Detoxified lipopolysaccharide protects mice against pyelonephritis caused by *Klebsiella pneumoniae*

Hina1✉, Chhibber S2

1. Research Scholar, Department of Microbiology, Basic Medical Sciences, Panjab University, Chandigarh 160014, India
2. Professor, Department of Microbiology, Basic Medical Sciences, Panjab University, Chandigarh 160014, India

✉ Corresponding author: Department of Microbiology, Basic Medical Sciences, Panjab University, Chandigarh 160014, India, e-mail: hina14s@gmail.com

Publication History
Received: 21 January 2014
Accepted: 12 March 2014
Published: 26 March 2014

Citation
Hina, Chhibber S. Detoxified lipopolysaccharide protects mice against pyelonephritis caused by *Klebsiella pneumoniae*. *Indian Journal of Science*, 2014, 9(22), 26-34

ABSTRACT

*Klebsiella pneumoniae* infects urinary tract, especially in nosocomial settings with multi-drug resistant strains leading to treatment failure. Lipopolysaccharide (LPS) has been found to be protective against *K. pneumoniae* infections; however, the toxicity of LPS restricts its prospects for vaccine development. Hence, an attempt was made to detoxify LPS of *K. pneumoniae* B5055 by alkaline hydrolysis and study its prophylactic potential in a mouse model of ascending pyelonephritis. Detoxified lipopolysaccharide (D-LPS) provided significantly more protection against bacterial challenge in comparison to purified LPS through reduction in renal bacterial load and histopathology (p<0.001). An effort was made to delineate the underlying mechanism of protection. Since macrophages form the first line of defense against infection, their functions were assessed in the test and control groups. The macrophages obtained from D-LPS immunized group have shown increased phagocytic activity coupled with decreased nitric oxide (NO) production (p<0.001). Assessment of renal pathology correlated with diminished malondialdehyde (MDA) levels in the homogenized tissues of D-LPS immunized animals (p<0.01) confirming that treatment with D-LPS vis-a-vis LPS not only resulted in lowering of bacterial counts but it also protected the host from exaggerated immune response following infection. The efficacy of any vaccine preparation is greatly affected by the route of antigen delivery. Three different routes of immunization (intranasal, intraurethral and intramuscular) were tried and intranasal route was found to providemaximum protection. We suggest that D-LPS administered through intranasal route is protective and immunogenic against urinary tract infections (UTI) caused by *K. pneumoniae*.

Keywords: Detoxified lipopolysaccharide; Immunization; Intranasal; *Klebsiella pneumoniae* B5055; Urinary tract infection
1. INTRODUCTION

Urinary tract infection (UTI) is a leading cause of morbidity in the community as well as in hospital settings. This alone accounts for over 8 million episodes in the United States annually and more than 1 million hospitalizations, with an overall annual cost in excess of $2.4 billion (Ayegor et al., 2007; Litwin et al., 2005). *Escherichia coli* frequently encountered is the most common pathogen followed by other gram-negative organisms (Ronald, 2002). *Klebsiella pneumoniae*, however, is an important human pathogen which accounts for 6-17% of all nosocomial UTIs and shows higher incidence in specific groups like patients with nephropathic bladder or diabetes mellitus (Ojo and Anibijuwon, 2010; Podschun and Ullmann, 1998). *K. pneumoniae* among the top eight pathogens isolated from hospital patients (Vincent, 2004). The high rate of nosocomial *Klebsiella* colonization is correlated with the emergence of multi-drug resistant *K. pneumoniae* isolates leading to treatment failures with antimicrobials (Khanfar et al., 2009; Regue et al., 2004). The development of new immunophylactic and immunotherapeutic agents has been emphasized for the management of nosocomial UTI with *K. pneumoniae* (Chathley et al., 1996; Uehling et al., 2003). Efforts have been made to evaluate the surface antigens of *K. pneumoniae* and their potential for vaccine development. The main focus has been on the capsular polysaccharide (CPS) (Cazy et al., 1986a; Cazy et al., 1986b). However, type-specific protection afforded by CPS coupled with prevalence of 90 different K serotypes with no significant clinical predominance limits the potential of K-antigen based vaccines (Podschun and Ullmann, 1998; Regue et al., 2004). The lipopolysaccharide (LPS) molecule, which is another cell surface component of the organism, is an important virulence factor (Chhibber et al., 2003). LPS appears to be a promising antigen since only 9 serotypes have been reported for this genus (Vinogradov and Perry, 2001). Although LPS has been shown to be highly protective in animals (Chathley et al., 1996; Rani et al., 1990), the toxicity of the preparation limits its potential for use as vaccine candidate. The toxicity of LPS antigen has been reported to be associated with the lipid A component of this molecule (Rietschel et al., 1984). However, lipid A, in addition to its toxic action, has an adjuvant effect. For this reason, retaining and simultaneously reducing the toxicity of lipid A are of special interest for the development of new LPS-based vaccines (Chhibber et al., 2004).

