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Review

Antibiotic-Induced Endotoxin Release from Bacteria and Its Clinical Significance

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Introduction

In patients with Gram-negative bacterial infection, the administration of appropriate antibiotics is well recognized as an important chemotherapeutic strategy to reduce morbidity and mortality. The requisite use of antibiotics, however, is thought to contribute, in some cases, to serious side effects such as endotoxin shock, because of the release of biologically active endotoxin from the bacteria due to the action of the antibiotics. Infection-associated sepsis, followed by septic shock, is known to be one of the leading causes of death in immunocompromised patients and in the elderly. It is often stated that the incidence of sepsis is estimated to be approximately 300,000 patients per year in the United States, with an approximate 20-30% mortality from septic shock caused by Gram-negative bacterial infection (5, 54). Septic patients with severe underlying illnesses have a high rate of mortality independent of intensive antibiotic treatment (42). The inadequate choice of antibiotics may also enhance mortality caused by the release of large amounts of endotoxin from microbes (19, 63). In this review, we summarize some of the characteristics and biological activities of endotoxin released from microbes by the action of antibiotics, and discuss the potential clinical significance of this endotoxin in the pathophysiological manifestations of sepsis.

Structure and Characteristics of Endotoxin

Endotoxin has been well established to be a major cell wall constituent of Gram-negative bacteria. It can be extracted from either intact microbes or isolated cell wall membranes as lipopolysaccharide (LPS) by treatment with hot phenol-water (80) or other procedures (23). LPS consists of polysaccharide and a covalently associated lipid constituent, the lipid termed lipid A (Fig. 1). LPS extracted from the so-called smooth or S-form organisms of *Enterobacteriaceae* (S-LPS) forms high molecular weight aggregate structures containing both *O*-polysaccharide-R-core-lipid A subunits and R-core-lipid A subunits. Although LPS extracted from R-form organisms (R-LPS) also forms high molecular weight aggregates, the aggregates are significantly less heterogeneous and consist exclusively of R-core-lipid A (72). Lipid A has been well documented to be the chemical principally responsible for the endotoxic activity of LPS (29, 41, 58). The primary structure of lipid A is well conserved among different species of Gram-negative microbes although minor differences in the lipid A structure are known to exist among species. These differences may reflect on the toxicity of different preparations of endotoxin (7, 48).

Purified LPS administered to experimental animals or human volunteers evokes a number of pathophysiological effects characteristic of those observed in severe sepsis, including fever or hypothermia, tachycardia, tachypnea, leukopenia or leukocytosis, and depression of blood pressure (66). The last decade has witnessed a vast increase in knowledge of the molecular mechanisms of LPS-mediated pathogenesis *in vivo* and *in vitro*, including LPS-induced release of a variety of biologically active pro- and anti-inflammatory mediators, such as

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Abbreviations: CAZ, ceftazidime; CSF, cerebrospinal fluid; CTX, cefotaxime; IPM, imipenem; LAL, *Limulus* amebocyte lysate; LBP, LPS-binding protein; LPS, lipopolysaccharide; NO, nitric oxide; PBP, penicillin-binding protein; PW-LPS, phenol-water extracted LPS; TNF, tumor necrosis factor.

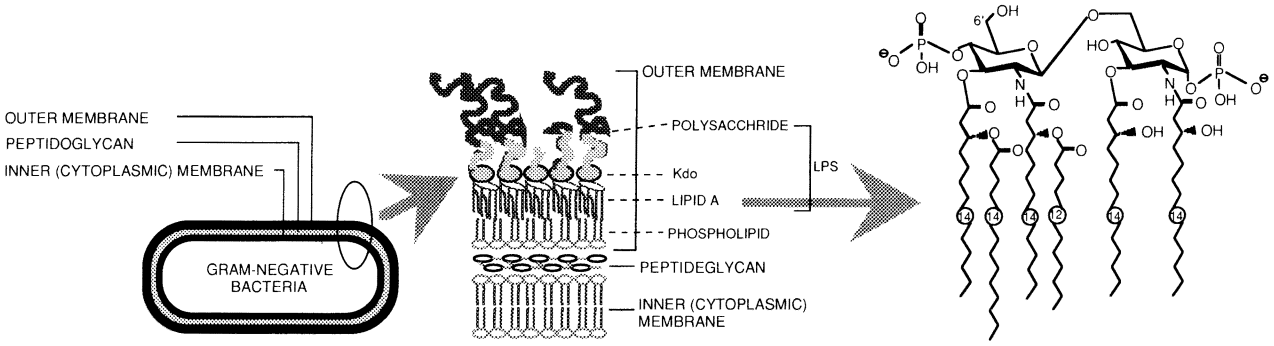

Fig. 1. Architecture of outer membrane of Gram-negative bacteria, and chemical structure of lipid A. LPS resides in the outer membrane of Gram-negative bacteria, and consists of a polysaccharide chain and phosphoglycolipid called lipid A which binds to 2-keto-3-deoxy-octonic acid of the polysaccharide at position 6′.

