ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Oct. 1996, p. 2410–2412 Vol. 40, No. 10
0066-4804/96/$04.00+0

NOTES

Differential Release of Smooth-Type Lipopolysaccharide from *Pseudomonas aeruginosa* Treated with Carbapenem Antibiotics and Its Relation to Production of Tumor Necrosis Factor Alpha and Nitric Oxide

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Received 29 April 1996/Returned for modification 1 July 1996/Accepted 27 July 1996

Endotoxin release from *Pseudomonas aeruginosa* treated with cell wall-active carbapenem antibiotics and its effect on the production of tumor necrosis factor alpha and nitric oxide were examined. Treatment of bacteria with imipenem induced much lower levels of endotoxin release than treatment with meropenem. The endotoxin released was demonstrated to be of the smooth type and O-specific polysaccharide-rich. The exposure of the filtrates of *P. aeruginosa* treated with imipenem to physiologically relevant cells caused low-level production of tumor necrosis factor alpha and nitric oxide, while similar treatment with meropenem induced high levels of production.

Recently, many in vitro and in vivo studies have shown antibiotic-induced endotoxin release when gram-negative bacteria are exposed to antibiotics (3–8, 11–20, 22), although lipopolysaccharide (LPS) is shed spontaneously during bacterial growth in culture. β-Lactam antibiotics are considered the antibiotics most responsible for the liberation of excessive amounts of LPS. It has been reported that the differences in the propensities of β-lactam antibiotics to release LPS exist among and within the subclasses of β-lactam antibiotics. Of the many β-lactams studied, imipenem (IPM), an antibiotic of the carbapenem subclass, has been shown to cause lower amounts of endotoxin release during bacterial exposure (7, 14, 15). Furthermore, several studies have included exploring the LPS release capabilities of IPM and antibiotics of other β-lactam subclasses (aminoglycosides, etc.) and the subsequent influence of liberated endotoxin on the production of tumor necrosis factor alpha (TNF-α) (2, 5, 17, 21). In the latter studies, filtrates of ceftazidime (CAZ)-treated bacteria were shown to induce high levels of production of TNF in mononuclear cells, whereas filtrates of bacteria similarly treated with IPM induced low levels of TNF-α production. No such studies, however, have been conducted in which the relative abilities of various carbapenems to induce the release of LPS, TNF-α, and NO are compared. In the current studies, we compared two carbapenems, IPM and meropenem (MEPM), with a cephalosporin antibiotic, CAZ, against infection with *Pseudomonas aeruginosa*. The production of proinflammatory mediators such as TNF-α and NO were determined with physiologically relevant cells. Additionally, the relative type of LPS (i.e., smooth versus rough) liberated by antibiotic treatment is also reported.

Stock solutions of CAZ (Tanabe Pharmaceutical Co.), IPM (Banyu Pharmaceutical Co.), and MEPM (Sumitomo Pharmaceutical Co.) were prepared by the methods supplied by the manufacturers. The MICs for *P. aeruginosa* were 0.63 μg/ml for CAZ, 1.25 μg/ml for IPM, and 2.0 μg/ml for MEPM. A single colony of *P. aeruginosa* PAO-1 was selected, suspended in L broth (5 ml), and placed in a test tube with shaking at 37°C overnight. The bacterial suspension (approximately 10 μl) was diluted in Mueller-Hinton broth (1 ml) and was cultured for 2 h with shaking. Log-phase bacteria (approximately 10^5 CFU/100 μl) were added to each 1 ml of decomplemented fetal calf serum containing IMP, MEPM, or CAZ at 2× or 0.5× the MICs and were incubated with shaking for 8 h at 37°C. After incubation, the bacterial suspensions were passed through a 0.22-μm-pore-size filter and the filtrate was collected. The LPS level was determined with Endospecy ES-6 and Toxicolor system DIA-MP (Seikagaku Corp., Tokyo, Japan) instruments. The concentrations of free endotoxin released in the culture fluids are presented in Table 1. Treatment with IPM at 2× the MIC resulted in much lower levels of release of endotoxin in the culture fluid compared with that resulting from treatment with the other antibiotics. Treatment with MEPM and CAZ caused a more than 10-fold increase in the concentration of free endotoxin in the culture fluid compared with treatment with IPM. Similarly, there were marked differences in endotoxin release among treatments with 0.5× the MIC of those antibiotics. Treatment of *P. aeruginosa* with 2× the MIC of IMP, MEPM, or CAZ for 8 h showed marked differences in the resulting bacterial morphologies. IPM treatment induced rod-shaped bacteria to become rounded, whereas exposure to CAZ induced the formation of long filaments. MEPM treatment resulted in elongated rods (filamentous structures) containing round center regions (data not shown). The LPS preparation

