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EXTRACTION OF KLEBSIELLA PNEUMONIAE CAPSULAR POLYSACCHARIDE AND EVALUATION OF ITS ADJUVANTICITY

(With 3 Tables)

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استخلاص كبسول الكلبسيلا نيموني عديد السكريات وتقييم كفاءته المناعية

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تمت دراسة التنبيه المناعي والكفاءة المناعية المستحثة من كبسول الكلبسيلا نيمونـــي عديـد السكريات الخام المعالج بالفورمالين (سي بي أس-ك١) والبكتريا الغير معالجة (ســـى بـــى أس-ك٢) على خنازير غينيا المحصنة بلقاح طاعون الخيل الفيروسي الميت أو المســتضعف نوع ٩ بواسطة اختبار المصلى التعادلي لعينات أمصال هذه الحيوانات. أوضحت النتـائج أن (سي بي أس-ك١) أكثر كفاءة في تحفيز استجابة الأجسام المناعية للقـاح طـاعون الخيـل الميت بينما (سي بي أس-ك٢) لم يعط أي استجابة للأجسام المناعية (شلل منـــاعي) لنفـس اللقاح وكذلك إحداث انخفاض ملحوظ في استجابة الأجسام المناعية للقـاح طـاعون الخيـل المستضعف في خنازير غينيا المحصنة.

SUMMARY

Adjuvanticity and immunomodulating activity of crude capsular polysaccharide (CPS) prepared from *Klebsiella pneumoniae* treated with formaldehyde (CPS-K.1) and from untreated bacteria (CPS-K.2) was investigated in Guinea pigs immunized with freeze dried vaccine of inactivated or attenuated African Horse Sickness virus (AHSV) of serotype 9 as measured by assaying AHSV-neutralizing antibodies in their serum samples. The obtained results demonstrated that CPS-K.1 was highly effective in enhancing antibody response to inactivated

AHSV; conversely, CPS-K.2 induced an antibody unresponsiveness (immunological paralysis) to inactivated AHSV, and a marked depressed antibody response to attenuated AHSV in immunized Guinea pigs.

Key words: *Klebsiella pneumoniae capsular polysaccharide.*

INTRODUCTION

Klebsiellae are Gram-negative bacilli, that characteristically possess a thick polysaccharide capsules. The capsular polysaccharide (CPS) are composed of three regions differing in their chemical and biological properties. The O-specific polysaccharide (region I) carries the main serologic specificity of bacteria. It is linked to a core polysaccharide (region II) that is common to groups of bacteria. This polysaccharide core is linked through a 2-keto-3-deoxyoctanate di-or trisaccharide to the lipid component (region III), term lipid A (Ribi, 1986).

The ability of *Klebsiella (K) pneumoniae* to cause lethal burn wound sepsis in mice is dependent upon the amount of CPS produced (Cryz et al., 1984). Several hypothesis exist as to how *Klebsiella pneumoniae*. (CPS) acts to increase virulence: (1) antiphagocytic activity (Ehrenworth and Baer, 1956 and Yokochi et al., 1977); (2) induction of immune paralysis (Batshon et al., 1963) and (3) providing a barrier against non-specific host defense system (Domenico et al., 1982). This virulence property is mostly conferred by the lipopolysaccharide (LPS) constitute in *K. pneumoniae* - CPS which correlated closely with their pyrogenicity. Chemical modification of such LPS has been reduced their toxicity while retaining adjuvanticity (Ribi et al., 1979). While, purified CPS is a thymus-independent antigens (mainly induce production of IgM antibodies) (Dresser, 1968). LPS is a well-known adjuvant as its capability to enhance the formation of antibodies to non-related antigens. Investigators credit the adjuvant effect of LPS to the suggestive activation of T-lymphocytes (McGhee et al., 1979), macrophages (Louis and Lambert, 1979), polyclonal lymphocytes (Nakashima et al., 1980) and B-lymphocytes (Jacobs, 1982 and Tomas et al., 1986).