2. OBJECTIVE OF THE STUDY

In the present study, an attempt was made to reduce the toxicity associated with LPS preparation by subjecting it to chemical detoxification through alkaline hydrolysis. This detoxified preparation was then evaluated and compared with LPS preparation for its protective efficacy against *K. pneumoniae* induced UTI in mice.

3. MATERIALS AND METHODS

3.1. Bacterial strain

*Klebsiella pneumoniae* B5055 (K2, O1) standard strain procured from Dr. MathiaTrautmann (Department of Medical Microbiology and Hygiene, University of Ulm, Germany) was employed in this study. The strain was maintained in nutrient agar stabs at 4°C. The bacteria was grown in nutrient broth at 37°C for 18h under static conditions and harvested by centrifugation. Cells were washed three times with phosphate buffered saline (PBS, 0.1M, pH 7.2) and suspended in PBS so as to achieve a concentration of 10⁶ CFU (colony forming units) and 50μl of this was used for induction of pyelonephritis.

3.2. Animals

Female BALB/c mice, 4 to 6 weeks of age, each weighing 20-30 g, procured from Central Animal House, Panjab University, Chandigarh, were used in the study. The mice were fed on standard antibiotic free synthetic feed (Hindustan Levers Ltd., Mumbai, India) and water *ad libitum*. New Zealand white rabbits (body weight 1.5±0.2kg) were used for biological study of antigens. The animals were housed in clean polypropylene cages and experiments were performed in conformity with the protocol approved by the institutional animal ethics committee.

3.3. Preparation of LPS and Detoxified LPS (D-LPS)

LPS from *K. pneumoniae* B5055 was extracted by the conventional hot phenol extraction procedure of Westphal and Jann (1965) as modified by Morrison and Leive (1975). LPS was further purified by sequential ultracentrifugation as described by Johnson and Perry (1976). Purified LPS was then detoxified by alkaline hydrolysis as described by Svenson and Lindberg (1978). Both preparations (LPS and D-LPS) were analyzed for DNA, RNA and protein content by standard methods (Burton, 1956; Lowry et al., 1951; Munro and Fleck, 1966).

Hina et al.


Discovery Publication. All Rights Reserved
3.4. Biological study

Both the antigens were tested for local Schwartzman reaction. The hair on the back of healthy rabbit was removed by scissors following the application of hair removing cream. The area was cleaned with methylated spirit and divided into different sectors. The test samples in 0.1 ml sterile distilled water were injected intradermally (i.d.) at different sites. After 18 h, the animal was given provocative intravenous (i.v.) injections each of 50 µg and 100 µg of both purified and detoxified LPS. The intensity of local skin reaction in the rabbit was determined 5 h after the provocative injection. The intensity of the reaction was recorded as follows:

- 0 : No reaction
- + : Induration without haemorrhage
- ++ : Induration with petechial haemorrhage
- +++ : Induration with slight haemorrhage and necrosis

3.5. Protection studies

The mice were divided into groups of five mice each. An equal number of mice were taken as controls. The following preparations were injected through intranasal (i.n.), intramuscular (i.m.) as well as intraurethral (i.u.) routes in each animal of the respective group: (i) 25 µg of LPS (ii) 50 µg of LPS (iii) 100 µg of LPS (iv) 25 µg of D-LPS (v) 50 µg of D-LPS (vi) 100 µg of D-LPS. Fourteen days post immunization; the animals were challenged with K. pneumoniae B5055 to induce acute pyelonephritis.

