Aminoglycosides Modify the In Vitro Metachromatic Reaction and Murine Generalized Shwartzman Phenomenon Induced by Salmonella minnesota R595 Lipopolysaccharide

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Endotoxin-neutralizing activity may be an important property for antibiotics to be used in severe sepsis. Several antibiotics, belonging to different classes, were evaluated as to their endotoxin-neutralizing ability, using the inhibition of an in vitro metachromatic assay for lipopolysaccharides and a murine generalized Shwartzman reaction model. Gentamicin, amikacin, and sisomicin have been found to share significant in vitro antitoxin activity at an antibiotic/endotoxin ratio as low as 1.0/5 (by weight) and to reduce the murine generalized Shwartzman reaction at an antibiotic/endotoxin ratio of 3.3/5.

It has been reported that acute vasomotor collapse could follow clinical use of proper bactericidal antibiotics in gram-negative sepsis (3, 21). Both in vitro and in vivo experimental evidence of antibiotic-induced massive bacteriolyis associated with release of a substantial amount of endotoxin (lipopolysaccharide [LPS]) has been published (4, 22). In septic patients treated with antibiotics, a sudden increase in the level of free LPS in plasma has been detected and correlated with the number of dead and alive bacteria in plasma samples and with clinical outcome (21). Also, non-lethal experimental endotoxemia may cause a self-promoted translocation of bacteria and their endotoxins from the gut (8). Unlike spontaneously released LPS, it has been suggested that antibiotic-released LPS bears a well-exposed toxic moiety (4), called lipid A, that is thought to be the innermost and least-variable portion of the LPS molecule (18). Therefore, an antibiotic with anti-lipid A activity would be useful in the control of endotoxin-dependent sepsis sequelae induced by different species of gram-negative organisms. The Limulus amoebocyte lysate gelation assay (LAL assay) has been used to evaluate endotoxin neutralization by antibiotics in vitro (1, 4, 5). However, several limitations of the LAL assay (10) warrant a different approach to this problem.

An endotoxin assay using the metachromatic dye 1,9-dimethyl-methylene blue (DMB assay) has been reported to be reproducible, specific, positively correlated to LPS toxicity, and less laborious and expensive than the LAL assay and other previous endotoxin tests (11). The DMB assay has already been used to evaluate the in vitro reactivity of Salmonella minnesota R595 LPS (11). It has been reported that polymyxin B binds to lipid A and reduces or abrogates most of the biological activities and chemical properties of LPS (15), including in vitro metachromatic reactive in the DMB assay (11).

The purpose of this study was to examine the LPS-neutralizing ability of antibiotics from several classes by comparing the effects of antibiotic-LPS mixtures with those of antibiotic-free LPS, both in vitro by the DMB assay and in vivo by a model of the generalized Shwartzman reaction in the mouse (2).

S. minnesota R595 LPS was obtained from Calbiochem Corporation and dissolved in sterile water for injection (Bioindustria).

Chloramphenicol succinate (Carlo Erba), polymyxin sulfate (Burroughs-Wellcome), gentamicin sulfate (Schering-Plough), amikacin sulfate (Bristol), tobramycin sulfate (Lilly), sisomicin sulfate (Menarini), dibekacin (Logifarm), kanemomycin sulfate (Crinos), netilmicin sulfate (Menarini), ofloxacin (Sigma Tau), cefixime (Menarini), and aztreonam (Menarini) were dissolved and diluted in sterile water for in vitro experiments and in sterile saline (Bioindustria) for in vivo experiments. Aminoglycoside concentrations are given for the free base. The dye 1,9-dimethyl-methylene blue was obtained from Serva, and the DMB reagent for the LPS metachromatic assay was prepared as previously reported (11).