African horse sickness virus (AHSV) is an icosahedral non-enveloped virus of 10 segments-double stranded RNA genome, a

member of Reoviridae family (Joklik, 1974), identified in nine serotypes (Howell, 1962), and cause fatal disease in horses (Erasmus, 1973). Attenuated and/or inactivated vaccines of AHSV were recommended for saving the horses in risk of AHS epizootics (Hazrati and Ozawa, 1965 and Bourdin, et al. 1970). Guinea pigs could be taken as a laboratory animal model instead of horses for assaying the immunogenicity of such vaccines (Erasmus, 1963; Parker, 1975 and Abdel Baky, 1995). From a practical point of view, the search for an effective adjuvant of a very little or no undesirable local and systemic effect to be used in horses offer an exciting but difficult challenge. The present investigation has therefore attempted to examine the immunomodulating activity and adjuvanticity of *K. pneumoniae* capsular polysaccharide to AHSV in Guinea pigs inoculated with inactivated or attenuated vaccine of AHSV type 9.

MATERIAL and METHODS

Bacterial capsular polysaccharide:

The capsular polysaccharide of clinical isolate of *Klebsiella pneumoniae* (locally identified isolate) (CPS-K) was prepared from the grown organism on nutrient agar medium in Roux bottles mainly according to the method of Wilkinson et al. (1955). Bacteria were removed from cultures by suspending in a sterile normal saline solution (50ml/bottle). Twice washing of bacteria in saline solution were done by suspending and centrifuging at 4500 rpm for 30 minutes. The pooled bacteria suspension was divided into two equal portions. The first portion was treated with a final concentration of 0.5% formalin at room temperature for 24 hours; while the second portion was leaved without treatment. The bacterial capsules in both portions were separated in their aqueous phase by heating in 100°C water bath for 10 minutes and centrifuged at 4500 rpm for 30 minutes. The supernatants were retained and the CPS-K was precipitated in each portion by addition of two volumes of chilled acetone with stirring and keeping at 4°C for 18 hours. The precipitates were harvested as a moist pellets of CPS-K by centrifugation at 3000 rpm for 30 minutes, then dehydrated by keeping in desiccator for 3 days. The dehydrated polysaccharide was weighed and dissolved in acetate buffer solution, pH 4.8 [4% (w/v) sodium acetate and 2% (v/v) acetic acid]. Two preparations of crude-CPS-K were

obtained (1) 0.005% (w/v) CPS-solution of bacteria treated with formaldehyde, termed (CPS-K. 1); and (2) 0.005% (w/v) CPS-solution of bacteria did not treated with formaldehyde, termed (CPS-K.2). The two preparations were kept at -20°C until used. pH of CPS-K.1 and 2 was adjusted to 6.8-7.2 just before their application by using 0.1M NaOH.

Vaccines:

(1) Binary ethyleneimine (BEI) inactivated freeze dried vaccine of AHSV, type 9 and; (2) attenuated freeze dried vaccine of AHSV type (9) were prepared respectively as described by Abdel Baky (1995) and Hazrati and Ozawa (1965) from mouse adopted neutropic strain (S2) propagated in VERO cell culture (a cell line derived from African green monkey kidney).

Sterility and safety assays:

Sterility and safety of CPS-K.1 and 2 were examined by their inoculation in cultures duplicate of nutrient and blood agar media (incubated aerobically and anaerobically) and in adult Swiss mice (6 mice/each CPS-K) of approximately 20-35 grams weight by intraperitoneal route of injection (0.1ml/mouse). Inoculated cultures and mice were observed daily for 7 days after inoculation to determine the culture sterility and CPS-K lethality in inoculated mice (Mackie and McCartney, 1989).