3.6. Induction of ascending pyelonephritis with Klebsiella pneumoniae

A soft intramedic-non-radiopaque polyethylene catheter with an outer diameter of 0.61 mm (Clay Adams, USA) was inserted in the bladder through the urethral meatus, and 50 µl of bacterial inoculum (10^7 CFU) was slowly injected into the bladder to avoid leakage (Hagberg et al., 1983; Mittal et al., 2004). Later the catheter was withdrawn carefully. No obstruction or further manipulation of the urinary tract was performed.

3.7. Bacterial quantification

The mice were sacrificed 3 days post-infection by cervical dislocation. One half of both the kidneys was excised, weighed and homogenized in 1 ml of sterile PBS under aseptic conditions. The number of viable bacteria was determined by quantitative culture of serial dilutions on nutrient agar following incubation at 37°C for 24 h. The results were expressed as CFU/g of kidney tissue.

3.8. Histopathological examination

The other half of the kidney tissues was fixed in 10% buffered formalin and dehydrated in ethanol gradient of 30-100%. Tissues were then embedded in paraffin wax blocks and 5 µm thin sections were stained with haematoxylin and eosin (H and E) and graded according to the method of Garget et al., (1987).

3.9. Phagocytosis assay

The mice were divided into groups of five mice each. An equal number of mice served as controls. The following preparations were injected through intranasal (i.n.), intramuscular (i.m.) as well as intraurethral (i.u.) routes in each animal of the respective group: (i) 50 µg of LPS (ii) 100 µg of LPS (iii) 50 µg of D-LPS (iv) 100 µg of D-LPS. The mice were sacrificed at 14 days after the day of injection. Peritoneal macrophages were collected from all the groups of mice as described by Bjornson and Michael (1971). Uptake and intracellular killing of K. pneumoniae B5055 by mouse peritoneal macrophages was studied according to the method of Allen et al., (1987).

3.10. Biochemical studies

Kidney tissue homogenates from different experimental groups were centrifuged at 1500 x g for 10 min. The supernatants were collected, passed through a 0.45 µm filter and stored at -70°C. These were employed for analysis of following biochemical parameters:

3.10.1. Malondialdehyde (MDA) production

Lipid peroxidation was assessed through MDA production in kidney homogenate supernatants. It was estimated by the method of Anjaneyulu and Chopra, (2004).

---

**Comparison:** This study stresses on the problem of emerging multi-drug resistance especially with reference to nosocomial pathogen K. pneumoniae and suggests the prophylactic approach using bacteria’s cell surface component (LPS) and its detoxified preparation in an experimental UTI mouse model.

**Content:** The literature for this work has been collected from books and articles.

---

**Phagocytosis**

Phagocytosis forms the first line of host defense mechanism against invading pathogens and involves a process of removing pathogens and cell debris from the body.
3.10.2. Nitrite assay
Nitric oxide (NO) production in kidney homogenate supernatants was measured by nitrite estimation following the method of Rockett et al., (1994).

3.11. Statistical analysis
All experiments were carried out in triplicates to validate the reproducibility of the experiments. Results were analyzed statistically by the two-tailed Student’s t-test to calculate probability (p-values). p<0.05 was taken as statistically significant. All results were expressed as Mean±SD.

4. RESULTS
4.1. LPS and D-LPS preparations
The purified LPS preparation was found to contain virtually trivial amounts of DNA (0.25%, w/w), RNA (1.02%, w/w) and protein (2.8%, w/w), thereby confirming the purity of the preparation. The preparation was evaluated for its biological activity employing Schwartzman reaction. i.d. injection of purified LPS at a higher dose of 100µg was found to induce local skin reaction in rabbit showing second degree of induration with petechial haemorrhage. D-LPS also showed very low levels of DNA (0.12%, w/w), RNA (0.91%, w/w) and protein (1.7%, w/w). D-LPS did not induce Schwartzman reaction in both the doses tested on rabbit.

