ORIGINAL ARTICLE

Rapid Method of Observing LPS Neutralization by Polymyxin B and Polymyxin B Nonapeptide with Light Microscope

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ABSTRACT

Introduction: **The Lipopolysaccharide (LPS) of gram negative bacteria is present at the outer leaflet, and its lipid contains endotoxin. The toxin often causes fatal septic shock. Polymyxin B (PMB) is a peptide antibiotic that could nutralize endotoxin activity. Its analougue Polymyxin B Nonapeptide (PMBN) which lacks antibactrial activity but may also nutralize endotoxins.**

Objective: **This study examined the effect of PMB and PMBN in neutralization of LPS. Crystallized LPS (E.coli K1) was stained with Sudan Black and observed under light microscope.**

Results/Discussion: **The control LPS (not exposed to PMB or PMBN) formed loop, curled or ribbon-like strands. The exposed LPS to PMB at concentrations of 5 µg/ml for 10 minutes caused the LPS to degrade and form tiny black fragments. The exposure to 5 µg/ml of PMBN reduced the LPS to short strands and branch-like fragments. The degradation of the lipid moiety, particularly the lipid A, is an essential step in the endotoxin neutralization process by both polymyxins.**

Conclusion: **PMB and PMBN degraded the LPS structure which indicates nutralizing of the endotoxins. The method used to observe the neutralizing activity is simple and fast .**

KEY WORDS

endotoxin, lipopolysaccharide, polymyxin B, polymyxin, B nonapeptide

INTRODUCTION

The outer surface membrane of practically all gram-negative bacteria contains Bacterial lipopolysaccharide (LPS), often known as endotoxin. The major component of gram-negative bacterial envelopes, i.e., LPS, is macromolecular amphiphiles that are present exclusively in the outer leaflet of the bacterial outer membrane[1]. LPS is comprised of lipid A, which is the endotoxin and the main virulence factor, the repeating hydrophilic distal oligosaccharide or O-antigen and the hydrophilic core polysacharride. As the LPS molecules envelopes the entire bacterial cell it creates a barrier and protection to the bacteria. Besides providing the cell integrity[2], the LPS protects against outside environment of harmful stimuli. These includes bile salts and other chemicals, such as antibiotics. Some Gram negatives such as *Pseudomonas aeruginosa*, Helicobacter *pylori* and *Salmonella enterica*, adopt changes on the LPS synthesys modulation[3]. These changes resulted in immune system evasion, increased in virulence and antimicrobial resistance. The Gram negatives A moiety of the LPS constitutes the active components of the endotoxin[4]. It often leads to a common but fatal condition known as septic shock. Hypertension, coagulopathy, and circulatory failure are all symptoms of endotoxic shock, which can lead to multiple organ system failure[5] LPS complexes could start a chain reaction that leads to sepsis while still in circulation[6]. Sepsis is a complicated combination of local and systemic inflammatory reactions that necessitates a multifaceted management strategy to control the disease's course through its various stages[7] The treatment for endotoxic shock has a non-specific and remains supportive in the absence of a specific method to treat fatal endotoxicosis. For many decades, the methods of neutralizing the LPS, mainly the lipid A portion, have been investigated. It was found that some peptide molecules, namely, Polymyxin B, could carry out this function[8]. Despite the fact that it has a lot of negative side effects, polymyxins, it occupies a premier position in the armamentarium for combating endotoxicosis[9].

Polymyxin has become an acceptable and widely used model for studying interaction with LPS, particularly the binding process[10]. However, a single binding can be a misleading predictor for LPS neutralising synthetic compounds with proclivities have been shown to neutralise the toxin. As a result, identifying additional physical factors that explain the outcome of the PMB-endotoxin interaction is critical[11]. As the mechanisms of interaction between endotoxins and polymyxins are increasingly being understood, it has been noted that the degradation of LPS structure was possible[12]. It is believed that degradation, particularly of the lipid A region, is an important additional parameter that contributes to the LPS neutralization[5]. In conjunction with Plasmon Resonance studies, Thomas and co-workers[6], demonstrated that PMB solubilized the endotoxin, which lead to LPS neutralization. This research aims to investigate the contribution of PMBN towards the neutralization of LPS along with PMB. A suitable and simple light microscope technique was developed to examine and verify the fate of the LPS after exposure to the polymyxins.

