Test of efficacy of capsular polysaccharide antigen of *Klebsiella pneumonia* in stimulation of immune response in albino mice

Mayada F. Darwesh*

الخلاصة

صممت الدراسة الحالية لتقييم كفاءة مستضد عديد السكرايد المحفظي المعزول من بكتريا *Klebsilla Pneumonia* في تحفيز الاستجابة المناعية من خلال قياس عدد من المعايير المناعية في الفأر الابيض .

حضر مستضد عديد السكرايد المحفظي من بكتريا *Klebsilla Pneumoniae* ، ثم حضرت سلسلة من التراكيز المخففة لكل من العالق البكتيري والمستضد بالاعتماد على قيمة الجرعة القاتلة النصفية لكل منهما والتي كانت 1×10^3 cfu /ml للعالق البكتيري و 1×10^5 cfu/ ml لمستضد عديد السكرايد المحفظي ، ثم حقنت في 0.1 ml داخل غشاء البريتون للفئران .

قيمت الاستجابة المناعية بأستخدام الاختبارات الاتية : (معامل البلعمة ، تفاعل آرثس ، فرط الحساسية الآجل و معدل الخلايا المكونة للـويحات بأستخدام قيمة LD50 للعالق البكتيري و قيمة 0.1 LD50 , LD50 للمستضد ، لتقييم التركيز الاكفأ في تحفيز الاستجابة المناعية ، في ثلاث حيوانات لكل اختبار بالأضافة الى مجموعة سيطرة والتي حقنت بالمحلول الملحي المتعادل .

اكدت نتائج الدراسة الحالية ان مستضد عديد السكرايد المحفظي بتركيز 1×10^6 cfu/ ml هو الاكفاء حيث اعطى اعلى معدل لمعامل البلعمة ، تفاعل آرثس ، و فرط الحساسية الآجل اما بالنسبة للمناعة النوعية والتي قيست من خلال معدل الخلايا المكونة للـويحات فقد كانت الزيادة عالية المعنوية لنفس التركيز . من ذلك نستنتج ان مستضد عديد السكرايد المحفظي بتركيز 1×10^6 cfu/ ml هو الأكفاء والافضل في حث الاستجابة المناعية .

Abstract

The study was designed to assess *in vivo* the effects of capsular polysaccharide (CPS) antigen of *K. pneumonia* on some immunological parameters in albino mice.

CPS antigen from *K.pneumonia* was prepared, then series concetration of wild type of bathogenic bacteria and from CPS antigen were prepared depending on LD50 value which was;1×10^3 cfu /ml of wilde type and 1×10^5 cfu/ ml of CPS antigens ,these concentration injected in 0.1 ml intraperetonial in mice .

***Collage of Science –Kufa university**

Immune response in mice evaluated by using the following assay: (phagocytosis , Arthus reaction , delayed hypersensitivity and plaque forming cells) by use two concentration LD50 and 0.1 LD50 which was(1×10^5 , 1×10^6) cfu/ ml in 0.1 ml from CPS antigens« to evaluate the best concentration in stimulate immune response» and bacterial suspention in 3 mice to each test in addition to control group injected by normal saline .

The results of this study confirmed that , the CPS antigen in1×10^6 cfu/ ml concentration after 14 days from injection is the best efficient and gave highest mean in phagocytosis , delayed hypersansativity and arthus reaction , specific immunity in term of plaque forming cells was significantly increase in 1×10^6 cfu/ ml .It is concluded that efficient immune response can be obtained by CPS from *K. pneumoniae*

Introduction

Klebsiella pneumoniae (K. pneumoniae) is one of the known species of Klebsiella genus, a member of the family Enterobacteriaceae. *K. pneumoniae* is an encapsulated gram-negative bacteria , facultative anaerobic, It is found naturally in the soil, water and vegetables. In humans, it can be found in the skin, pharynx and gastrointestinal tract (1) .

K. pneumoniae is responsible for a variety of diseases in humans and animals. Its a prominent nosocomial pathogen mainly responsible for urinary tract, respiratory tract or blood infections (2) . Isolates from hospitals often display antibiotic resistance phenotypes (3). *K. pneumoniae* infections that are severe but more rarely reported include meningitis, necrotizing fasciitis and prostatic abscess (4) .

Multiple Klebsilla components (e.g., fimbriae , siderophores ,LPS , and capsule) have been considered to be potential virulence factors , Among these factors ,LPS induce TNF-∞ (5) . Also , the capsuler polysaccharide (CPS) has ability to stimulate antibody production (6) .