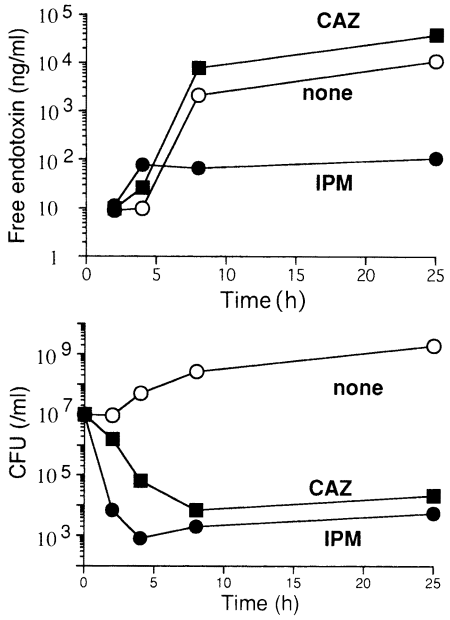

Fig. 2. Time course of bacterial growth and antibiotic-induced release of endotoxin from *Pseudomonas aeruginosa* PAO1 cultured in synthetic M9 medium in the absence (○) or presence of 2×MIC of CAZ (■) or IPM (●) for the indicated time. The amounts of endotoxin in the supernatants (top) and the number of CFUs in the cultures (bottom) were determined.

immunologic cytokines, arachidonic acid metabolites and nitric oxide (NO) (reviewed in (55)).

Release of Endotoxin from Gram-Negative Bacteria

Endotoxin can be released from the outer membrane of bacteria, not only under conditions that are deleterious to the microbes but also from viable growing cells (Fig. 2) (13, 27, 38). Phagocytic cells and a spectrum of antibacterial substances, such as antibodies, complements and lysozymes, are all known to kill the microbes and can result in the release of endotoxin. Antibiotics, especially those that target microbial cell walls, disrupt the integrity of the cell walls and subsequently allow the release of endotoxin. Endotoxin released by any of these mechanisms has, at least, the potential to contribute to the inflammation and tissue damage that are observed in septic patients (19).

Antibiotic-Induced Endotoxin Release

The amount of endotoxin released from bacteria exposed to antibiotics can be variable depending on: a) the bacterial species, because of different sensitivities of individual bacterial species to various antibiotics; b) the type and target of the antibiotic; c) the concentration of the antibiotic; and d) the duration of exposure of the bacteria to the antibiotic (17, 36). In addition, the presence of antibodies and/or other serum constituents that can interact with endotoxin affects the activity of the released endotoxin (43).

Beta-lactam antibiotics bind various penicillin-binding proteins (PBPs), cell wall synthesizing enzymes, on the surface of the bacteria and inhibit cell wall biosynthesis. Penicillin, moxalactam, cefotaxime, ceftriaxone (15) and meropenem (36) have been shown to induce relatively high levels of endotoxin release from Gram-negative bacteria *in vitro*. Cephalosporins induce the release of larger amounts of endotoxin from bacteria as compared with carbapenem antibiotics. Among the beta-lactam antibiotics, imipenem (IPM) induces the release of lesser amounts of endotoxin, a level similar to that induced by antibiotics of the aminoglycoside and quinolone groups (63, 65).

