

A study of the immune response of *Salmonella typhi* - LPS in rabbits

دراسة الاستجابة المناعية لـ LPS المعزول من البكتيريا *S.typhi* فى الارانب

Dr. Mahdi H. M.Al- Ammar

Coll. Sci – Dept . Biol ,

University of kufa

www. mhmal@Yahoo.com

Summary

The study included isolation of *S. typhi* from a diagnosed cases of typhoid patients, which dependent on the morphological and biochemical reactions in addition to the serological tests .LPS antigens were prepared and assuaged . as well as cell free culture filtrate antigen besides the ready prepared *S. typhi* agglutinogens. Such antigens were made available for plotting the immune status of the rabbits. In animals, *S.typhi* specific agglutinins titers(S.TH,S.TO) were measured and found to be within the range of 160-640 in peripheral blood respectively. The cellular immune response were determined by performing , leukocyte inhibition factor LIF. In case of *S. typhi* infection the LIF percentage raised up to 40% in systemic LIF when CFCF were used as a sensetizer . *S. typhi* immune protection was determined in animals ; the immune protection rate with LPS vaccine was 90% while the death rate was 10% .

الخلاصة :

تم خلال هذه الدراسة عزل وتشخيص بكتيريا الاختبار من المرضى المصابين بالتيفوئيد اعتمادا على الصفات الشكلية والأختبارات الكيموحياتية بالاضافة الى الفحوصات المصلية ، وكذلك تحضير عالق LPS من راسح مزرع الخلية الحر المعزول والمنقى من البكتريا المرضية *S. typhi* المسببة لمرض التيفوئيد ، وتم أيضا "دراسة المناعة الخلطية للحيوانات المعاملة ، حيث سجلت العيارات المصلية S-TH , S-TO . 160 , 320 على التوالي LPS . بعد ذلك حددت المناعة الخلوية غير المتخصصة والتي شملت تثبيط الخلايا البلعمية على المستوى الجهازى حيث كان معدل LIF % 40 قبل وبعد التجريع بـ *S. typhi* . فضلا عن ذلك حددت الوقاية المناعية لحيوانات المختبر حيث كان معدل الوقاية المناعية لـ LPS 90 % فيما كانت نسبة الوفيات كانت 10 % .

Introduction

Endotoxins are part of the outer membrane of the cell wall of Gram negative bacteria . The lipopolysaccharide complex associated with the outer membrane of Gram – negative bacteria such as *E. Coli* , *Salmonella* , *Shigella* , *Pseudomonas* , *Neisseria* , *Haemophilus* , and other leading pathogens (Kenneth ,2002). The biological activity of endotoxin is associated with the lipopolysaccharide (LPS). Toxicity is associated with the lipid component (Lipid -A) and immunogenicity is associated with polysaccharide components . The cell wall antigens (O antigens) of Gram – negative bacteria are components of LPS . LPS elicits a variety of inflammatory responses in an animal . Because it activates complement by the alterantive (properdin) pathway , it is often part of the pathology of Gram – negative infections (Michetti ,et al 1993) . The function of the outer membrane of Gram – negative bacteria is to act as permeability barrier . The outer membrane is impermeable to large molecules and hydrophobic compounds from the environment . It may play several roles in the pathogenesis of Gram – negative infections . It is a permeability barrier that is permeable only to low molecular weight , hydrophilic molecules . It also a barrier to lysozyme and many antimicrobial agents . It impedes destruction of the bacterial cells by serum

components and phagocytic cells. LPS may be involved in adherence (colonization) , or resistance to phagocytosis (Robbins et al ,1994; Kenneth ,2002). LPS consists of three components or regions , Lipid A , R polysaccharide and O polysaccharide. Both lipid-A (the toxic component of LPS) and the polysaccharide side chains (the nontoxic but immunogenic portion of LPS act as determinants of virulence in Gram – negative bacteria(Collee et al ,1996) .It is thought that LPS released into the bloodstream by lysed– Gram negative bacteria is first bound by certain plasma proteins identified as LPS – binding proteins . The LPS – binding protein complex interacts with CD14 receptors on monocytes and macrophages and other types of receptors on endothelial cells . In monocytes and macrophages several types of events are triggered during their interaction with LPS (Kenneth , 2002).

Production of cytokines , including IL-1 , IL-6 , IL-8 , tumor necrosis factor (TNF) and platelet – activating factor stimulate the production of prostaglandins and leukotrienes .These are powerful mediators of inflammation and septic shock that accompanies endotoxin toxemia . LPS activates macrophages to enhanced phagocytosis and cytotoxicity . Macrophages are stimulated to produce and release lysosomal enzymes , IL-1 (endogenous pyrogen) and tumor necrosis factor (TNF alpha) as well as other cytokines and mediators(Blanden *et al* ,1966; Kenneth , 2002). Activation of the complement cascade . C3a and C5 a cause histamine release (leading to vasodilation) and effect neutrophil chemotaxis and accumulation which result is inflammation(Blanden *et al* ,1966) .

LPS also acts as a B cell mitogen stimulating the polyclonal differentiation and multiplication of B-cells and the secretion of immunoglobulins , especially IgG and IgM . (Schumonn *et al* ,1990; Michetti *et al*,1993; Schiff *et al*,1998; Kenneth , 2002).

The aims of this study included the purification (LPS) of *S. typhi* in addition to the role of LPS to elicited the immune response in rabbits.

