapolis). Mice were housed 5 per cage and allowed to rest ≥5 days prior to experimentation.

Bacteria. E. coli O111:B4 (List Biological Laboratories, Campbell, CA) was used throughout the experiments. Bacteria were cultured and prepared exactly as described by Bucklin and Campbell [9].

Antibiotics. Stock solutions of ceftazidime (Eli Lilly, Philadelphia), imipenem-cilastatin (Merck, West Point, PA), and meropenem (Zeneca Pharmaceuticals, Wilmington, DE) were prepared according to manufacturers’ instructions and stored at −70°C. The MICs for E. coli O111:B4 were determined as previously described [8]. They were 0.25 μg/mL for both ceftazidime and imipenem and 0.03 μg/mL for meropenem.

Whole blood stimulation. Mice were anesthetized intraperitoneally with 60 μg/g of body weight with pentobarbital sodium (Abbott Laboratories, Abbott Park, IL). Blood was collected with tuberculin syringes containing ~0.05 mL of 50 U of heparin (Eli-Lilly, Cherry Hill, NJ) by cardiac puncture and pooled in 50 mL of sterile conical tubes on ice under pyrogen-free condition. Aliquots of 0.9 mL of blood were dispensed into borosilicate glass tubes that had been baked at 230°C overnight before use. After samples were warmed to 37°C, 0.05 mL of log-phase bacterial suspension (2 × 10^7 cfu/mL) was added as was 0.05 mL of 180 μL of trypticase soy broth in microtiter plates and plated onto MacConkey agar (Difco, Detroit) in duplicate. After overnight incubation at 37°C, colony-forming units (cfu) were counted. The detection limit in blood was 10^2 cfu/mL.

Endotoxin assay. Total endotoxin present in PRP was determined by a chromogenic limulus amebocyte lysate (LAL) assay with a 0.01 EU/mL detection limit (BioWhittaker, Walkersville, MD) in accord with the manufacturer’s instructions. The plasma was diluted 10-fold with pyrogen-free water and incubated at 70°C for 5 min as a pretreatment before the LAL assay.

Interleukin-6 (IL-6) assay. We measured mouse IL-6 by an ELISA with a 15 pg/mL detection limit (PharMingen, Cambridge, MA) following the manufacturer’s instructions. Plasma was diluted 5-fold for the assay.

Tumor necrosis factor-α (TNF-α) assay. TNF-α was measured by a mouse ELISA kit (Genzyme, Cambridge, MA) with a 35 pg/mL detection limit. Again, we followed the manufacturer’s instructions. Plasma was diluted 5-fold for the assay.

Endotoxin levels. Blood (20 μL) was collected at 4 or 8 h of incubation from the blood cultures incubated with 10^7 cfu/mL at 4 h of incubation and 10^6 cfu/mL at 8 h of incubation, confirming the inability of mouse plasma to kill E. coli. At 50× the MIC of all 3 antibiotics, bacteria were undetectable in all cultures at 4 and 8 h of incubation. At 5× the MIC, viable bacteria were undetectable at 4 h of incubation, but some detectable cfu were present at 8 h of incubation from the blood cultures inoculated with imipenem or meropenem. No bacteria were recovered from the culture treated with 5× the MIC of ceftazidime.

Results

Bacterial recovery. Our experiments were designed to assess the relative efficacy of 3 antibiotics—imipenem, meropenem, and ceftazidime—in killing E. coli in whole mouse blood and the consequences of bacterial killing on endotoxin release and induction of cytokine secretion. For these studies, E. coli at 10^7 cfu/mL were incubated in 1.0 mL of whole anticoagulated mouse blood in the presence of 5× or 50× the MIC of the antibiotics. Control bacteria were incubated in blood in the absence of antibiotics. After 4 and 8 h, samples were assessed for cfu of viable organisms and for endotoxin and the cytokines TNF-α and IL-6 as described in Materials and Methods.

As shown by the data in figure 1A, the viable bacteria in the control cultures inoculated with bacteria in the absence of antibiotics increased to 10^7 cfu/mL at 4 h of incubation and 10^6 cfu/mL at 8 h of incubation, confirming the inability of mouse plasma to kill E. coli. At 50× the MIC of all 3 antibiotics, bacteria were undetectable in all cultures at 4 and 8 h of incubation. At 5× the MIC, viable bacteria were undetectable at 4 h of incubation, but some detectable cfu were present at 8 h of incubation from the blood cultures inoculated with imipenem or meropenem. No bacteria were recovered from the culture treated with 5× the MIC of ceftazidime.

