Enhancement of Pro-inflammatory Cytokine by Partial Purified Lipopolysaccharide Extracted from Invasive *Klebsiella Pneumoniae*

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Abstract

Lipopolysaccharide was extracted from invasive local isolate *Klebsiella pneumonia* previously isolated from neonate blood samples. LPS was extracted by phenol hot water method and partial purified by gel filtration chromatography on sepharose CL-6B gel. The results showed that the percentage of carbohydrate amount in the partially purified LPS extract was 9.5% while the percentage of protein in the same extract was 0.06% with no nucleic acids. Intravenous administration of (0.1and1)µg/kg of partial purified LPS in the rabbit led to increase body temperature in dose dependent manner (38.8° and 40.2°)C respectively. Intraperitonial administration with the dose 10µg/mouse cause an increasein serum cytokine (IL-1α, IL-6 and TNFα) level after 4 hr of the injection. Conclusion: LPS extracted from pathogenic isolate of *K.pneumoniae* with high purity, has immunomodulator effect by increasing serum cytokine (IL-6 & IL-1α) level which play a role in the elevation of body temperature, and TNFα after 4hr of intraperitonial administration of LPS.

Keywords**:** *K.pneumoniae, LPS, cytokine.*

Introduction

Klebsiella pneumoniae, are important causes of nosocomial infections [1]. These bacteria occur as saprophytes at almost all the body site, but the highest incidence found in the nasopharynx and intestinal tract, leading causes of community acquired pneumonia. This opportunistic pathogen responsible for sever nosocomial infection in neonate and adult in intensive care. It could cause blood sepses [1], liver sepses [2] in addition to respiratory and urinary tract infection [3].

Klebsiellapneumoniae typically expresses both lipopolysacharide (LPS,O antigen) and capsule polysaccharide (K antigen) on their surface which play critical role in their virulence and contribute to the pathogenicity of this species and LPS is an important structural component of the outer membrane complex of Gram-negative bacteria consists of lipid A, the core and O- polysaccharide antigen.LPS elicits a variety of inflammatory responses in animal and could activate complement by the alternative pathway [4]. Many previous studies showed that LPS isolated from gram-negative bacteria has modulator effect on through innate immunity activation and complement manifestation on cellular and humeral immune response [5]. These studies explain the role of LPS isolated from invasive *K.pneumoniae* of neonate with blood sepses in the elevation of serum cytokine.

Materials and methods

Bacterial strains and growth condition

K.pneumoniae was isolated from neonate blood sepses (from Baghdad medical city, in Baghdad)and identifiedby VITEK 2 system (Bio Merieux) according to company instruction. *K. pneumoni*wasgrown in brain-heart infusion broth at 37ºC in shaker incubator overnight. Bacterial cells harvested by centrifugation, sediment bacteria washed twice with phosphate buffer saline (PBS pH=7.2) containing 0.15mM of $CaCl_2$ and 0.5mM $MgCl_2$ in order to used in LPS extraction and purification.

LPS extraction and purification

LPS extraction was done by phenol hot-water, the method was describe by Wetsphal [6] and modified by Rezania [7]. Briefly, bacterial pellet (17mg)was suspended in10 ml PBS and sonicated for 10 minute with 50 Hz on ice (10 second on and one minute off). The mixture was treated with proteinase K, DNase, RNase for elimination contaminating protein and nucleic acid prior to extraction step. Proteinase K (120µ/ml) was added to the cell

mixture at 37°C for 4hrwith stirring, then the mixture was boiled for 10 min, subsequently treated with DNase (20µg/ml) and RNase (20µg/ml) in the presence of 1µ/ml 20% $MgSo_4$ and 4µl/ml chloroform. The mixture was then incubated at 37°C overnight. Next, an equal volume of hot (70°C)90% phenol was added to the mixture followed by vigorous shaking at (70°C) for 15 min. The suspension was then cooled on ice, finally centrifugation at 10000rpm for 15 min was carried out. Watery and phenol phase were put in separated tubes, phenol phase reextracted by distilled water and added to previous collected watery phase. Sodium acetate at 0.5M final concentration and 10 volumes of 95% ethanol were added to the extract. The mixture was centrifuged at 3000rpm at 4°C for 10 min, and the pellet were resuspended in 1m distilled water. Extensive dialysis against distilled water at 4°C until the residual phenol in aqueous phase was totally eliminated was preformed (0 absorbance at 260nm in the water outside the dialysis tube).

Partial purification

This was done according to the method which was previously describe[8] using gel filtration column (20×2.5cm) of sepharose CL-6B gel, washed and equilibrated with 0.2M NaCl. Ten mg/ml of the LPS extract was applied on surface column. Separation was performed at 70ml/hr, fraction of 3ml were collected. Chemical analysis of collected samples were performed by estimating protein content [9], nucleic acid content [10] and the total quantity of carbohydrate [11].