4.2. Protection studies
Three doses of purified LPS as well as D-LPS (25µg, 50µg, 100µg) were administered through i.n., i.u. and i.m. routes. Mice were challenged intrauretherally with K. pneumoniae 14 days post immunization. No significant protection was observed for ascending pyelonephritis in immunized mice with 25µg and 100µg doses of LPS (Figure 1). In mice administered 50µg of purified LPS, quantitative decrease in renal bacterial counts of 2.6, 1.2 and 1 log cycles was observed following i.n. (p<0.01), i.u. (p<0.05) and i.m. (p<0.05) routes of immunization. Vaccination of mice with 25µg and 50µg doses of D-LPS did not result in significant protection in experimental model of UTI (Figure 2). However, the group in which 100µg of D-LPS was administered showed extremely significant decrease in renal bacterial load of 6.6, 5.6 and 5 log cycles following i.n. (p<0.001), i.u. (p<0.01) and i.m. (p<0.01) route of immunization.

4.3. Histopathological examination
Examination of kidney sections obtained at day 3 post infection (p.i.) from infected mice challenged with K. pneumoniae showed periglomerular infiltration, capillary haemorrhage along with destruction of tubules confirming the establishment of ascending pyelonephritis (Severity Score +10, Figure 3a). In protection experiments with purified LPS, immunization of mice with 50 µg dose and subsequent challenge was found to show capillary haemorrhage, shedding of cells and infiltration of neutrophils (Severity score +7, Figure 3b). In contrast, immunization with a higher dose (100 µg) of D-LPS followed by K. pneumoniae challenge resulted in mild inflammation in the interstitial region, thus confirming protection (Severity score +3, Figure 3c).
4.4. Phagocytosis

Non-specific protection in terms of phagocytosis was studied in mice immunized with both the preparations. A significant increase in uptake of bacteria and killing capacity of peritoneal macrophages obtained from mice immunized with 50 µg of LPS administered through intranasal route was observed (p<0.01, p<0.05) as compared to control group (Table 1). However, results from D-LPS immunized mice via intranasal route at a higher dose of 100 µg showed furthermore increase in uptake as well as killing of bacteria by peritoneal macrophages (p<0.001, p<0.01).

4.5. Biochemical analysis

The biochemical events were assessed in terms of quantitation of kidney MDA and nitrite levels in different experimental groups. A significant decrease in MDA levels of kidney homogenates was observed in mice immunized with D-LPS through i.n. (p<0.01), i.u. (p<0.01) and i.m. routes (p<0.05) compared to group treated with purified LPS (Figure 4). Similarly, kidney nitrite levels showed significant reduction in mice immunized with D-LPS through i.n. (p<0.01), i.u. (p<0.01) and i.m. routes (p<0.05) compared to results obtained with purified LPS (Figure 5).