Immunization of Guinea pigs:

Groups of 6-8 adult Guinea pigs of approximately 300-400 grams weight were obtained from Vet. Serum and Vaccine Research Institute, Abbasia, Cairo) and inoculated subcutaneously as follows :

	Group	Inoculum (1ml/animal)
(A)	(8 guina pigs)	Three injections of inactivated vaccine of AHSV, reconstituted in saline solution (1ml/injection) with one week apart.
(B)	(8 guina pigs)	Three injections of inactivated vaccine of AHSV, reconstituted in CPS-K.1 with one week apart.
(C)	(8 guina pigs)	Three injections of inactivated vaccine of AHSV, reconstituted in CPS-K.2 with one week apart.
(D)	(8 guina pigs)	One injection of attenuated vaccine of AHSV, reconstituted in saline solution.
(E)	(8 guina pigs)	One injection of attenuated vaccine of AHSV, reconstituted in CPS-K.1.
(F)	(6 guina pigs)	One injection of attenuated vaccine of AHSV, reconstituted in CPS-K.2.

Adverse reactions to inoculation of CPS-K including the local reactions (any notable pain swelling, tenderness or induration at the sites of injection were recorded over a seven days period.

Bleeding of Guinea pigs:

To determine the AHSV, type 9-neutralizing antibody level in sera of immunized Guinea pigs, blood was taken by heart puncture. For kinetics studies of antibody production, samples were obtained repeatedly from the same group at 21th, 42th and 63th days after the first injection.

Estimation of antibodies to AHSV, type 9:

Neutralizing antibody indices to AHSV, type 9 in sera of immunized Guinea pigs were measured by the serum neutralization test (SNT) as performed by Parker (1974) using VERO cell cultures and AHSV, type 9, strain (S2).

RESULTS and DISCUSSION

Straight forward choice of 0.005% crude CPS-K (5ug/dose/mouse and 50ug/dose/Guinea pig) to examine safety immunomodulating activity and adjuvanticity of *K. pneumoniae*. CPS was based on the available study of Nakashima et al. (1971) who concluded that injection of CPS-K in mice (5ug/dose) was efficient to induce a high antibody titre to unrelated antigen (bovine serum albumin). Sterility and safety assays of 0.005% crude CPS-K.1 and CPS-K.2 are shown in table (1). The two preparations were free from any viable *K. pneumoniae* or other aerobic bacteria. CPS-K.1 derived from klebsiella treated with formaldehyde was completely safe in inoculated mice, whereas, CPS-K.2 derived from untreated bacteria was recorded 33.3% of lethality in mice. It is clear that treatment of *K. pneumoniae* with 0.5% formaldehyde before extraction of crude CPS markedly reduced its toxicity probably as a result of detoxification of the containing LPS. The possibility of using chemical modification techniques to reduce selectively the toxicity of LPS while retaining its adjuvanticity was recommended (Ribi et al., 1979).

The safety, adjuvanticity and immunomodulating activity of CPS-K were next evaluated in Guinea pigs immunized with inactivated vaccine or attenuated vaccine of AHSV, type 9, reconstituted in CPS-K.1

or CPS-K.2 as measured by detection of AHSV type 9, neutralizing antibodies in their serum samples obtained on intervals.

Approximately 100% of Guinea pigs received one or three injections of CPS-K.1 exhibited a very similar mild local reactions characterized by a small swellings of slight tenderness at the sites of injection that were faded after few days; whereas Guinea pigs inoculated with one or three doses of CPS-K.2 were showed no local reactions. On the other hand, no systemic reactions were noted by individuals of any group. Next, the immune response of Guinea pigs (Group B) to inactivated freeze dried vaccine of AHSV, type 9, reconstituted in solutions of 0.005% crude CPS-K.1 was markedly initiated and prolonged more than in Guinea pigs (control Group A) that received inactivated vaccine, reconstituted in saline solution. The mean values of neutralizing antibody indices were (≥ 3.75, ≥ 3.75 and 2.25) in group (B) and 3.0, 2.0 and 1.0 in control group at the 7th, 28th and 49th days after immunization respectively. In contrast, the most conspicuous finding was that Guinea pigs of group (C) which immunized with inactivated vaccine reconstituted in solution of 0.005% crude CPS-K.2, exhibited no or undetectable levels of AHSV, type 9 neutralizing antibodies in their serum samples obtained between the 7th and 49th days after immunization (Table 2). Thus, the crude CPS-K.2 of *K. pneumoniae* untreated with formaldehyde seems to induce immunological paralysis in inoculated animals. Theoretically, molecular configuration of LPS in capsule of *K. pneumoniae* by the action of formaldehyde changed the immunoparalyletogenic effect of crude CPS-K.2 to be immuno-stimulatogenic one. The configuration of tetanus toxin molecules by inactivation with formalin rendered to safe and immunogenic as well as immunostimulant. Tetanus toxin has an immunosuppressive action on the reticuloendothelial cells with concomitant hypoglobinaemia; no antibodies were produced in case of tetanus due to the damage of immunocomponent cells by tetanus toxin (Mose and Dostal, 1972).