MATERIALS AND METHODS

Antibiotics and Lipopolysaccharide (LPS)

The PMBN was obtained from Boehringer Mannheim and used for testing against LPS (Sıgma Aldrıch). For this experiment, PMB was used as a positive control. The commercial *E. coli* K1 LPS was used

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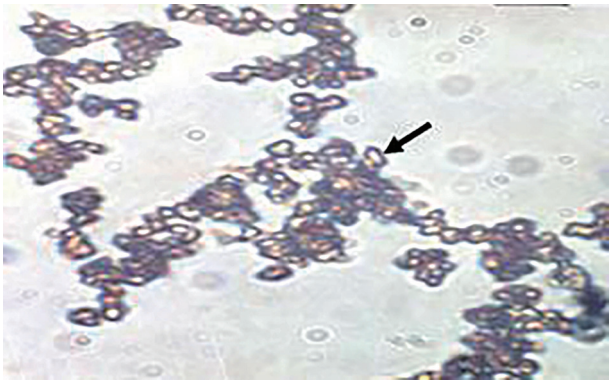

Figure 1: LPS Extracted From *E.coli* K12. Control LPS remained untreated and was stained by Sudan Black. The arrows indicate the normal structure of the LPS (magnification 100 x).

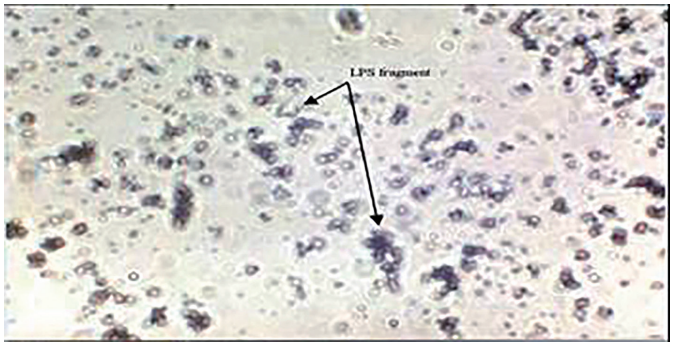

Figure 2: Effect of 5 µg/ml of PMBN on *E.coli* LPS. LPS after exposure to 5 µg/ml of PMB. Arrows indicate the fragment of LPS.

throughout this experiment.

Staining of LPS and microscopic observation

E. coli K1 was solubilized in a buffer of phosphate (0.01 M, pH 7.4) and 10 µl of the LPS solution was pipetted and dropped on the glass slide. The solution was then mixed with 5 µl of Sudan Black II. A cover slip was placed on top of the stained-glass slide, and the slide was observed under light microscope at 40 x magnification.

PMB and PMBN reaction and LPS staining

The LPS of K1 concentration of 0.2 mg/ml was diluted buffered in PBS (0.01 M, pH 7.4) and kept in an Eppendorf tube. At least three portions of the LPS solution were prepared. 5 µg/ml of PMB was added to one portion of the solution, and PMBN (5 µg/ml) was added to the second portion. The concentration of polymyxins used was established according to the authors previous research (Sahalan and Dixon 2008).

The third LPS portion served as control (without Polymyxin treatment). All three LPS portions were left for 10 minutes at 37°C for reactions to take place. Later, 10 µl of each LPS portion was pipetted and dropped on a separate glass slide. The portions with polymyxins and the control portion were mixed with 1 µl of Sudan Black II. Coverslips were placed on top of the stains. All the three slides were observed under a light microscope.

RESULTS

Light microscope study on the interaction of PMB and PMBN with LPS

All the LPS solutions (control LPS, PMB and PMBN) were observed under light microscope at the magnification of 100 x. The control LPS (without polymyxin) exhibits the formation of loops and curled or ribbon-like strands. Although, a rather low magnification of 100 x was used, a clear image of strands was observed. However, exposure of the LPS to 5 µg/ml of PMB for 10 minutes caused the formation of little fragments in the LPS structure (Figure 2). The black fragments were seen to be scattered in the sample. The exposure of the LPS to 5 µg/ml PMBN leads to morphological changes distant in the LPS structure. The LPS appeared as short strands or branch-like fragments as seen in Figure 3.