The initial interaction between bacteria and the host mucosal immune system probably occurred through recognition between

these surface components and dendritic cells. Indeed,the mucosal immune response is orchestrated by a network of surveillance based on dendritic cells (DCs), which are professional antigen-presenting cells (7). The role of DCs in the development of *K. pneumoniae* infections has not yet been clearly elucidated. DCs are a critical component of early lung inflammation elicited in a murine *K.pneumoniae* infection model and play a role in regulating lung barrier integrity (8). In another murine model of invasive bacterial pneumonia, it has been shown that DCs expressing TLR9 are required for effective innate immune response against *K. pneumoniae*, in particular to allow functional activation of lung macrophages and NK and T cells (9). In humans, little is known about the contribution of DCs to the host response to *K. pneumoniae* infections. Only in vitro experiments have been performed, and they indicate that *K. pneumonia* induces maturation of immature human DCs and production of interleukin 12 (IL-12) (10).

K. pneumonia infections have a high rate of mortality, sencie this organism has a rapidly progressive clinical course that often complicated multilobular involvement and lung abcesses , leaving little time to institute effective antimicrobial treatment (11) . This problem is further complicated by the emergence of multidrug resistant *K. pneumoniae* isolates (12) . As a result , development of new approaches to treatment, such as immunoprophylactic and immunotherapeutic agents against *K. pneumonia* infection has been sugested .Therefore, number of immunological parameter was conducted to evaluated the effect of *K.pneumonia* antigen in activation of immune response which include : Phagocytosis , Arthus Reaction , Delayed Type Hypersensitivity and Plaque forming cell

Materials and Methods

A- Bacterial isolate : *Klebsiella pneumoniae* was obtained from Biotechnology Research Centre (Al-Nahrain University), and further biochemical test (table 1) was carried out to ensure from the strain . The concentration of bacteria was determined by measuring

the absorbance at 600 nm on a Spectrophotometer compared to a standard curve of absorbances. The bacteria were pelleted by centrifugation at 6000 rpm were washed twice in normal saline , and were resuspended at a concentration of 1×10^{3} cfu /ml. One thousand CFU was used as the inoculation dose(13) . The capsular polysaccharide antigens were prepared by (14) then , the purification by (15) . The carbohydrate concentration according to (16). The 50% lethal dose(LD50%) was determined by (13) .

B- **Laboratory Animals** :Albino male mice were supplied by the Biotechnology Research Centre (Al-Nahrain University). Their age at the start of experiments was 8-10 weeks, and their weight was 23-27gm. They were divided into groups, and each groups was kept in separate plastic cage .The animals were maintained at a temperature of 23-35º C, and they had free excess to food (standard pellets) and water .

C- **Experimental Design** :The experiments were designed to evaluate (in vivo) the immunological effects of CPS antigens of *K. pneumoniae* in albino mice, as well as, their role in modulating the immunological effects, such evaluations were carried out through the following parameters :-

1-phagocytic index

The evaluation of phagocytosis was carried out on phagocytes obtained from the peritoneum of mice. The procedure of (17) was followed with some modifications.

2-Arthus reaction

After immunization protocol,the left foot pad was injected intradermally with 0.05 ml of 10% sheep red blood cell (SRBC) , while the right foot pad was injected with 0.05ml of normal saline. After four hours, the thickness of both pads was measured using a vernier, and the difference represented Arthus reaction index, which was given in units of millimeter (mm) (18).

3- Delayed Type Hypersensitivity

The index of delayed type Hypersensitivity (DTH) was carried out as outlined for Arthus reaction index, but the difference between the thicknesses of both pads was measured after 24h (18) .

4- Plque forming cells

The plaque forming cells(PFC) assay was carried out on B-lymphocytes obtained from the spleen of mice after performing the assay of DTH , following the procedure of (19) .

Statistical analysis : Data (mean ± standard deviation) for each experimental group were derived from three experiments , and analyzed statistically using Student's *t* test.mean with *p* of □ 0.05 were considered statistically significant.

Results and discussion.

The development of vaccines based on CPS obtained from *k.pneumonia* and other gram negative bacteria is now attracting much attention . The underlying immunological mechanism providing protection against *k.pneumonia* was studied in term of specific and non-specific immunity .