The given results are in agreement with previous studies. The capsular polysaccharide of *K. pneumoniae* induces immunologic paralysis in a way similar to that in which *K. pneumoniae* causes lethal infections (Batshon et al., 1963, Yokochi et al., 1977 and Domenico et al., 1982). In mice paralyzed with the capsular polysaccharide of *K. pneumoniae*, there is some inhibition of antibody formation to unrelated

antigens such as influenza virus, *Salm. typhosa* and sheep erythrocytes (Kobayashi, 1969). In mice, immunized with bovine serum albumin simultaneously with purified CPS-K, the amounts of CPS-K less than 10ug act immunologically whereas amounts larger than 100 ug act paralytogenically (Nakashima, 1971).

On the other hand, inhibition of the antibody response to living attenuated vaccine of AHSV, type 9 was recorded in Guinea pigs of Group (F), that administered with one injection of the vaccine reconstituted in 0.005% crude CPS-K.2 solution; at the 21th day after immunization, the mean value of AHSV-neutralizing antibody indices of 1.5 was significantly lower than those of mean value ≥ 3.75 in sera of the control Guinea pigs (Group D) that administered with attenuated vaccine (Table 3). Comparing these results with the negative antibody response to AHSV in Guinea pigs received inactivated vaccine reconstituted in CPS-K.2, it is evident that the antibody response to the virus was much more depressed in Guinea pigs that had repeatedly been injected with 3 doses of CPS-K.2 than in Guinea pigs that received one dose. Also this difference in antibody response to AHSV might occurred as a result of difference in the nature of immune response to inactivated and attenuated AHSV in immunized animals. In contrast to the successful adjuvanticity of CPS-K.1 to enhance the antibody response to inactivated vaccine of AHSV; attenuated vaccine of AHSV reconstituted in solution of 0.005% crude CPS-K.1 could not be shown to have any advantage over attenuated vaccine reconstituted in saline solution as judged at the 21th, 42th and 63th days after immunization by the serum neutralizing antibody indices with the mean values of (≥ 3.75, ≥ 3.75 and 2.0) and (≥ 3.75, 3.75 and 3.0), respectively in groups E and D of Guinea pigs (Table 3). In conclusion, the data presented in this paper suggest that (1) applicable safe and effective adjuvant could be prepared as a crude CPS of K. pneumoniae treated with formaldehyde, but we should have further more studies to achieve its application value; and (2) Problems associated with what is called immunosuppression or immunodeficiency looks like vaccination failure "immune unresponsiveness" in some individuals of the farm animals might circumstancely attributed to their suffering from subclinical infections of Gram negative bacteria as well as exotoxogenic bacteria.

Table 1: Sterility and lethality of crude capsular polysaccharide of *K. pneumoniae,* inoculated in nutrient agar medium and adult mice.

Preparation	Sterility	Lethality in mice	
		-/-	%
CPS-K.1	Sterile	0/6	0
CPS-K.2	Sterile	2/6	33.3 %

CPS-K.1Derived from *K. pneumoniae* treated with formaldehyde.
CPS-K.2Derived from *K. pneumoniae* untreated with formaldehyde.
-/- Number of dead mice/total number of inoculated mice.