Endotoxin levels. For all of the studies, great efforts were made to ensure that there was no contribution of exogenous environmental endotoxin. After incubation for 4 or 8 h, whole blood was centrifuged, and endotoxin levels in platelet-rich plasma were assessed. As shown in figure 1B, in control cul-
Figure 1. Effect of antibiotics on bacterial growth, endotoxin level, interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) at 5× and 50× MICs at 4 and 8 h of incubation (○, control; △, meropenem [MERO]; ▲, ceftazidime [CEF]; ◆, imipenem [IPM]). All data points are average of duplicate determinations that routinely differed by <10% from each other. Data are representative of 3 experiments with ceftazidime and imipenem and 2 with meropenem. All gave same essential findings.

A. Growth of E. coli O111:B4

![Graph showing bacterial growth at 5x MIC and 50x MIC for MERO, CEF, and IPM compared to control.]

B. ENDOTOXIN

![Graph showing endotoxin levels at 5x MIC and 50x MIC for MERO, CEF, and IPM compared to control.]

C. IL-6

![Graph showing IL-6 levels at 5x MIC and 50x MIC for MERO, CEF, and IPM compared to control.]

D. TNF-α

![Graph showing TNF-α levels at 5x MIC and 50x MIC for MERO, CEF, and IPM compared to control.]

tures not treated with antibiotics, endotoxin levels increased during the 8-h observation period. In contrast to these results, the levels of endotoxin detected in cultures treated with the various antibiotics were strikingly different at 4 and 8 h of incubation, depending on the MIC. At 5× the MIC, the endotoxin levels in cultures incubated with meropenem or ceftazidime were much higher than in controls or in cultures treated with imipenem, even 4 h after treatment. Of interest, the endotoxin levels in cultures treated with meropenem at 5× the MIC at both time points were higher than those treated with
Cytokines in the white blood cells present in the ex vivo culture, and endotoxin levels shown in Figure 1B. Endotoxin levels with antibiotics-treated cultures, although at the MIC, were significantly lower than with cultures incubated with antibiotics imipenem or meropenem. Definitive conclusions are difficult because of the propensity of ceftazidime to cause filamentation that may be misleading with respect to precision quantitation of cfu. Of importance, the levels of endotoxin, IL-6, and TNF-α varied significantly, depending on antibiotic: The lowest levels of endotoxin and cytokines were observed with imipenem. At both 5× and 50× the MIC, the levels in cultures inoculated with antibiotics alone were ∼560–680 pg/mL. TNF-α levels. As shown in Figure 1D, TNF-α was detected only after 8 h of incubation in all of the cultures. In the absence of antibiotics, only very low minimally detectable levels of TNF-α were observed. At both antibiotic concentrations (50× and 5× the MIC), the levels of TNF-α in cultures with meropenem or ceftazidime were again significantly higher than those observed with imipenem treatment. At 5× the MIC, the level with meropenem was much higher than with ceftazidime. The levels in cultures inoculated with antibiotics alone were below the detection limit.

Correlation analysis. The general concept that might explain these observations is that endotoxin released from E. coli by the action of the antibiotics stimulates white blood cells to secrete TNF-α and IL-6. As a consequence, it would be predicted that cytokine levels should correlate with levels of free endotoxin generated by the antibiotic treatment. We therefore assessed whether such a correlation exists by comparing cytokine levels with measured endotoxin levels in all of the antibiotic-treated cultures. (For reasons discussed below, we excluded nonantibiotic-treated cultures from analysis.) On the basis of the postulated time lag between the release of endotoxin and production of cytokines, correlation analyses were sought between endotoxin levels at 4 h of incubation and cytokine levels at 8 h of incubation. A significant correlation among antibiotic-treated cultures was detected between the levels of endotoxin and IL-6 (Figure 2: \( r = .91, n = 9, P = .0002 \)), endotoxin and TNF-α (Figure 2: \( r = .96, n = 9, P < .0001 \)), and IL-6 and TNF-α (Figure 3: \( r = .90, n = 9, P = .0004 \)).