1- Biochemical tests

Biochemical tests of the strain are summarized in table (1) , result in morphology, characteristic of culture and biochemical test explain that the strain was *k.pneumonia* (20)

Table (1) Biochemical tests

Characteristic	*K. pneumonia*
Gram stain	-
Esculin	+
Inositol	+
Lactose	+
Sorbitol	+
Sucrose	+
MR	-
VP	+
H2S	-

(-) negative reaction; (+) positive reaction

2- Phagocytosis

The results in the Table (2) show that the significant increase in phagocytic activity in animal treated with (1×10^5) cfu/ ml CPS of *k.pneumonia* comared with control group , in the same table was highly significant ($P \leq 0.01$) increase in (1×10^6) cfu/ ml CPS of *k.pneumonia* while significant decreased in Bacterial suspension compared with control group .

Table (2)Phagocytic activity in animal treated with CPS antigens and Bacterial suspension of *k.pneumonia* compared with control group .

study groups	No.of animals	Phagocytosis (mean±st.d.)
Control	3	0.57±21.67
Bacterial suspension(1×10^3) cfu/ml	3	0.55±18.98
CPS antigen (1×10^5) cfu/ml	3	0.58 ± 27.68
CPS antigen(1×10^6) cfu/ml	3	1.91 ± 42.35

The alveolar macrophage (AM) is the primary means of innate host defense against bacterial pathogens that attempt to reach the alveolar space (21) . PMNs play a critical role in clearing encapsulated bacteria , such as *K.pneumonia* , from the lung and in preventing bacterial dissemination to the systemic circulation (22) . The ability of these cells to perform this function can be enhanced by inflammatory mediators that activate the PMN for increased phagocytosis and killing of ingested bacteria . Mechanistic studies revealed that the CPS augmentation of phagocytosis in AMs was largely Fc receptor (FcR) mediated and protein kinase C dependent in AMs (23) . In preliminary studies , the addition of antigens of *K.pneumonia* is able to augment the phagocytic index (PI) of alveolar macrophage . It has been shown previously that incubation of peritoneal macrophages with CPS from *K.pneumonia* stimulated the release of TNF-∞ , IL-1 and IL-6 (24) . CPS can increase *Trypanosoma cruzi* uptake and parasite killing by human macrophages in a nitric oxide – dependent manner (25)

3-Arthus reaction and delyed hypersensitivity

The study revealed that the CPS antigen of *k .pneumonia* in 1×10^6 cfu / ml concentration was high significant in arthus reaction and delayed hypersansativity and 10^3 was significant in phagocytosis while the Bacterial suspension decreased the phagocytosis in two test .

Table (3) Arthus reaction and delyed hypersensitivity in animal treated with CPS antigen and bacterial suspention of *k .pneumonia* compared with control group .

Study groups	No.of animals	Arthus reaction (mean±st.d.)	Delyed hypersensitivity (mean±st.d.)
Control	3	0.017 ± 0.48	0.01 ± 0.50
Bacterial suspension(1×10^3) cfu/ml	3	0.039 ± 0.36	0.03 ± 0.35
CPS antigen (1×10^5) cfu/ml	3	0.044 ± 0.92	0.06 ± 1.002
CPS antigen(1×10^6) cfu/ml	3	0.13 ± 1.52	0.09 ± 1.91

Intradermal injection of SRBCs causes production of IgG antibodes that react with antigen forming immune complex this cause local inflammation as a result of complement activation (26). DHT is one type of cell mediated immune response in which the macrophage stimulate the activation of T-cells and these cells produce cytokine that stimulate phagocytosis and activate the enzymes responsible to kill and destroy the tissue (27) .

4- Plaque Forming Cells

The result in the table (4) indicate that both (1×10^5 , 1×10^6) cfu/ ml were found to induce the formation of PFCs . The number of PFC observed in (1×10^5 , 1×10^6) cfu/ ml treated animals was (53.3 ± 1.52 ; 70 ± 2) , whereas those observed in bacterial suspention and control group at 14 days after treatment were (10.6 ± 1.52 ; 27.3 ± 0.57) respectively .

Table (4) Plaque Forming Cell in animal treated with CPS antigen and bacterial suspention of *k .pneumonia* compared with control group .

Study groups	No.of animals	Plaque Forming Cells
Control	3	27.3 ± 0.57
Bacterial suspension(1×10^3) cfu/ml	3	10.6 ± 1.52
CPS antigen (1×10^5) cfu/ml	3	53.3 ± 1.52
CPS antigen(1×10^6) cfu/ml	3	70.0 ± 2.