Samples of 0.1 ml of the antibiotic to be tested were incubated at 37°C in a sterile Falcon 96-well culture plate (Becton Dickinson) with 0.1 ml of LPS solution to obtain final dilutions of antibiotic of 10.0, 33.3, 100.0, 333.3 μg/ml (reported in Fig. 1 as 1.0, 1.5, 2.0, and 2.5 log μg/ml, respectively) against a final concentration of 500 μg/ml of LPS. Thus, we tested the following antibiotic/LPS ratios: 0.1/5, 0.3/5, 1.0/5, and 3.3/5. Control samples with only 0.2 ml of sterile water as well as control combinations of LPS solvent (sterile water) plus antibiotic or antibiotic solvent (sterile water) plus LPS were incubated, in addition to other experimental combinations. After 3 h of incubation, 0.1-ml samples were taken from each well, immediately mixed with 2.0 ml of DMB reagent, and spectrophotometrically read against a reference cuvette containing 2.0 ml of sterile water (11). Experiments were done at least in triplicate.

The optical density (OD) at 535 nm of each antibiotic plus sterile water sample was subtracted from the corresponding OD value of antibiotic plus LPS sample, and the resulting values were averaged and compared with averaged OD values of antibiotic-free LPS samples. Data were shown as means and subjected to analysis of variance. Fisher’s protected least squares differences test was used to determine significant differences between groups.

Figure 1 shows that polymyxin B, gentamicin, amikacin,
tobramycin, sisomicin, dibekacin, and kanamycin shared the ability to reduce significantly \( P < 0.05 \) versus antibiotic-free LPS the reactivity of \textit{S. minnesota} R595 LPS with 1,9-dimethyl-methylene blue. In contrast, chloramphenicol and netilmicin, as well as the remaining antibiotics tested (data not shown), did not interfere with LPS metachromatic assay, not even at the highest concentration used.

For in vivo experiments, the generalized Shwartzman reaction model in the mouse (2) was used, with some changes. Female 6-week-old NMRI mice, bred under nonspecific pathogen-free conditions (Nossan), were used. Groups of four to five mice were injected twice, 24 h apart, with several combinations of an antibiotic plus LPS or with control combinations. The treatment schedule as well as results are shown in Table 1. For both preparative injection in the footpad and for provocative intravenous (i.v.) injection, we used an antibiotic/LPS concentration ratio of 3.3/5, which was the highest one we used during in vitro experiments. Within each group the percentage of animals developing footpad swelling and/or macroscopically visible hemorrhagic, petechial, or echymotic lesions (5 and 18 h after i.v. injection) was recorded for at least one of the following mucocutaneous sites: nose tip, mouth, conjunctiva, ears, tail end, nail beds, and anus.

Table 1 summarizes the results of the antibiotic-LPS interaction evaluated by a model of the generalized Shwartzman reaction in the mouse. Animals injected twice with sterile saline did not show any pathological signs. In contrast, every mouse treated with LPS plus saline in the footpad, followed by an i.v. injection of LPS plus saline 24 h later, showed an obvious swollen and hemorrhagic footpad, particularly 5 and 18 h after the second injection. At the same time, most of the mice also showed hemorrhagic lesions on the ears, nose tip, conjunctiva, and nail beds. There were no dead mice; however, some animals of this group developed transient paralysis of the hind legs.

The mice treated twice (first in the footpad and then i.v.) with antibiotics plus LPS showed a reduced incidence of both footpad swelling and generalized hemorrhagic symptoms (Table 1). No animals developed paralysis of the hind legs in these groups.

Therefore, we demonstrate the neutralizing potential of three aminoglycosides on LPS from \textit{S. minnesota} R595 both in vitro and in vivo. Two models are also suggested for quick evaluation of the anti-LPS activity of antibiotics.