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TABLE 1. Endotoxin release from *P. aeruginosa* treated with β-lactam antibiotics and its effect on production of TNF-α and NO

Antibiotic	MIC	Concn of endotoxin released (ng/ml)	TNF-α concn (pg/ml)[a]	NO concn (μM/ml)[a]
IPM	2	34	18.7 ± 7.5[b]	19.1 ± 1.2[b]
	0.5	14	87.2 ± 35.4[b]	20.8 ± 2.1[b]
MEPM	2	314	1079.1 ± 39.4	54.3 ± 3.8
	0.5	329	1153.3 ± 72.1	52.9 ± 2.2
CAZ	2	337	1430.9 ± 49.5	42.4 ± 3.2
	0.5	337	1026.5 ± 68.2	41.3 ± 1.8
None		311	1341.4 ± 50.3	49.3 ± 2.0

[a] For each group, the mean ± standard deviation for three dishes is expressed.
[b] $P < 0.01$ versus MEPM and CAZ by Student's *t* test.

was extracted from the filtrates (10 ml) of antibiotic-treated bacterial suspensions by the phenol-water method as described previously (23, 24). The extracted LPS was separated on a 15% homogeneous polyacrylamide gel in the presence of sodium dodecyl sulfate (sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE]) under reducing conditions, and LPS bands were detected by the silver staining method of Hitchcock and Brown (10). The high-molecular-weight ladder pattern of the endotoxin released by treatment with MEPM and CAZ suggested that the endotoxin released by the treatments described above was of the O-specific, polysaccharide-rich, smooth-type LPS (Fig. 1). The LPS released from the control bacteria showed a typical ladder pattern of both high- and low-molecular weight endotoxins, while hardly any LPS was observed following IPM treatment by this detection method. Next, spleen cells from BALB/c mice were exposed to bacterial culture filtrates previously treated with IPM, MEPM, or CAZ, and the level of production of TNF-α was determined. A spleen cell suspension (10^7 cells) in 100 μl of RPMI 1640 medium was mixed with the filtrates (100 μl) of the bacterial suspensions and cultured in vitro in a microplate for 3 h. TNF-α levels in the culture supernatant were measured by an enzyme-linked absorbent assay (mouse TNF-α cytoscreen immunoassay kit; Biosource International, Camarillo, Calif.). The filtrates of the bacterial suspensions treated with 2× the MICs of MEPM and CAZ induced a significant level of production of TNF-α in cultures of spleen cells (Table 1). On the other hand, the filtrate of the bacterial suspension treated with IPM resulted in a low level of production of TNF-α. Treatment with MEPM and CAZ caused an approximately 60-fold increase in the level of production of TNF-α compared with treatment with IPM. Similar results were produced by the filtrates of the bacterial suspensions treated with 0.5× the MICs of those antibiotics. The level of production of NO in cultures of mouse peritoneal cells with the filtrates of the bacterial supernatants from *P. aeruginosa* treated with 2× the MICs of carbapenem antibiotics was estimated. Mouse peritoneal cells were obtained by washing out the peritoneal cavity with RPMI 1640 medium. One million peritoneal cells in 150 μl of RPMI 1640 medium were mixed with the filtrates (50 μl) and were cultured in vitro in the microplate for 16 h. The concentration of NO_2^- in culture supernatants was measured with the Griess reagent (9). The filtrates of the bacterial suspensions treated with MEPM and CAZ induced significant levels of production of NO in the cultures of peritoneal cells (Table 1). On the other hand, the filtrate of the bacterial suspension treated with IPM resulted in a low level of production of NO. The NO concentration obtained by exposure of peritoneal cells to filtrates of IPM-treated *P. aeruginosa* cultures was less than half of that observed by exposure to MEPM- or CAZ-treated culture filtrates.