The differences in the extent of effect of different beta-lactam antibiotics on the induction of endotoxin release can be, in part, explained by their differential binding to and inhibition of the different PBPs (60). *Escherichia coli* is known to have a number of PBPs among which the three named PBP-1, PBP-2 and PBP-3, are most important. The binding of antimicrobial

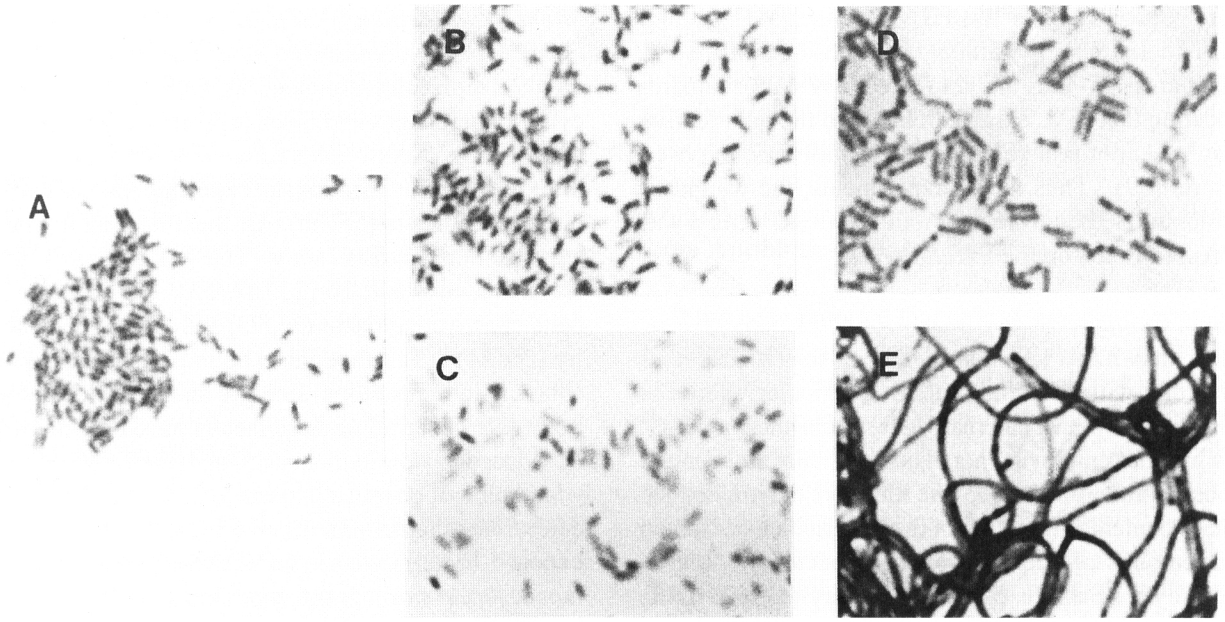

Fig. 3. Photomicrographs of *P. aeruginosa* cultured in the absence (A) or presence of 2 × MIC of IPM for 4 (B) and 8 hr (C), or 2 × MIC of CAZ for 4 (D) and 8 hr (E).

agents to one or more of these PBPs inhibits the PBP function and results in the killing of bacteria and release of endotoxin. Inhibition of the PBP-1 function causes rapid killing and lysis of bacteria, while inhibition of the PBP-2 function results in the formation of spherical non-growing cells, spheroplasts. The binding of beta-lactam antibiotics to PBP-3 causes striking changes in bacterial morphology, characterized by the emergence of long filamentous forms of non-septated bacteria (60, 77). The filamentous cells have been demonstrated to release significantly larger quantities of endotoxin than the spheroplasts do (14, 15, 36, 53, 59, 60, 77). Among beta-lactam antibiotics, IPM binds preferentially to PBP-2 in bacteria and causes inhibition of the growth of the bacteria, spheroplast formation and subsequent bacteriolysis, as described. Ceftazidime (CAZ) binds to PBP-3 at lower concentrations (25, 60). Figures 2 and 3 show the results of one of our studies (59) that demonstrated the relation between PBP selectivity of antibiotic and amount of endotoxin released from or morphological changes in bacteria exposed to the antibiotic. As shown in Fig. 2, *Pseudomonas aeruginosa* cultured in the chemically defined medium M9 (3) in the presence of a low concentration of either IPM or CAZ showed a rapid decrease of viable numbers, as determined by cfu assay; indicating that IPM and CAZ are equally effective in killing the *P. aeruginosa* strain used in the study. However, the amounts of endotoxin released in the culture supernatants determined by *Limulus* amebocyte lysate (LAL) assay, which has been proved to be the most sensitive method for the detection of endotoxin (34) and reliable for the assay of endotoxin released in cultures of antibiotic-exposed bacteria (as to be described in the following section), were quite different between the cultures with IPM and those with CAZ. Thus, *P. aeruginosa* cultured in the presence of CAZ released large amounts of endotoxin, whereas *P. aeruginosa* cultured in the presence of IPM released small amounts of endotoxin and, at 8 hr of culturing, far smaller amounts than those released by the bacteria growing in the absence of the antibiotic (59). Figure 3A–E shows photomicrographs of *P. aeruginosa* in these cultures. The bacteria exposed to IPM, which bound to PBP-2 and caused the release of only small amounts of endotoxin, show spheroplast formation, while the bacteria exposed to CAZ, which bound to PBP-3 and caused the release of large amounts of endotoxin, show the formation of long filamentous non-septated cells.