Discussion

All 3 β-lactam antibiotics inhibited bacterial growth during the first 8 h of incubation relative to control nonantibiotic-treated cultures, although at 5× the MIC, the ceftazidime appeared to be somewhat more bactericidal than either imipenem or meropenem. Definitive conclusions are difficult because of the propensity of ceftazidime to cause filamentation that may be misleading with respect to precise quantitation of cfu. Of importance, the levels of endotoxin, IL-6, and TNF-α varied significantly, depending on antibiotic: The lowest levels of endotoxin and cytokines were observed with imipenem. At both 5× and 50× the MICs, ceftazidime, a cephalosporin, and PBP-1 and -3 inhibitors of E. coli induced ∼3 times as much endotoxin as imipenem, a carbapenem, and PBP-1 and -2 inhibitor [15]. Of interest, meropenem (a carbapenem known to inhibit the growth of E. coli by binding to PBP-2 and -3) induced ∼3 times (at 50× the MIC) and 4 times (at 5× the MIC) more endotoxin than did imipenem. Jackson and Kropp [4] reported that at high meropenem concentrations (10× the MIC), the PBP-2 enzyme was primarily inhibited, whereas PBP-3 was inhibited more effectively at lower or sub-MIC concentrations (0.25–2.0× the MIC) in Pseudomonas aeruginosa. The same underlying mechanism may serve to explain the results described here; however, until additional studies are done, these findings cannot be extrapolated to all gram-negative bacteria.

**Figure 2.** Correlations between levels of endotoxin at 4 h of incubation and cytokines at 8 h of incubation among antibiotic-treated bacterial cultures: endotoxin vs. tumor necrosis factor-α (TNF-α): \( r = .96, n = 9, P < .0001 \); endotoxin vs. interleukin-6 (IL-6): \( r = .91, P = .0002 \) by analysis of Pearson’s correlation coefficient and Fisher’s z for calculation of probability. Regression lines were made by least-squares method.
microorganisms. In addition, since recent studies from our laboratory showed that different clinical isolates of gram-negative organisms have LPSs that differ significantly in biological potency [18], the effect of differential LPS release may not be reflected in cytokine generation.

The levels of IL-6 and TNF-α, both well-known proinflammatory cytokines in sepsis [19], were significantly correlated with the level of endotoxin in antibiotic-treated cultures but not with bacterial growth indicated by cfu. Of potential importance, cytokine levels (especially between antibiotic-treated groups and untreated controls) did not always parallel those of endotoxin. For example, relatively high levels of endotoxin were observed after 8 h in the control nonantibiotic-treated cultures; however, this was not accompanied by significant production of either IL-6 or TNF-α. These findings suggest that the biologic activity of endotoxin may differ between antibiotic-treated and nontreated bacteria and/or free and cell-bound endotoxin.

Earlier studies from our laboratory [20] found that soluble LPS is significantly more active than particulate (microbe-associated) LPS in inducing procoagulant activity in human monocytes. These findings suggest that the primary source of endotoxin activity measured in the present studies in the platelet-rich plasma was, in fact, free endotoxin. However, differences in biologic activity among endotoxins induced by different antibiotics remain to be established. We have also found a high correlation between total TNF-α and IL-6 levels. Our ex vivo data therefore correspond well with previous in vitro data showing that differential levels of endotoxin can be induced according to differential affinities of antibiotics for PBP's and that immunologic responses, such as TNF-α and IL-6 production, are highly correlated with endotoxin levels.

We previously reported in vivo data using the identical strain of mice and classes of antibiotics as used here [8]. In that study, imipenem showed a much higher protective efficacy than did either ceftazidime or meropenem at doses recommended for clinical use and equivalency in the inhibitory effects on bacterial growth. Considering that meropenem is the same subclass as imipenem but also has an affinity for PBP-3 similar to that of ceftazidime, the present ex vivo data support the concept that higher mortality in experimental models of *E. coli* sepsis may result from differential levels of endotoxin release induced by antibiotics with differential modes of action.

Regarding potential cellular sources of cytokine production in whole blood, Matsumoto et al. [21] reported data suggesting that neutrophils may be the major cells producing TNF-α in response to LPS and determining mouse mortality after LPS challenge in the carrageenan-pretreated mouse model. Others have reported that the liver plays a central role in the regulation of multiple host defense during sepsis [22]. In our ex vivo system, the precise origin of cytokines is not clear.

The outcome of septic deterioration is complicated, and a subtle balance between microbial factors and the immunologic responsiveness of the host may be critical. Nevertheless, our ex vivo system using mouse whole blood is useful for analysis to clarify what may transpire during treatment with antibiotics in vivo during sepsis and for interpreting accumulating in vitro and in vivo data by facilitating extrapolation with each other. Collectively, our data provide clear evidence that, at least in this relatively straightforward environment, antibiotics may affect the bacterial endotoxin level according to differential affinities for PBPs and corresponding immunologic responses, such as TNF-α and IL-6 production.

**References**


18. Luchi M, Worley P, Morrison DC. Lipopolysaccharides (LPS) from clinical isolates of gram-negative bacteria (GNB) have diverse levels of activity as measured by the limulus amebocyte lysate (LAL) assay [abstract B-85]. In: Program and abstracts of the 35th Interscience Conference on Antimicrobial Agents and Chemotherapy. Toronto: American Society for Microbiology, 1997:42.