Evaluation of partial purified LPS pyrogenic activity and serum cytokine measurement

Animals

White New Zealand rabbits weighting between 1.5Kg and 1.8Kg were used. The rabbits were housed in plastic cages and feed on a conventional diet in an air conditioned room. Pyrogenicity of LPS was measured according to the method [8]. The test was done by intravenous injection with the dose (0.1 and 1) µg/kg separately and recording rectal temperature before and after 4 h of injection.

BALB/c mice (from Al Nahrain University) ranging from 6-8 month of age were used to detect serum cytokine level in the experiment. Serum were collected from the blood of the retroorbital venous pexus at the zero time and after 4hrs following intraperitonial injection with 10µg/mice LPS (12). Serum cytokineswere measured by using Enzyme-LinkedImmuno-Sorbant Assay (ELISA Biokit. Spain) according to supplied instruction.

Experimental design and statistical analysis

The effects of partial purified LPS isolated from *K.pneumoniae* on the body temperature and serum cytokines were evaluated by intravenous injection of (0.1 and 1)µg/kg LPS in the rabbit and compare body temperature before and after 4 hours while effects on serum cytokine were measured after 4 hours of intraperitonial administration of 10µg/mouse. result was statistically analyzed by t-test and $p \leq 0.05$ was considered statistically significant difference.

Result and discussion

LPS is the main outer membrane components of Gram negative bacteria. LPS play a major role in the immune modulation [5].The basic method for LPS extraction found by westphal [6], and still the most frequent procedure employed for LPS extraction. This method depends on hot phenol water extraction with simple modification by [7] to get high purity and less contaminants. In this method 1.4 g LPS was recovered from 10.5g of dried cell. Carbohydrate and protein was (2.3%and 0.9%) respectively in crud extraction and (9.5% and %0.06) in partial purified LPS. No nucleic acid was detected in crude and partial purified LPS due to effective destructive action of nucleic acid enzyme on bacterial nucleic acids.

Table (1)
Percentage of carbohydrate, protein, nucleic acids in the crude and partial purified LPS.

LPS extraction acid % (260nm)	*Carbohydrate % (490nm)*	*Protein % (280nm)*	*nucleic*
Crud	**2.3**	**0.9**	**0**
Partial purified	**9.2**	**0.06**	**0**

Partial purification of LPS on sepharose CL-6B gel Fig.(1) showed three peaks. The biggest one represented carbohydrates and the others two were protein. The carbohydrate and protein percentage in partial purified LPS were 9.2 and 0.06 respectively. These components were linked with LPS which was difficult to separate. We note that carbohydrate percentage in partial purified LPS was 9.2% while in crude extract was 2.3%. This evidence of the removing of some cellular components that contaminated LPS extraction and raised purity of LPS [5].

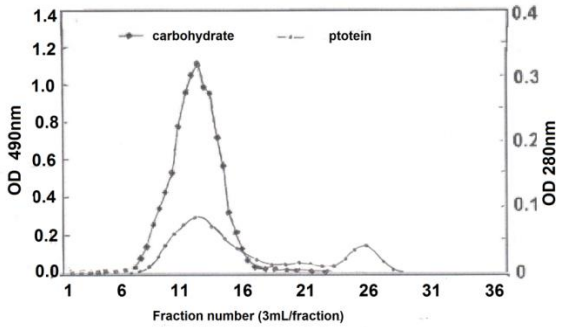

Fig. (1) Partial purification of LPS on gel filtration chromatography on sypharose CL-6B column (20×2.5 cm) fraction volume 3ml, follow rate 70ml/hr .elution buffer PBS 0.025M pH 7.5.

Results revealed that 0.1 and1µg/kg intravenous adminstration of partial purified LPS showed an elevation in body temperature (1.2 and 2.8)°C and body temperature were (38.8 and 40.2)°C respectively. In another study results revealed that intravenous adminstration of 5 ng/kg LPS led to elevation in body temperature due to activation of lymphocyte to produce cytokines [14 and 7] and both IL-1α and IL-6 induce the production of protein that led to elevated in body temperature.

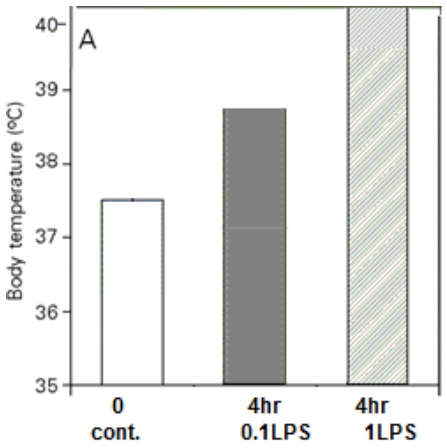

Fig.(2) Effect of intravenous adminstration of (0.1and1)µg/kg of partial purified K.pneumoniae LPS on rabbit temperature compared with control.