Humoral immune response to CPS antigen can be described in terms of the systems ability to function as an antigen depot (28) . This percent of PFC was found to be significant at $P \leq 0.01$, thereby indicating that CPS antigen 1×10^6 cfu/ml concentration produce a better humoral immune response by effect on lymphocytes in spleen that produce specific antibodes to SRBCs , this antigen increase immunoglobuline production especially IgG , IgM this agree with (29) , the increase in arthus reaction and DHT together with PFC this emphasis the effect of immunological function of spleen .

Over the past two decades, CPS has been the obvious vaccine candidate for *Klebsiella*-induced pneumonia studies. Cryz et al. (30) have demonstrated that active immunization with purified CPS protected rats against lethal *Klebsiella*-induced pneumoniae , anti-CPS antigens are the potential therapeutic agents to protect host against the complications induced by *k .pneumonia* , Onther study demonstrates that *K. pneumoniae* CPS antigens can agglutinate all of the K1 strains tested and prevent liver abscess formation induced by *magA_ K. pneumoniae* (31) . This indicates that the K1 epitope is a promising target for vaccine development .

References

1-Sahly, H., and Podschun, R. (1997): Clinical, bacteriological, and serological aspects of *Klebsiella* infections and their spondyl arthropathic sequelae. Clin. Diagn. Lab. Immunol. 4:393–399.

2- Podschun, R.,and Ullmann ,U. (1998) : Klebsiella spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. Clin Microbiol Rev 11: 589–603.

3- Paterson, D.L.; Ko ,W.C.; Von Gottberg ,A.; Mohapatra, S.; Casellas, J.M.; et al. (2004): Inter national prospective study of *Klebsiella pneumoniae* bacteremia: implications of extended-spectrum beta-lactamase production in nosocomial Infections. Ann Intern Med 140: 26–32.
4- Bergogne-Berezin, E.(1995). Nosocomial pathogens: new pathogens, incidence, prevention. Presse Med. 24:89–97.
5- Henderson, B.; S. Poole, and M. Wilson. 1996. Bacterial modulins: a novel class of virulence factors which cause host tissue pathology by inducing cytokine synthesis. Microbiol. Rev. 60:316–341.(15) .
6- Fung, C.; Hu, F, ; Change, S. ; Lee,B. ; Siu, L.; Kuo,M. and Lie, C. (2000) : A 5-year study of the seroepidemiology of klebsilla pneumonia : high prevalence of capsular serotype K1 in Taiwan and implication for vaccine efficacy . J. Infec. Dis. 181: 2075-2079.
7- Cools, N..; Ponsaerts, V. F.; Van Tendeloo,p. and Berneman, Z. N. (2007) :Balancing between immunity and tolerance: an interplay between dendritic cells, regulatory T cells, and effector T cells. J. Leukoc. Biol. 82:1365–1374.
8- Von Wulffen, W.; Steinmueller, S.; Herold, L. ; Marsh, P.; Bulau, W.; Seeger, T. ;Welte, J.; Lohmeyer ,S. and Maus, U.S.(2007) : Lung dendritic cells elicited by Fms-like tyrosine 3-kinase ligand amplify the lung inflammatory response to lipopolysaccharide. Am. J. Respir. Crit. Care Med. 176:892–901.
9- Bhan, U; Lukacs, J. ; Osterholzer, M. ; Newstead, X.; Zeng, T. ; Moore, T. ; McMillan, A. ; Krieg, S.; Akira, W. and Standiford, T.(2007) : TLR9 is required for protective innate immunity in Gram-negative bacterial pneumonia: role of dendritic cells. J. Immunol. 179:3937–3946.
10- Braat, H.; de Jong, J. ; van den Brande, M. ; Kapsenberg, M. ; Peppelenbosch, E. ; van Tol, A. and van Deventer, S. (2004) : Dichotomy between *Lactobacillus rhamnosus* and *Klebsiella pneumoniae* on dendritic cell phenotype and function. J. Mol. Med. 82:197–205.
11- Jay,S. (1983) : Nosocomial pneumonia , nosocomial infectiob ,74, 221-224.

12- Mackenzie , F.; Forbes , K . ; Dorai , J. ; Amyes , S. (1997) : Emergence of a carbapenem – resistant *klebsiella pneumonia* . Lancet , 358-370 .