Table 2: AHSV, type 9, neutralizing antibodies in sera of Guinea pigs inoculated with inactivated freeze dried vaccines of AHSV, type 9, reconstituted in saline solution or solutions of crude capsular polysaccharide CPS-K.1 and .2 of *K. pneumoniae*.

Group	Inoculum	Mean neutralizing antibody index		
		7th *	28th	49th
A	Inactivated vaccine, reconstituted in saline solution (Control)	3.0 (1-5)	2.0 (1-4)	1.0 (0-2)
B	Inactivated vaccine, reconstituted In solution of CPS-K.1	≥ 3.75 (2-7)	≥ 3.75 (3-5)	2.25 (1-4)
C	Inactivated vaccine, reconstituted in solution of CPS-K.2	0	0	0

CPS-K.1, derived from *K. pneumoniae* treated with formaldehyde.
CPS-K.2Derived from *K. pneumoniae* untreated with formaldehyde.
* Days after immunization.
0 Undetectable.

Table 3: AHSV, type 9, neutralizing antibodies in sera of Guinea pigs inoculated with attenuated vaccine of AHSV, type 9, reconstituted in saline solution or solutions of crude capsular polysaccharide CPS-K.1 and .2 of K. pneumoniae.

Group	Inoculum	Mean neutralizing antibody index		
		21th *	42th	63th
D	Attenuated vaccine, reconstituted	≥ 3.75	≥ 3.75	3.0
	in saline solution (Control)	(3-5)	(2-5)	(2-4)
E	Attenuated vaccine, reconstituted	≥ 3.75	≥ 3.75	2.0
	in solution of CPS-K.1	(2-6)	(2-7)	(1-3)
F	Attenuated vaccine, reconstituted	1.5	Nt	Nt
	in solution of CPS-K.2	(0-3)		

CPS-K.1, derived from *K. pneumoniae* treated with formaldehyde.
CPS-K.2Derived from *K. pneumoniae* untreated with formaldehyde.
* Days after immunization.
Nt Not tested.

REFERENCES

Abdel Baky, M.H. (1995): Studies on production and application of AHS polyvalent inactivated freeze dried vaccine. Ph.D. Thesis, Virology, Fac. Vet. Med., Cairo Univ.

Batshon, B.A.; Ber, H. and Schaffer, M.F. (1963): Immunological paralysis in mice by Klebsiella pneumoniae type 2 polysaccharide. J. Immunol, 90: 121 - 126.

Bourdin, P.; Monnier-Combon, J.; Rioche, M. and Laurent, A. (1970): Vaccination against AHS in tropical Africa : Evaluation of an inactivated vaccine. Proceed. 2nd Int. Conf. Equine Infectious Disease, Paris, 1969, pp. 202-206 (Karger, Basel, Munchen, New York, 1970).

Cryz, S.J., Jr; Furer, E. and Germanier, R. (1984): Experimental Klebsiella pneumoniae burn wound sepsis: Role of capsular polysaccharide. Infect. Immun., 43 (1): 440 - 441.

Dresser, D.W. (1968): An assay for adjuvanticity. Clin. Exp. Immunol., 3: 877-888.

Domenico, P.; Johanson, W.G., Jr. and Straus, D.C. (1982): Lobar pneumonia in rats produced by clinical isolates of K. pneumoniae. Infect. Immun., 37: 327 - 335.

Ehrenworth, L. and Baer, H. (1956): The pathogenicity of Klebsiella pneumoniae for mice: the relationship to the quantity and rate of production of type-specific capsular polysaccharide. J. Bacteriol., 72 : 713.

Erasmus, B.J. (1963): Preliminary observation on the value of the Guinea pigs in determining the innocuity and antigenicity of neurotropic attenuated AHS virus. Onderstepoort. J. Vet. Sci., 30: 11 - 22.

Erasmus, B.J. (1973): The pathogenesis of AHS. Proc. 3rd Int. Conf. Equine Infect. Dis., Paris, 1972, pp. 1 - 11.