DISCUSSION

Degradation of the LPS by PMB and PMBN

For many years, the effectiveness of polymyxins coupling with the LPS or endotoxic molecules has been studied in detail[13]. The present study suggests that the structural disorganization of LPS by PMB or PMBN constitutes an important factor of endotoxin neutralization. The LPS was stained with Sudan Black and observed using a light microscope. The normal isolated LPS appeared as ribbon-like strands[14]. The Sudan Black stain was specifically used to stain lipid or fat molecules. Therefore, the black string formations were the lipid portion of the LPS, which formed the monolayer strands. The saccharide region of the LPS may not be stained with Sudan Black and therefore, was not seen through the microscope.

The strands were broken down when the LPS was exposed to 5 µg / ml of PMB. This indicates that an advanced disorganization or degradation of the lipid assembly in the LPS occurred. Similar observations were also made in electron microscope studies of LPS from *E.coli*[6] and *Salmonella sp*[14]. The strands of LPS were broken down into small fragments after the interaction with PMB. Although, all the tests were performed on isolated LPS (free LPS), intact LPS on the bacterial membrane may undergo a similar phenomenon. Other researchers viewed the phenomenon as solubilization of the endotoxin components in the lipid A region[15] or merely as the lateral separation of lipid molecules[16].

In this work, PMBN has also been observed to induce unusual lipid assembly in the LPS. Figure 2 shows the short strands and branch-like formation of the stained lipid after exposure to PMBN. Although the exact mechanism is unknown, this experiment confirmed the PMBN's disorganizing effect on the lipid part of the LPS, namely the lipid an ingredient. Some researchers have claimed that PMBN changed the chemical structure of LPS, particularly the lipid A region, thereby disorganizing the lipid A region ultra-structure[18].

The degradation or solubilization of the lipid A region has not been known to occur with LPS-PMBN interaction. The solubilizing phenomenon may be possible. The number in lipids A solute to a molecule by PMBN may be much lower than PMB. An initial study has demonstrated that PMBN retained the antiendotoxin activity[17] by blocking the endotoxin-induced neutrophil priming, but it is less potent than PMB. Nonetheless, PMBN-induced disruption of lipid construction has measurable effects on the bacterial membrane. This includes increase on the outer layer of the membrane, which causes leakage and a sensitization of microorganisms that are gram-negative in the numerous hydrophobic antibiotics[21], as well as increased permeability of human neutrophils[22]. Despite this, PMBN alone lacks bactericidal activity and therefore, it does not cause any serious damage to the cells, which undergo membrane repair.

The method used in this study provide some useful information regarding the degradation of the LPS structure and neutralization of endotoxins. Some authors have studied the neutralization of LPS through animal model, biochemical and imuno based experiments. Limulus amebocyte lysate test or LAL[19] and murine models[20] were also used to justify the endotoxins neutralization. However, the method used in this study allows the observation of the true degradation of the LPS. Besides, the method is simple and fast and cost-effective. The degrada-

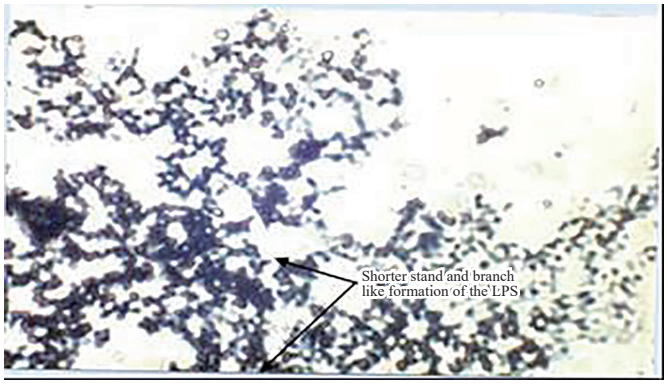

Figure 3: Effect of 5 µg/ml of PMB on *E.coli* LPS. LPS after exposure to 5 µg/ml of PMBN. Arrows indicate the remaining LPS.

tion of LPS could be observed by simple light microscope.

CONCLUSIONS

In conclusion, this work has certainly helped in understanding the role of polymyxin particularly, its interaction with the LPS's lipid component. The degradation of the lipid moiety, particularly the lipid A is an essential step in the endotoxin neutralization process by both polymyxins. The findings in this work also have helped to introduce a new method which is simple, fast and less costly to monitor the degradation of LPS.

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