13- Sinha , S.; Gabriel , A. ; Brian , K. (2004) : The *klebsiella pneumonia* O antigen contribute to bacteremia and lethaly during murine pneumonia .infection and immunity , 72,3 p1423-1430 .

14- Facklam , R. and Carey , R. (1985) : Streptococci and aerococci . In Lennette , E.H. .; Balom,A. ; Husler,W. J. and Shadom,H.J. (1985) : Manual of clinical microbiology . 3ed . American society for microbiology , Washington , D.C. 145-173

15- Garvey ,J. ; Cremer,N.; and Sussdorf , D. (1977) : Methods in immunology .3ed .W.A. Benjamin , Inc . Massachusetts . 518, pp: 356-359.

16- Bishop , M. ; Dben-Von Laufer , J.; Fody , E. and Thirty three contributors (1985) : Clinical chemistry principles , procedures and correlation . The murray printing company . Philadelphia , pp : 181-182 .

17- Metcalf, J.A.; Gallin, M.D.; Nauseef, M.D. and Root, R.K. (1986). Laboratory Manual of Neutrophil Function . Raven Press New York, U.S.A

18- Triolo, A.J. ; Osteholm, L. and Kratky, M.T. (1989): Enhancement of the Arthus and suppression of delayed type hypersensitivity (DTH) by bluronic f 68. A detergent frequently used to prepare perf carbon emulsion. International Journal of Immunopharmacology ,11:41-48.

19- Myers,R. L. (Editor) (1995). Immunology, a laboratory manual, 2nd Editor. Wm. C. Brown Communications, Ine. U.S.A. Pp. 109-112.

20- MacFaddin , J.F.(2000) : Biochemical test for identification of medical bacteria tested ; the Williams and Wilkins , U.S.A. baltimor .

21- Nelson ,S. ; Mason, C. ; Knolls , J. and Summer,W. (1995) : Pathophysiology of pneumonia . Clin . Chest.Med. 16: 1-12 .

22- Standifford , T. (1997) : Cytokines and pulmonary host defenses . Curr. Opin . Pulm. Med.3:81-88.

23- Mancuso , P. and peters-Golden ,M. (2000) : Modulation of alveolar macrophage phagocytosis by leukotrienes is Fc receptor –

mediated and protein and protein kinase C dependent . Am . J. Respi.Cell Mol. Bio. 23: 727-733.

24- . Fahey, T.; Tracey, P. ; Tekamp-Olson, L.; Cousens, W.; Jones, G. ; Shires, A.; Cerami,W. and B. Sherry. (1992) : Macrophage inflammatory protein 1 modulates macrophage function. J. Immunol. 148:2764–2769.

25- . Villalta, F.; Zhang, Y. ; Bibb, J. ; Kappes, J.and M. Lima (1998) : The cysteine-cysteine family of chemokines RANTES, MIP-1_, and MIP-_ induce trypanocidal activity in human macrophages via nitric oxide. Infect.Immun. 66:4690–4695.

26- Huebner ., Wang , Y. ; Krueger ,W. ; Madoff ,L. and Pier , G. (1999) : Isolation and chemical characterization of a capsular polysaccharide antigen shared by clinical isolation of Enterococcus faecalis and vancomycine resistance . Infec. Immu. , 67(3) :1213-1219 .

27- Miettinen, A.; Huses, J.;Toomi,J. (1990) : Serum antibody response to listerial excration , and clinical characteristic in experimentally infected goats . J. cl. Mic. 28 (2) : 340-343 .

28- Gregoridadis , G. (1990) : Immunological adjuvant , role for liposome. Immun. Toda. 11:89-97.

29- Salvadori , L.G. ; Blake ,M.; McCarty, M. ; Tai, J. and Zabriskie , J. (1995) : Group A streptococcus- liposome ELISA antibody titers to group A polysaccharide and opsonophagocytic capabilities of the antibodies . J Infec. Dis. 171 : 593-600.

30- Cryz, S.; Furer, E. and R. Germanier (1986) : Immunization against fatal

experimental *Klebsiella pneumoniae* pneumonia. Infect. Immun. 54:403–407.

31- Wu, M.; Yang , C.; Lin, T.; Bore-shin ,H.; Th-ying , Ch.; and Hsieh , Sh.(2009) : Humoral immunity against capsular polysaccharide protects the host from *magA Klebsilla pneumonia* induced lethal disease by evading toll-like receptor 4 signaling . Infec.and Immun. 77(2) : 615-621.