5. DISCUSSION

UTIs caused by K. pneumoniae continue to be a significant clinical problem, especially in nosocomial settings (Nickel, 2002). The situation demands the development of a vaccine that will help in controlling these infections. The present study, therefore, was planned to target such population for immunoprophylaxis with D-LPS and the protection afforded by D-LPS was compared with purified LPS in mice. Rani et al., (1990) studied the immunoprotective potential of LPS and its related antigens in a lobar pneumonia model developed in rats. All the preparations, namely, reduced LPS, the LPS-BSA complex, and the lipid A-BSA complex, were found to afford protection against bacterial challenge. In order to use the preparation on large scale, an attempt was made to lower the toxicity associated with LPS molecule by subjecting it to detoxification through alkaline hydrolysis with sodium hydroxide. Treatment of LPS with sodium hydroxide has been shown to reduce the LPS toxicity without destroying its O-polysaccharide antigenic determinants (Niwa et al., 1969; Seid and Sadoff, 1981; von Eschen and Rudbach, 1976). Alkaline hydrolysis of LPS rendered the detoxified preparation non-toxic at a higher dose (100µg) as compared to native LPS. Mild-alkali treatment of LPS has been reported to remove ester-linked fatty acids but preserve amide-linked fatty acids of lipid A, thus retaining its antigenic determinants (Gu et al., 1996). Ding et al., (1990) and Rioux et al., (1998) have shown effective protection following immunization with D-LPS against Salmonella typhimurium infection in mice and porcine pleuropneumonia in pigs. Similarly, active immunization with a complex comprising of detoxified Escherichia coli J5 LPS and group B meningococcal outer membrane protein vaccine has been shown to effectively protect immunocompromised animals.
from experimental sepsis (Cross et al., 2001). Another study with an analog of D-LPS from Neisseria meningitidis (LpxL1) has shown that D-LPS functions as a potent adjuvant for an influenza H5N1 virosomal vaccine (de Vries et al., 2008). Recently, Kong and colleagues have shown Salmonella expressing D-LPS to be immunogenic and protective in mice both as an attenuated vaccine and for delivery of foreign antigens (Kong et al., 2011). In our study, with D-LPS, the overall protection rate was significantly higher in experimental model of UTI by K. pneumoniae and these results corroborate the findings reported earlier.

The ultimate establishment of infection depends on the host-parasite interaction in the form of non-specific defense mechanisms operative through production of reactive oxygen and nitrogen species and phagocytes which form the first line of host defense. Tsai et al., (1997) demonstrated that the production of nitric oxide (NO) in lung homogenates following infection with K. pneumoniaematched with the level of lung bacterial load. In relation to UTI, Nowicki et al., (1999) found an inverse relationship between severity of infection with E. coli and NO production in C3H/HeJ mice. However, in the present study, NO level in kidney homogenates was found to decrease significantly following immunization with D-LPS as compared to purified LPS. The activation of peritoneal macrophages in terms of bacterial uptake and killing was enhanced effectively with D-LPS immunized animals in comparison to purified LPS. Both the biochemical events mentioned above were associated with lower renal bacterial load. The basis of such non-specific defense could be the combined effect of the biochemical events as well as increased bacterial clearance from the kidneys through phagocytic system.

Route employed for immunization significantly affects the efficacy of any vaccine preparation. Chathley et al., (1996) reported effective local protection against UTI employing both i.u. as well as i.m. routes. However, administration of vaccine through i.u. route especially in humans is not only cumbersome but will also not be acceptable in large scale population. Alteri et al., (2009) demonstrated that i.n. immunization of CBA/J mice with outer membrane iron receptors of uropathogenic E. coli (UPEC) elicited a systemic and mucosal response against experimental UTI by UPEC. Lately, Scavone et al., (2011) have shown i.n. immunization with attenuated S. typhimurium expressing MrpA-TetC fusion protein effectively protected mice against an experimental P. mirabilis UTI. The results of the present study revealed i.n. route of immunization to offer maximum protection against UTI compared to traditional, i.u. and i.m. routes, thus corroborating the earlier findings.

### 6. CONCLUSION

The present study brings out that detoxified LPS preparation holds promise for protection against UTI with an organism like K. pneumoniae for which non-invasive intranasal route of administration can be gainfully employed.
SUMMARY OF RESEARCH
The study compared the protective efficacy of LPS and D-LPS preparation in vivo and established that D-LPS preparation could prevent ascending pyelonephritis by K. pneumoniae.

FUTURE ISSUES
Whether immunization, following coupling of D-LPS to an effective carrier molecule/adjuvant, is able to provide better enhancement in protection, warrants further investigation in order to employ prophylactic approach opposed to the curative approach. This is specifically desirable in nosocomial situations and immunocompromised hosts.

DISCLOSURE STATEMENT
There is no financial support for this research work from any funding agency.

ACKNOWLEDGEMENT
The authors gratefully acknowledge Dr. Mathia Trautmann for providing the standard strain of Klebsiella pneumoniae B5055 and Dr Jaspal Singh for proof-reading of the manuscript.

REFERENCE

Hina et al.


