

SHORT COMMUNICATION

Effect of antibiotic concentration and exposure time on the release of endotoxin by microorganisms

Alssum, R. M.,* Alharby, A. and Omar, H. M. Shair

Department of Botany and Microbiology, Faculty of Science, King Saudi University
P.O. Box 2455, Riyadh 11451, Saudi Arabia.
E-mail: oshair@hotmail.com

Received 15 June 2009; received in revised form 30 September 2009; accepted 3 October 2009

ABSTRACT

Antibiotics represent a key component of treatment for severe bacterial infection. Bacterial lipopolysaccharide from Gram-negative organisms is now well recognized to be a potent microbial toxin that plays a critical role in the initiation of the proinflammatory events that contribute to the pathogenesis of the disease. *Pseudomonas aeruginosa*, *Haemophilus influenza* and *Escherichia coli* were exposed to 0.5 MIC and 5 MIC of Imipenem, Aztreonam, Gentamicin and Polymyxin B for 2 and 5 h. Variable amounts of endotoxin were released after exposing the organism to antibiotic. Aztreonam at a concentration of 0.5 MIC with exposure period 5 h, induced the release of substantial amounts of endotoxin from all organisms and decreased amounts of endotoxin by Gentamicin while Imipenem and Polymyxin B induced the release of negligible values under the same conditions.

Keywords: bacteria, infection, endotoxin, antibiotics, neuroinflammatory

INTRODUCTION

Shock is a serious medical condition caused by decreased tissue perfusion and oxygen delivery as a result of infection and sepsis, though the microbe may be systemic or localized to a particular site. It can cause multiple organ dysfunction syndrome (formerly known as multiple organ failure) and death (Tslotou *et al.*, 2005). It is most common victims are children, immunocompromised individuals, and the elderly, as their immune systems cannot deal with the infection as effectively as those of healthy adults. The mortality rate from septic shock is approximately 50 to 60% of these cases. It is widely accepted that the majority of the cases are caused by Gram-negative rod species. (Annane *et al.*, 2007).

Bacterial lipopolysaccharide from Gram-negative organisms are now well recognized to be a potent microbial toxin that has been approved to play a critical role in the initiation of the proinflammatory events that contribute to the Pathogenesis of the disease (Cheng *et al.*, 2005). Inflammation is the body's defense mechanism against threats such as bacterial infection, undesirable substances, injury, or illness. The process is complex and involves a variety of specialized cells that mobilize to neutralize and dispose of the injurious material so that the body can heal (Theresa and Gioannini, 2003). In the brain, a similar inflammation process occurs when glia, especially astrocytes and microglia, undergo activation in response to stimuli such as injury, illness, or infection. Like peripheral immune cells, glia in the central nervous

system also increase production of inflammatory cytokines and neutralize the threat to the brain. This brain inflammation, or neuroinflammation, is generally beneficial and allows the brain to respond to changes in its environment and dispose of damaged tissue or undesirable substances. Unfortunately, this beneficial process sometimes gets out of balance and the neuroinflammatory process persists, even when the inflammation- provoking stimulus is eliminated (Deborah *et al.*, 2006). Uncontrolled chronic neuroinflammation is now known to play a key role in the progression of damage in a number of neurodegenerative diseases. Thus, overproduction of proinflammatory cytokines offers a pathophysiology progression mechanism that can be targeted in new therapeutic development for multiple neurodegenerative diseases. (Linda *et al.*, 2007). The systemic resultant from the inflammatory response is antibiotic treatment represents a key component of treatment for severe bacterial infection but endotoxin in various amounts and potency were released when Gram-negative bacteria were exposed to certain antibiotics such as β -lactam antibiotic (Lotz *et al.*, 2006). Bacterial lipopolysaccharide (LPS) elicits inflammation and endotoxic shock by inducing proinflammatory cytokine gene expression (Zhou *et al.*, 2003). More recently, in vivo and in vitro studies have shown that endotoxin released can be detected when Gram-negative bacteria are exposed to antibiotics. Our investigation was initiated in response to the new trend known as pharmacodynamic which explains the efficiency of an antibiotic as a

consequence of concentration and time of pathogen exposure.