Our results are consistent with most of the previously reported data on the inhibition of LPS by polymyxins and aminoglycosides, using in vitro cell-free systems (1, 5, 11). In addition, aminoglycosides and polymyxins are known to interact with \textit{Pseudomonas aeruginosa} (14) as well as with \textit{Escherichia coli} and \textit{Salmonella typhimurium} (17) LPS. Evaluating the inhibition by polycations of dansyl-polymyxin binding to LPS and lipid A, Moore et al. (14) suggested the important role played by the fatty acyl tail of...
polymyxin B. The lack of lipidic moiety among aminoglycosides might account for their reduced affinity for LPS in comparison to polymyxin B. Furthermore, the use of aminoglycosides with different chemical structures allows us to suggest that at least four primary amino groups may be necessary to ensure an anti-LPS effect. Netilmicin, unlike the other aminoglycosides used, has only three primary amino groups and did not show a significant anti-LPS effect within the range of antibiotic concentrations used for this study. Chloramphenicol (1, 5), as well as tetracycline, ampicillin, carbenicillin, and sulfoxazole (5), has been reported as an antimicrobial agent without demonstrable LPS-neutralizing potential, as shown by the lack of inhibition of the LAL assay for LPS.

The hypothesis of the absence of anti-LPS effect for chloramphenicol and β-lactams is further supported by our data, which also suggest the lack of anti-LPS effect for a monocyclic β-lactam such as aztreonam.

Quinolone antimicrobial agents such as ciprofloxacin have been reported to cause a rapid and uncontrolled in vitro release of LPS following their prompt bacteriolytic effect (4). In the same study, polymyxin B and gentamicin effectively controlled the antibiotic-induced release of free LPS in broth and cultured in vitro. In our studies, ciprofloxacin, a quinolone derivative, did not alter in vitro recovery of isolated LPS, suggesting the lack of an anti-LPS effect for quinolone.

The results of in vivo experiments were consistent with in vitro data on the anti-LPS effect of polymyxin B and aminoglycosides, thus allowing us to correlate the reduction of an in vitro metachromatic reaction of LPS with the decrease of LPS-induced in vivo toxicity by antibiotics, as suggested by Keler and Nowotny (11).

A different origin and extraction procedure as well as major structural differences between the LPS used in this study and the LPS used by Billiau et al. (2) may account for the lack of mortality in control mice injected with antibiotic-free LPS. However, obvious signs of hemorrhagic lesions appeared at many sites in mice treated with antibiotic-free LPS, while both the number and the intensity of such lesions were reduced in antibiotic plus LPS-injected animals and completely absent in control mice injected with antibiotic-LPS vehicle (sterile saline). Therefore, in this murine generalized Shwartzman reaction model, polymyxin B, gentamicin, amikacin, and sisomicin may reduce the signs of LPS toxicity, as reported for polymyxin B by other workers, who used a Shwartzman reaction model in the rabbit (6, 7).

The heptoseless LPS used in the present study was isolated from a deep rough strain and has been reported to contain mainly 2-keto-deoxy-octonic acid plus lipid A, the LPS toxic moiety (18). Although smooth strains are often reported as more virulent than rough strains (19), our data may have a significant clinical impact. First, antibiotic-induced LPS liberation makes the toxic innermost fraction of bacterial LPS readily available to interact adversely with host cells (4, 9). Second, since lipid A structure shows minimal variability among endotoxins from different organisms (18), our results might extend to antibiotic-released LPS from many gram-negative bacterial species. Third, the innermost portion of LPS has been reported to be less inhibited by human serum components than a smooth type LPS (16), thus the anti-LPS effect of antibiotics could be important.

Moreover, the concentrations of aminoglycosides (20) and polymyxin B (13) in plasma which may be safely achieved during therapy are within the range of a few micrograms per milliliter (1 to 8 μg/ml, depending on the molecule). In antibiotic-treated humans, the highest level of free LPS in plasma was recently found, 5 ng/ml (21). Thus, the present report demonstrates that both in vitro and in vivo, an antibiotic/LPS ratio of 3.3/5 (1,000-fold less than the ratio achievable in patient plasma) may be able to significantly decrease LPS activity. Also, a recent study suggested that gentamicin, tobramycin, amikacin, and kanamycin may work as inhibitors of LPS synthesis in gram-negative bacteria (12).

In conclusion, the present contribution may be of clinical usefulness in the choice of an antibiotic therapy for severe sepsis, since we demonstrate that aminoglycosides behave as endotoxin-neutralizing molecules both in vitro and in vivo.

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REFERENCES