The current study shows that IPM, MEPM, and CAZ differentially induce endotoxin release from *P. aeruginosa* and that IPM liberates lower amounts of smooth-type endotoxin than either MEPM, CAZ, or untreated controls. The low level of release of free endotoxin induced by IPM has been reported by several investigators (7, 14, 15). Treatment with MEPM, also a carbapenem, caused a high level of release of endotoxin at 0.5× and 2× the MICs. Jackson and Kropp (15) have reported that the ability of MEPM to release LPS is antibiotic concentration dependent and that lower MICs and sub-MICs of MEPM liberate large amounts of free LPS from *P. aeruginosa*. Although they reported that there were no significant differences in the level of endotoxin release between IPM and MEPM at 2× the MICs, we found that MEPM at 2× the MIC caused a higher level of release of endotoxin than IPM. Differences in the bacterial isolates used in the studies may be responsible for these findings. It has been suggested that the release of endotoxin may be related to morphological changes observed during antibiotic treatment (i.e., spheroplast and filament formation [6, 14, 16, 17, 19]). In fact, *P. aeruginosa* cells treated with IPM became round (spheroplasts), whereas those treated with CAZ formed long filaments. MEPM induced the characteristic morphology, showing both filament formation and spheroplast formation as well as a third morphology consisting of a combination of the two previous morphologies on a single structure (e.g., a filament and a spheroplast). The morphological differences described above seem to be explained by the specificity and binding affinity of penicillin-binding proteins associated with IPM and MEPM binding (14, 15, 19).

A few reports have described the biochemical nature of the LPS released during antibiotic treatment of gram-negative bacteria (3, 4). The SDS-PAGE analysis conducted in the current studies indicates that endotoxin was predominantly of the high-molecular-weight, smooth type (O-specific, polysaccharide-rich). These data suggest that the LPS released may be

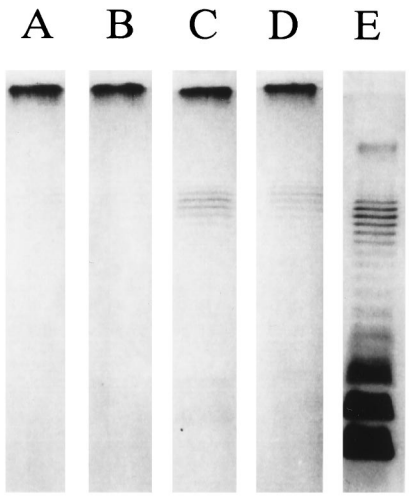

FIG. 1. SDS-PAGE analysis of endotoxin released from *P. aeruginosa* treated with β-lactam antibiotics. Endotoxin extracted from the filtrates of the culture fluid by the phenol-water method was separated in SDS-polyacrylamide gels under reducing conditions and was detected by the silver staining method. Lanes: A, no antibiotics; B, IPM; C, MEPM; D, CAZ; E, whole bacteria.

derived from the outer membranes of bacteria since preparations derived from the extraction of whole bacteria was of low molecular weight and showed the typical ladder pattern.

The differential release of endotoxin induced by IMP and MEPM affected the production of TNF-α and NO. TNF-α is a major mediator of septic shock associated with gram-negative bacterial infection. NO is also an important mediator of it. IPM induced a significantly lower level of release of TNF-α and NO than did CAZ and MEPM, despite the use of equivalent antibiotic concentrations. The low level of production of TNF-α by IPM was consistent with the findings reported by several investigators (2, 5, 17, 21). On the other hand, the lower level of production of NO by IPM treatment was described first in the present study. NO causes death of the host cells via apoptosis and damages the host as a result of the NO-mediated cytotoxicity (1). Because the excessive release of TNF-α and NO in response to LPS is believed to be major contributing factors to mortality and morbidity in patients with gram-negative bacterial sepsis, our present data suggest that the initial antibiotic choice may influence the degree of endotoxin release and the resultant induction of TNF-α and NO. However, it remains to be further clarified whether our findings could be applied to choosing the appropriate antibiotics for treating patients with gram-negative bacterial infections.

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