MATERIALS AND METHODS

Materials

Clinical isolates of *Pseudomonas aeruginosa*, *Haemophilus influenza* and *Escherichia coli* were obtained from the Central Hospital Riyadh, Saudi Arabia. Tryptecase Soy Agar, Chocolate Agar, antibiotics (Imipenem, Aztreonam, Gentamicin and PolymyxinB), limulus lysate, reference endotoxin, pyrogen free water and chromogenic substrate were purchased from Sigma. Mueller-Hinton and Mueller-Hinton with X and V factors. Chromogenic limulus amoecocyte lysate (CLAL), standard endotoxin curve (0.025-0.1 ng/mL = (25-100 pg/mL) was obtained with reference endotoxin (Sigma).

Methods

The isolates were re-identified, using the nitrate test for aerobe, facultative anaerobes, Gram stain method and growth inhibition test. *P. aeruginosa*, *H. influenza* and *E. coli*, were chosen in this study according to their sensitivity to antibiotics and bacterial lipopolysaccharide from Gram-negative organisms are now well recognized to be a potent microbial toxin that has been approved to play a critical role in the initiation of the proinflammatory events that contribute to the Pathogenesis of the disease.

The isolates were maintained at 4 °C and sub cultured every after two week in Mueller-Hinton and Mueller-Hinton with V and X factors. Baselines of endotoxin for each organism were constructed by measuring the spontaneously released endotoxin in the absence of antibiotics after 2 and 5 h of incubation reaction mixture for each organism. The MICs for the antibiotics against each isolates were determined by the dilution method.

Antibiotic Exposure

Organisms were grown in mueller-hinton and mueller-hinton with X and V factors for 12 h, and used to inoculate four replicate sets of four ependorf tubes containing mueller-hinton, 0.5 MIC/ml (two tubes) and 5 MIC/mL (two tubes) of each antibiotic, in final volume of 2 mL containing 10⁷/mL cfu of the required isolate. Control tubes had either the organism or the antibiotic omitted. Incubation was performed at 37 °C for 2 h and 5 h. Supernatants were obtained by centrifugation, passed through 0.4 Millipore filter and stored at – 20 °C.

Measurement of endotoxin concentration

Endotoxin concentration was determined by Chromogenic Limulus Amoecocyte Lysate (CLAL) and standard endotoxin curve (0.025 ng-0.1 ng/mL) was obtained with

reference endotoxin. Quantitative endotoxin CLAL assays were done by mixing 20 µL of reference endotoxin and 20 µL of CLAL in 96-microwell plate incubated for 10 min at 37 °C, 40 µL of chromogenic substrate were added, the plate was gently shaken and re-incubated at 37 °C for additional 3 min. The reaction was stopped by addition 30 µL of 50% acetic acid and absorbance was read at 405 in Enzyme-linked immunosorbent assay (ELISA) LP 400 spectrophotometer. The minimum concentration of endotoxin per milliliter needed to yield positive CLAL test was determined by two fold dilutions in pyrogen free water starting at 1:2 until negative result was obtained.

RESULTS

Preliminary experiments were devoted to the control to establish baseline for negative reading that have been defined as the reading obtained due to spontaneous release of endotoxin from the organisms in the absence of antibiotics (Table 1). The corresponding amount of endotoxin was calculated from standard curve and consequently subtracted from amount obtained from experiment.

Table 1: The baseline of endotoxin released by the microorganism before treatment of antibiotics

Microorganisms	Amount of endotoxin released in 2 h without addition of antibiotic (ng)			Amount of endotoxin released in 5 h without addition of antibiotic (ng)		
<i>P. aeruginosa</i>	12	11	7	25	6	3
<i>H. influenza</i>	15	15	9	31	12	6
<i>E. coli</i>	9	7	5	19	0	0

The antibiotic mediated release of endotoxin from the isolates varied substantially according to isolate, amount of antibiotic and incubation period (Fig. 1, 2 and 3). The peak values of endotoxin released were the results of 5 h exposure to 0.5 MIC of Aztreonam, however, in terms of isolates response it seems that *P. aeruginosa* was more responsive to the induced release of endotoxin than either *H. influenza* or *E. coli*. Decreasing the time of exposure from 5 h to 2 h reduced the amounts of endotoxin released, approximately by half, and the phenomenon had been noticed when organisms exposed to higher concentration of antibiotics 5 MIC. Gentamicin found to be less efficient than Aztreonam for inducing the release of endotoxin. However, it follows the same pattern in releasing more endotoxin during 5 h incubation in the presence of 0.5 MIC and the amount decreased with applying 5 MIC. Neither Imipenem nor Polymyxin was meritorious to release substantial amounts of endotoxin, even though; they obey the general pattern of dose and time of exposure.