Howell, P.G. (1962): The isolation and identification of further antigenic types of AHS virus. Onderstepoort J. Vet. Res., 29 (2) : 139 - 149.

Hazrati, A. and Ozawa, Y. (1965): Monovalent live-virus AHS vaccine. Bull. Off. Int. Epiz., 64: 683 - 695.

Joklik, W.K. (1974): Reproduction of Reoviridae. In: Comprehensive virology, ed. H. Franke-Cornat and R.R. Wagner, Plenum Press, New York. Vol. 2: 231 - 334.

Jacobs, D.M. (1982): Lipopolysaccharide and the immune response in D.R. Webb (ed) Immunopharmacology and the regulation of leukocyte function. Marcel Dekker, New York, pp. 231 - 251.

Kobayashi, T. (1969): Jap. J. Bact., 24: 602.

Louis, J.A. and Lambert, P.H. (1979): Lipopolysaccharides: From immunostimulation to autoimmunity. Springer Semin. Immunopathol., 2: 215 - 228.

Mackie, T.J. and McCartney, J.E. (1989): Practical Medical Microbiology. 13th Ed.Churchill Livingstone LTD., Edinburgh, London, U.K.

Mose, J.R. and Dostal, V. (1972): Die immunreaktion bei manifestem tetanus. Wien, Med. Wochen Schr, 122: 504 - 507.

McGhee, J.R.; Farrer, J.J.; Michalek, S.M.; Mergenhagen, S.E. and Rosenstreich, D.L. (1979): Cellular requirements for lipopoly-saccharide adjuvanticity. J. Exp. Med., 149 : 793 - 806.

Nakashima, I.; Kobayashi, T. and Kato, N. (1971): Alterations in the antibody response to bovine serum albumin by capsular polysaccharide of K. pneumoniae. J. Immun., 107 (4): 1112 - 1121.

Nakashima, I.; Nagas, F.; Matsuura, A. and Kato, N. (1980): Adjuvant actions of polyclonal lymphocyte activators. II. Comparison and characterization of their actions in initiation and potentiation of immune response to T-dependent and T-independent soluble antigens. Cell Immunol., 49 : 360 - 371.

Parker, J. (1974): AHS antibodies in Cyprus 1971 - 1977. Vet. Rec., 94: 370 - 373.

Parker, J. (1975): Inactivation of AHS virus by Beta-propiolactone and pH. Arch. Virol., 47: 357 - 365.

Ribi, E. (1986): Structure-function relationship of bacterial adjuvants; in advances in carriers and adjuvants of veterinary biologics. ed. Robert, M.; Nervig; Patricia, M. Gough, Merlin, L. Kaeberle and Cecelia A. Whetstone, the Iowa State Univ. Press, Chap. 4, pp. 35 - 49.

Ribi, E.; Parker, R.; Strain, S.M.; Mizuno, Y.; Nowotny, A.; Von Eschen, K.B.; Contrell, J.L.; McTaughlin, C.A.; Hwang, K.M.; Goren, M.B. (1979): Peptides as requirement for immunotherapy of the Guinea pig line-10 tumor with endotoxins. Cancer Immunol. Immunother., 7: 43 - 58.

Tomas, J.M.; Benedi, V.J. and Ciurana, Jofre, J. (1986): Role of capsule and "O" antigen in resistance of Klebsiella pneumoniae to serum bactericidal activity. Infect. Immun., 54 : 85 - 89.

Wilkinson, J.E.; Dudman, W.F. and Aspinall, G.D. (1955): The extracellular polysaccharide of Aerobacter aerogenes A3 (S1) (klebsiella type 54). Biochemical J., 59: 446 - 451.

Yokoch, T.; Nakashima, I. and Kato, N. (1977): Effect of capsular polysaccharide of Klebsiella pneumoniae on the differentiation and functional capacity of macrophages cultured in-vitro. Microbiol. Immunol., 21: 601 - 610.