Characteristics of Endotoxin Released from PBP-1/3 Binding Antibiotic-Exposed Bacteria

Firstly, we determined whether LAL assay is reliable in a quantitative assay of endotoxin released in the culture supernatant of antibiotic-treated bacteria, because we should compare the characteristics of endotoxin released from antibiotic-exposed bacteria with those of endotoxin chemically extracted from bacteria. Linear regression analyses (61) were done on the data of LAL assays performed on dilutions of culture supernatants of *P. aeruginosa* exposed to CAZ or IPM (CAZ-endotoxin and IPM-endotoxin) and compared with those on phenol-

water extracted LPS (PW-LPS). The lines of CAZ- and IPM-endotoxin were parallel with those of purified *P. aeruginosa* PW-LPS and an *E. coli* PW-LPS standard, indicating that LAL assay is reliable in the quantitative assay of endotoxin released from antibiotic-exposed bacteria (59). Thus, in all experiments, the weight of endotoxin released from antibiotic-exposed bacteria was expressed based on the LAL assay.

Protein Content

It is known that LPS has a tendency to associate noncovalently with bacterial proteins, particularly those in the outer membrane, and that vigorous chemical methods such as those employing hot aqueous phenol are required to obtain protein-free LPS. We therefore determined the protein content of the LPS in the supernatants obtained from cultures of antibiotic-exposed bacteria. As estimated by a sensitive DC protein assay (1), the CAZ-endotoxin of *P. aeruginosa* contained relatively large amounts of protein (3,200 μg/100 μg LPS). In contrast, PW-LPS was virtually protein-free, containing less than 0.5 μg protein/100 μg LPS (59).

Lethal Toxicity in Mice

D(+)-galactosamine (GalN)-sensitized mice are extremely sensitive to the lethal effect of LPS (22). CAZ-endotoxin was also toxic to GalN-sensitized mice. After the normalization of LPS content by LAL assay, the LD_{50} of the CAZ-endotoxin and PW-LPS in GalN-sensitized C3H/HeN mice was found to be 10 and 32 ng, respectively. These results suggest that the lethal toxicity of LPS released from antibiotic-exposed bacteria is greater than that of PW-LPS, and the findings are clinically worthy of noting. Neither preparation showed lethal toxicity to the LPS-hyporesponsive C3H/HeJ mice, and even in GalN-sensitized LPS-hyporesponder C3H/HeJ mice, the LD_{50} of CAZ-endotoxin and PW-LPS was more than 1,000 ng (59).

Monocyte/Macrophage Activation

There exists abundant evidence that proinflammatory effector molecules produced by LPS-activated monocytes/macrophages and other cells are largely responsible for the systemic inflammatory responses observed after LPS administration to experimental animals or human volunteers (55, 66). Of the inflammatory mediators, tumor necrosis factor (TNF) (12, 55, 76), interleukins 1, 6, 8 and 10 (21, 47, 55), NO (45, 75, 78), platelet-activating factor (33, 73) and procoagulant activity (26, 44) are all thought to be important factors contributing to the pathophysiologic manifestations of septic shock. Since all of these factors have been shown to be induced *in vitro* in monocytes and macrophages by the stimulation of chemically purified LPS, we attempted to determine whether endotoxin released from antibiotic-exposed bacteria also induces these factors in human monocytes *in vitro*. In this experiment, we found that the culture supernatant of *E. coli* exposed to CAZ contained many components other than LPS, and some of them might induce procoagulant activity and secretion of TNF in human monocytes *in vitro* (44). Therefore, the culture supernatants of CAZ-exposed *E. coli* were fractionated by velocity sedimentation, and each fraction was tested for the content of LPS by LAL assay and the *in vitro* biological activity on human monocytes. The predominant monocyte-stimulating activity (i.e., activity to induce inflammatory mediators in monocytes) in the supernatants was identified with LPS-containing fractions, but not protein-rich fractions (44). Thus, it was demonstrated that endotoxin released from antibiotic-exposed bacteria can exert biological activity similar to chemically purified LPS.