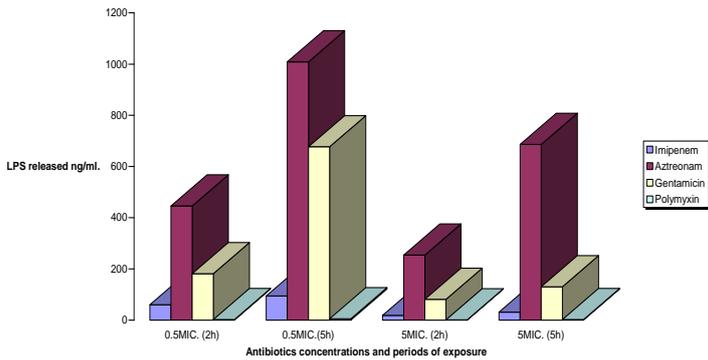


Figure 1: Indicates the amount of endotoxin released from *P. aeruginosa* due to the concentration of antibiotics and period of exposure.

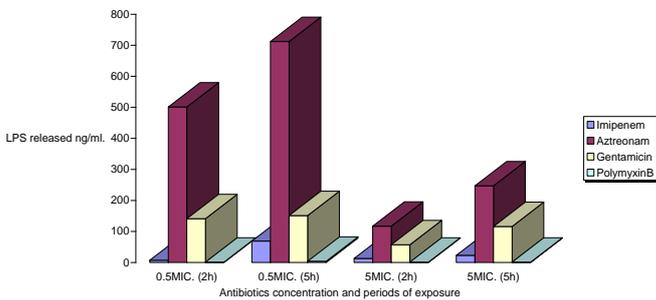


Figure 2: The amount of endotoxin released from *H. influenzae* due to the concentration of antibiotics and period of exposure.

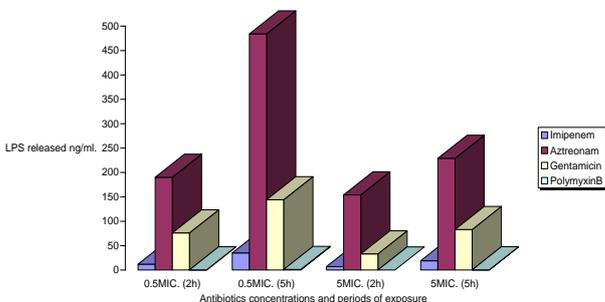


Figure 3: The amount of endotoxin released from *E. coli* due to the concentration of antibiotics and period of exposure.

DISCUSSION

The antibiotic mediated release of endotoxin from the isolates varied substantially according to isolate, amount of antibiotic and incubation period. The peak values of endotoxin released in our study were the results of 5 h exposure to 0.5 MIC of Aztreonam. However, in terms of isolates response it seems that *P. aeruginosa* was more responsive to the antibiotic inducing more endotoxin than either *H. influenzae* or *E. coli*. Decreasing the time of exposure from 5 h to 2 h reduced the amounts of endotoxin released, approximately by half. This is in accordance to the propensity of antibiotics to release variable amount of endotoxin due to its exposure of in different Gram-negative bacteria (Goscinski *et al.*, 2003). Aztreonam caused significant endotoxin release, correlating with its ability to affect cell-wall morphology, causing filamentation, cell wall breakage and cell lysis. The drug with long exposure promotes the eradication of the pathogen and the release of high amount of endotoxin bearing dual hazard (Homma *et al.*, 2007). In our study, after decreasing the time of exposure from 5 h to 2 h, the amount of endotoxin released, was reduced approximately by half, and this phenomenon had been noticed only when organisms were exposed to higher concentration of antibiotics 5 MIC. Gentamicin found to be less efficient than Aztreonam for inducing the release of endotoxin. However, it follows the same pattern in releasing more endotoxin during 5 h incubation in the presence of 0.5 MIC. Neither Imipenem nor Polymyxin was meritorious to release substantial amounts of endotoxin, even though; they obey the general pattern of dose and time of exposure.