It's known that TNFα has the ability to increase the permeability of local vessels to enhance the movement of immune cells and soluble molecules from vasculature into the tissue [14]. Many studies indicate the stimulatory effect of LPS on innate and adoptive immune response. LPS causes complement activation [8], T-Lymphocyte proliferation [15], cellular and humeral activation [5]. A significant increase in cytokine level in the serum after 4 hr of intraperitonial injection with dose 10µg/mouse was recorded (Fig.(3)), serum of IL-1α, IL-6 and TNFα were (43.2 ± 4.1, 200 ± 2.2 and 130±15) pg/ml respectively. While intraperitonial injection of 10µ of purified LPS of *E.coli* (sigma) was (39±5, 210±1 and 120±3) pg/ml respectively (Fig.(3)).

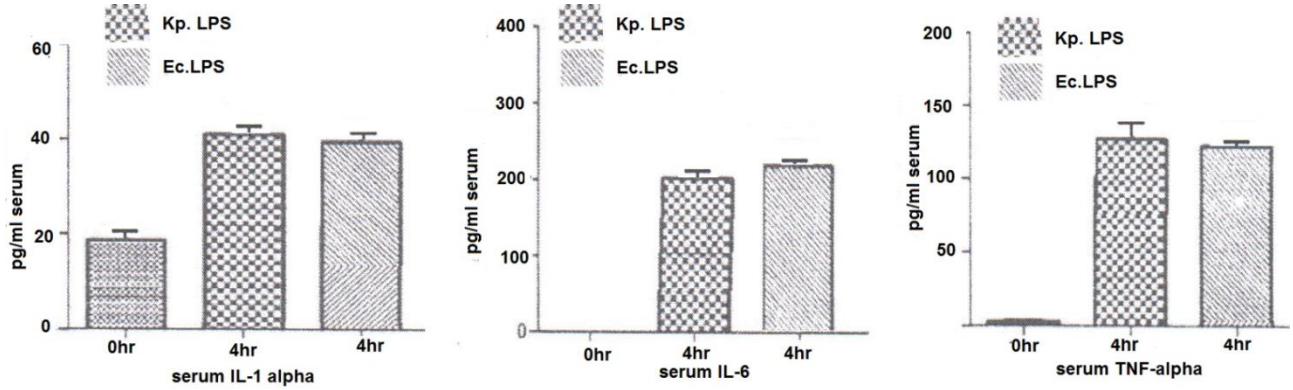

Fig.(3) Serum IL-1α, IL-6 and TNF-α level in mice injected itraperitonially with 10µ/mouse of K.pneumoniae and E.coli LPS.

The highest quantity of these cytokine was IL-1α, measured after 4hrs. There was no significant difference between IL-1α, IL-6 and TNFα in the serum of the mice injected with EcLPS and KpLPS. Many studies showed the effect of LPS isolated from Gram negative bacteria on proinflamatory cytokine [7] and its effect depend on many factors, include dose, time after antigen administration, microorganism and others [16]. Cytokine produced after LPS induction, alter transport rate for many peptide across the blood-brain barrier (BBB). Cytokine expression in both brain and serum compartment influence the BBB responses to an inflammatory stimulus and mediate changes in BBB transport[17] and showed that repeated dose caused more effective increase in cytokine level of serum and brain compartment, this could play an important role in the regulation of BBB. Elevation cytokine is benefitial in the protection and prevention against invaders, but the inappropriate cytokine production could led life-threating as septic shock, because these cytokine causes systemic changes and organ disorder and could lead to the death.

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الخلاصة

استخلص متعدد السكريد الشحمي باستعمال طريقه الفينول الساخن المحوره من عزله محليه لبكتريا *K.pneumoniae* من الدم. اجريت التنقية باستخدام عمود الترشيح الهلامي sepharoseCL-6B. اظهرت النتائج ان المستخلص على درجة من النقاوة ولم تتاثر الفعالية بعوامل التنقية وان كمية الكربوهدرات والبروتين كانت ٩,٥% و٠,٠٩% على التوالي. اظهرت النتائج ان حقن جرعتين من متعدد السكريد الشحمي المنقى جزئيا بمقدار ٠,١µg/kg و ١) داخل الوريد في الارنب ادى الى رفع درجة الحرارة جسم الارنب الى (٣٨,٨ و ٤٠,٢) درجة مؤويه على التوالي. اما حقن الفئران داخل البريتون بالجرعة ١٠µ g/mouse ادى الى زياده معنوية في مستوى المدورات الخلويه في المصل (IL-1α,IL-6 and TNF-α) مقارنة بالسيطرة بعد ٤ ساعات.

الاستنتاجات ان متعدد السكريد الشحمي المعزول من الكلبسيلا الرئويه الغازية له القابليه على تحوير الاستجابه المناعية ورفع السايتوكينات في المصل، والذي يؤدي بدوره الى رفع درجة حرارة الجسم بعد ٤ ساعات.