We also studied the responses of mouse peritoneal exudate macrophages to CAZ-endotoxin and PW-LPS *in vitro*. Macrophages from LPS-responsive C3H/HeN mice produced high levels of both TNF and NO in response to either of both stimuli, whereas LPS-hyporesponsive C3H/HeJ macrophages responded only mini-

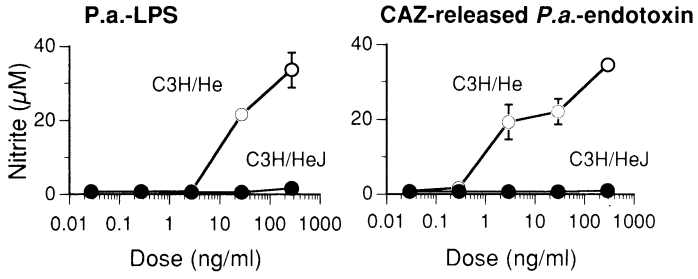

Fig. 4. NO production by LPS-responder (C3H/HeN) and -hyporesponder (C3H/HeJ) macrophages stimulated with endotoxin released from antibiotic-exposed bacteria. Thioglycollate-elicited macrophages from C3H/HeN (○) and C3H/HeJ (●) mice were incubated for 48 hr with varying doses of LPS extracted from *P. aeruginosa* with phenol-water (left) or CAZ-endotoxin from *P. aeruginosa* (right). NO production was measured as nitrite in the supernatants with Griess reagent.

mally to either of the stimulants (Fig. 4) (59). In the initial step of activation of macrophages by LPS, phosphorylation of cellular proteins takes place (4, 70, 79). We determined the protein phosphorylation in macrophages stimulated with CAZ-endotoxin by assaying the activities of MAP-kinases 1 and 2. The activation of MAP-kinases 1 and 2 was detected by immunoblot analysis in the macrophages from LPS-responsive C3H/HeN mice, but not in those from LPS-hyporesponsive C3H/HeJ mice, in response to CAZ-endotoxin (unpublished observation). Thus, it appears that CAZ-endotoxin induces the early cellular biochemical events in macrophages, which is known to take place following stimulation with PW-LPS.

Requirement of Co-Factors for Macrophage Activation by CAZ-Endotoxin

It is well documented that serum components, such as LPS-binding protein (LBP) (67) and septin (81), catalyze the molecular association of LPS with both soluble CD14 and cell membrane-bound CD14. This catalytic event is thought to be important for the CD14-dependent signaling response to the LPS in macrophages. C3H/HeN macrophages cultured in the absence of the serum showed a reduction in their ability to produce TNF and NO in response to CAZ-endotoxin as well as to PW-LPS (unpublished observation). For expression of the full potency of endotoxin released from antibiotic-exposed bacteria, serum factor(s) seems to be required like in the case of PW-LPS.

LPS-resistant J7.DEF3 cells are a mutant cell line established in our laboratory from the CD14-expressing murine macrophage-like cell line J774.1 (39, 40). The mutant cell line does not express CD14 antigen and manifests an altered response to LPS. The parental J774.1 cells cultured in the presence, but not in the absence of the serum produced TNF in response to CAZ-endotoxin like PW-LPS, while the mutant J7.DEF cells responded only minimally to either of the stimulants regardless of the presence or absence of the serum. These findings indicate that endotoxin released from antibiotic-exposed bacteria is mainly recognized by receptors on the macrophages after it is complexed with serum factor(s), presumably LBP and CD14 antigen, like in the case of PW-LPS.

Neutralization of the Endotoxic Activity of CAZ-Endotoxin by Polymyxin B

Polymyxin B has been well documented for its ability to neutralize the biological activity of LPS by binding to the lipid A moiety of LPS (52). We found that the ability of CAZ-endotoxin to induce TNF production by monocytes/macrophages was blocked by polymyxin B in a dose-dependent fashion (data not shown). Thus, it was indicated that the biologically active molecule in CAZ-endotoxin is most likely lipid A (44, 59).