The puzzling observations are that the exposures of an organism to 0.5 MIC for 5 h induce the release of higher amount of endotoxin that could be induced by 5 MIC for the same period of exposure. Our explanation to this observation is that the low concentration of antibiotic may promote the growth of the organism with defective envelope and continuous release of endotoxin while high antibiotic concentration tends to gradually kill the organism releasing less amount of endotoxin. However, pharmacodynamic may need to include the level of endotoxin that could be released under any desired condition, drug concentration, time of pathogen exposure and type of pathogen, to provide save guidance to save treatment. It may be early for such detailed information because sophisticated software and statistical analysis are needed.

ACKNOWLEDGEMENT

The authors are thankful to Prof. Dr. Ali Bahkali, Chairman of the Department of Botany and Microbiology for his encouragement and advice. They extend also their thanks to the members of the research Center, College of Science King Saud University for their financial support.

REFERENCES

- Annane, D., Vignon, P. and Renault, A. (2007).** Norepinephrine plus dobutamine versus epinephrine alone for management of septic shock: A randomised trial. *Lancet* **370**, 676-684.
- Cheng, J. J., Yang, C. J., Cheng, C. H., Wang, Y. T., Huang, N. K. and Lu, M. K. (2005).** Characterization and functional study of *Antrodia camphorata* lipopolysaccharide. *Journal of Agricultural and Food Chemistry* **53**, 469-474.
- Deborah, L. H., Jeff, J. S., Della, A. R., Matthew, D. B., William, B. S., Mark, W. B., Anatoli, M. M. and Howard, A. Y. (2006).** The proinflammatory cytokine interleukin-18 alters multiple signaling pathways to inhibit natural killer cell death. *Journal of Interferon and Cytokine Research* **26**, 706-718.
- Goscinski, G., Lundholm, M., Odenholt, I. and Sjolín, J. (2003).** Variation in the propensity to release endotoxin after cefuroxime exposure in different Gram-negative bacteria. Uniform and dose-dependent reduction by the addition of tobramycin. *Scandinavian Journal of Infectious Disease* **35**, 40-46.
- Homma, T., Hori, T., Sugimori, G. and Yamano, Y. (2007).** Pharmacodynamic assessment based on mutant prevention concentrations of fluoroquinolones to prevent the emergence of resistant mutants of *Streptococcus pneumoniae*. *Antimicrobial Agents and Chemotherapy* **51**, 3810-3815.
- Linda, V.J., Eldik, V., Wendy, L. T., Ranaivo, H. R., Heather, A. and Watterson, B. (2007).** Glia proinflammatory cytokine up regulation as a therapeutic target for neurodegenerative diseases: function-based and target-based discovery approaches. *International Review of Neurobiology* **82**, 277-296.
- Lotz, S., Starke, A., Ziemann, C., Morath, S., Hartung, T., Solbach, W. and Laskay, T. (2006).** β -lactam antibiotic-induced release of lipoteichoic acid from *Staphylococcus aureus* leads to activation of neutrophil granulocytes. *Annals of Clinical Microbiology and Antimicrobial* **82**, 277-296.
- Theresa, L. and Gioannini. (2003).** Regulation of interactions of endotoxin with host cells. *Journal of Endotoxin Research* **9**, 401-408.
- Tsilotou, A. G., Sakorafas, G. H., Anagnostopoulos, G. and Bramis, J. (2005).** Septic shock: Current pathogenetic concepts from a clinical perspective. *Medical Science Monitor* **11**, 76-85.
- Zhou, H. R., Islam, Z. and Pestka, J. J. (2003).** Kinetics of lipopolysaccharide-induced transcription factor activation/inactivation and relation to proinflammatory gene expression in the murine spleen. *Toxicology and Applied Pharmacology* **187**, 147-161.