Lipid A-Associated Protein and Endotoxin Released from Antibiotic-Exposed Bacteria

It has been reported that lipid A-associated protein or endotoxin-protein extracted from Gram-negative bacteria can exert lethal toxicity and biological activities similar to LPS in mice, and that its active principle is the protein and not lipid A (6, 11, 37, 46, 52). As described, endotoxin released from antibiotic-exposed bacteria that showed biological activities and characteristics similar to those of LPS contained larger amounts of protein. Therefore, the possibility that the active principle in the endotoxin released from antibiotic-exposed bacteria may be the lipid A-associated protein would arise. However, this possibility is ruled out by the findings described in the preceding sections; that is endotoxin released from antibiotic-exposed bacteria could not exert lethal toxicity or biological activities in LPS-hyporesponsive C3H/HeJ mice and their macrophages (59), whereas lipid A-associated protein could (6, 11, 37, 46, 52).

These findings provide further support that LPS is responsible for the lethal toxicity and biological activities of the endotoxin released from bacteria following antibiotic-induced bacteriolysis, and also suggest the possibility that the cell wall destroyed by antibiotics releases LPS as a free molecule which then binds with protein molecules, and not as a complex of lipid A-associated proteins.

Antibiotic-Induced Endotoxin Release in *In Vivo* Animal Models

A number of experimental studies in animals infected with Gram-negative bacteria have provided direct evidence that antibiotic treatment can promote endotoxin release *in vivo*. In a meningitis and sepsis model of rabbits infected with *E. coli*, both chloramphenicol (CM) and cefotaxime (CTX), a beta-lactam antibiotic, were shown to kill the infecting organisms equally; however, CTX treatment resulted in the emergence of a significantly greater level of endotoxin in the cerebrospinal fluid (CSF) than did CM (74). Treatment of experimental rabbit meningitis caused by *Hemophilis influenzae* with ceftriaxone was also shown to increase the TNF level in CSF (57). In a study of *E. coli* infection in rabbits, Shenep et al (68) showed interesting results that moxalactam induced higher levels of free- and total-endo-

toxin in the serum than those induced by gentamicin despite lower blood bacterial counts than those achieved by gentamicin. These various treatment strategies, however, did not show an obvious influence on the outcome of the infection. A more recent report on a rabbit *E. coli* meningitis model indicated that varying levels of endotoxin were released in CSF following treatment with different antibiotics, and especially high levels emerged by treatment with meropenem or CTX (20). It was also shown that the level of endotoxin correlated with the TNF level in CSF, but not with the clinical course of the disease.

Recently, Bucklin and Morrison (10) reported the results of a study in which the effects of IPM and CAZ on sepsis in *E. coli*-infected GalN-sensitized mice were determined. IPM and CAZ were equally effective in killing the bacteria *in vivo*. However, CAZ was less effective in protection of the mice from death as compared with IPM, implicating a higher level of endotoxin release from CAZ-exposed bacteria than from IPM-exposed bacteria. They also obtained similar results for *P. aeruginosa* infection (9, 10, 54). These results agree well with the results of *in vitro* experiments in that *P. aeruginosa* cultured in the presence of CAZ released far larger amounts of endotoxin as compared with the bacteria cultured in the presence of IPM; though both antibiotics showed equal killing activity on *P. aeruginosa in vitro* (Fig. 2) (59). In addition, Nakano and Kirikae reported that IPM treatment was 100% effective in eliminating mortality in *Pseudomonas*-infected GalN-sensitized mice, whereas the equivalent CAZ treatment actually increased mortality in mice infected with a LD_{50} dose of *P. aeruginosa* (59). Jackson and Kropp (36) also reported a higher efficacy of IPM than CAZ in the protection of cyclophosphamide-treated mice from *P. aeruginosa* infection. Along the line of these studies, Opal et al (62) studied the effects of IPM and CAZ on sepsis models of *E. coli*- or *P. aeruginosa*-infection in GalN-sensitized rats, and showed that IPM treatment resulted in reduced levels of blood-circulating endotoxin and TNF and a lower mortality of the rats as compared with CAZ treatment. They noted, however, that no significant differences were observed between these two antibiotic treatments in the effects on infection with *Klebsiella pneumoniae*.

Clinical Studies

Since early times in the antibiotic era, it has been suggested that, under some circumstances, shock might be caused by bacterial cell lysis and the sudden release of endotoxin from Gram-negative bacteria exposed to antibiotics (24, 30–32, 42, 71). In the late 1980s, Shenep et al (69) demonstrated antibiotic-induced endotoxemia during the treatment of human septicemia. McCartney et al (49) described several patients with a significant rise in circulating endotoxin levels during the treatment of septic shock with antibiotics, which might be, at least in part, caused by antibiotic therapy.

Mustafa et al (56) demonstrated increased endotoxin and interleukin (IL)-1β levels in the CSF of infants with meningitis caused by Gram-negative enteric bacilli during intraventricular gentamicin therapy. These patients had poor outcomes as compared with infants who received intravenous antibiotics alone and had lower endotoxin and IL-1β levels in the CSF. Parallel increases in endotoxin, lactate and lactate hydrogenase in CSF, but decreases in glucose levels in CSF were found after the initiation of treatment with ceftriaxone in children with *H. influenzae* meningitis (2), suggesting that the increased endotoxin level in CSF led to an enhanced inflammatory state.

Brandtzaeg et al (8) noted a strong relationship between total plasma endotoxin level and septic shock, organ failure or mortality in patients with meningococcal disease (septicemia and/or meningitis). They, however, reported that in this disease, the plasma endotoxin level decreased rather than increase after treatment with penicillin and chloramphenicol. Similarly, it was reported that no increase in plasma endotoxin level was found in patients with meningococcemia during the first 2 days after the initiation of antibiotic therapy (18). In contrast, Dofferhoff et al (16) reported an increase in plasma endotoxin levels, especially of free-endotoxin, in patients with sepsis caused by Gram-negative bacteria other than meningococcus after antibiotic therapy.

Recent comparative studies in uroseptic patients treated with IPM or CAZ (64) showed that colony-forming units detected in urine decreased equally in the patients after treatment with either IPM or CAZ. However, blood endotoxin levels in the patients at 4 hr after the treatment were found to decrease with IPM treatment but increase with CAZ treatment. Endotoxin levels in urine decreased with treatment by both antibiotics, but the level achieved using IPM was lower than that achieved using CAZ (63, 64). In addition, it was shown that the levels of cytokines in urine were increased with CAZ treatment but decreased with IPM treatment, suggesting inflammatory response due to endotoxin released by CAZ treatment. Further, they showed that the patients treated with CAZ experienced a slower defervescence (64). In a study of chronically bacteriuric patients (35), a decline in the viable number of bacteria and increase in endotoxin levels were shown in urine after treatments with beta-lactam antibiotics.

The most recent and large-scale clinical study address-

ing the issue of antibiotic-induced release of endotoxin is a retrospective analysis on 416 severely injured trauma patients treated with antibiotics in 9 hospitals (50, 51), and the results of analysis suggested that different types of antibiotics induced different levels of endotoxin release during antibiotic treatment for bacterial infections.

As an interesting finding, in a study of patients in surgical intensive care units, it was reported that a decrease in endotoxin-neutralizing units was found in the sera of patients after the administration of CTX, tobramycin, ceftriaxone or vancomycin, while the administration of IPM or ciprofloxacin increased the serum endotoxin-neutralizing units (28).

Conclusion

Many studies have documented that endotoxin is released both *in vitro* and *in vivo* from Gram-negative bacteria following exposure to antibiotics, particularly to those acting at the level of biosynthesis of cell wall constituents. In antibiotics of the beta-lactam group, PBP selectivity of the antibiotic determines the amount of endotoxin released from bacteria *in vitro*. Although endotoxin released *in vitro* from antibiotic-exposed bacteria contains significant amounts of protein, its biological activities and characteristics, including no lethal toxicity and biological activities in LPS-hyporesponsive C3H/HeJ mice, are quite similar to those of LPS extracted from bacteria by the hot phenol-water procedure, indicating that its biologically active principle is LPS and not lipid A-associated protein. Studies of animal model infections and clinical studies so far performed have indicated that the quantities of endotoxin released from antibiotic-exposed Gram-negative bacteria are quite different depending on the kind of antibiotics used for treatment, species of the infecting bacteria and the type of infectious disease. Further, the clinical studies suggest that therapy of septic patients using some kinds of antibiotics promotes endotoxin release, and consequently worsens symptoms; in some cases, the outcome of the patient, indicating the importance of the choice of antibiotics in the therapy of sepsis. For the selection of the most adequate antibiotics to control Gram-negative bacteria infection (i.e., antibiotics that are effective in killing the infecting bacteria and cause the least release of endotoxin), further studies are necessary.

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