*** Material and methods**

***Culture media and reagents**

Culture media and reagents are mentioned in table(1)

Seq	Name	Class	Use
1.	Brain heart infusion	Enrichment	Cultivation of bacteria for dry weight
2.	Tetrathionet broth	Selective media	Enriches <i>S. typhi</i>
3.	MacConky agar	Differential media	Cultivation of enteric bacteria
4.	S.S agar	Selective media	Isolation of salmonella
5.	Mast agar	Agar	For LIF test
6.	Normal saline	Solvent	Titration of antibody
7.	Basal medium Eagle	solution	As cell nutritive of LIF
8.	Methyl red	reagent	Acid production
9.	H2O2 product	reagent	Gas production
10.	Oxidase	reagent	Electron transport
11.	ONPG test	reagent	Late lactose ferment

***Determination of *S. typhi* infection dose**

The bacteria cells (from the culture) were emulsified in normal saline to a visible density approxmatly 1.5×10^5 cell /ml bacteria , compared with McFarland standard tube(0.5) (Shnawa and Thwaini,2002).

*** Lab animal inoculation .**

Rabbits (1000-1500 g) were orally infected using orogaolne tube (Shnawa and Thwaini , 2002) .

*** Ready prepared agglutinogens .**

Standard bacterial suspension of known agglutinogens were used for the two types of antigens involving *S typhi* O. Ag and *S typhi* H antigens . (Plasmetic company) .

*** Preparation of leukocyte sensitizer .**

Cell free culture filtrate(CFCF) was prepared by cultivation of *S. typhi* in brain heart infusion broth for 24 hr; and the liquid media was centrifuged at 5000 Rpm for 15 min.

The culture supernatant was membrane filtered through millipore filter in syringe device , the filtrate was collected and distributed in sterile bottles and preserved in refrigerator(4C) until use as sensitizer (Shnawa and Thwaini , 2002) .

*** preparation of bacterial dry weight**

Brain - heart infusion broth was inoculated by *S. typhi* using shaking incubator for overnight to obtain high density growth . The culture was coole - centrifuged at 5000 Rpm for 20 min. , the precipitate which represents the cells of bacteria was dried in an incubator(37 C) for 48 hr .

*** Preparation of LPS-antigen**

LPS- suspension prepared according the (Westphal's *et al* ,1952) method (Adoos 2005).

*** Bacteriology**

Laboratory diagnosis of salmonella infection depends mainly on the isolation and identification of salmonella from the specimens of patients blood and faeces (Collee et al 1996 MacFadin ,2000) .

*** Blood culture**

Blood samples were collected from patients using sterile disposable syring immediately cultured on blood agar , MacConkey and S.S. agar for isolation and biochemical identification of the bacteria (Collee *et al* , 1996) .

*** Agglutination**

Direct semi quantitative slide as well as standard tube agglutination using serial double dilution for sera , solutions were reacted with laboratory prepared antigens(Garvey *et al.*, 1977).

***Peripheral blood LIF**

Measurement of migration inhibition factor in systemic blood according to (Soberg ,1968):

*Preparation of agar-A medium in sterile plastic plates and 2 wells were made 2cm in diameter.

*Capillary tube containing systemic blood from rabbits infected was placed in each well after being centrifuged by haematocrite centrifuge for 10 min.

(Leucocytes and Buffy coat) .

*0.1 ml of Eagle basal medium was put in each well; one of the wells kept as control.

*0.1 ml of antigen (CFCF) of the causative bacteria was added in one well .

*Incubation was done at 37c⁰ for 24 hr in Jar humid environment .

*Measurement of LIF by ooulometer, same steps were used for control and normal saline was added instead of CFCF as mentioned (Soberg , 1968) .

*** LPS-immunization**

Five rabbits , exo-endo and haemo parasite free as well as anti. *S. typhi* antibodies pre rabbits were kept adlibitum condition and assigned in three groups(Table- 2) .

LPS-suspension was administrated in five doses respectively at four week intervals , while the saline injected once , blood was collected for immunological investigation . (Shnawa and Thwaini , 2002) .

***Results and Disscation**

*** Bacteriological diagnosis**

The result showed that the salmonella like character. Some isolate of bacteria showing character of some biochemically (table .3)

Table (3) Biochemical test of *S. typhi* enteric fever

Seq.	Tests	Experimental result
1.	Gram stain	-
2.	V-P- test	-
3.	Methyl red	+
4.	Citrate utilize	-
5.	Indole	-
6.	H2O2	-
7.	Oxidase	-
8.	Urease	-
9.	Motility	+
10.	ONPG test	-
11.	H ₂ S	+
12.	Acid	+

The clinically diagnosis typhoid fever patients were proved by culture and serological diagnosis (Collee *et al* ,1996;Gianella,2001).

The extracted and proved *S.typhi* -LPS antigen was assaged biochemically and immunologically (Kenneth ,2002).

* **LPS- immune protection.**

Lab animals were injected subcutaneously with IFA, then immunogen in saline containing 3 mg *S. typhi*-LPS- suspension was dosaged orally; the symptoms were scored as mild, the death ratio was 0/5, but the protection rate was 100%.

Animals with non-LPS- Vaccine treatment had, fever and diarrhea, then death . The death ratio was 0\4, while the protection rate was 90%,in compared with control group (Table - 4).

Table (4) *S. typhi* immune protection against live *S. typhi* challenge

Groups	No. of rabbits	Infection dose	Symptoms	Death ratio	Protection ratio
LPS-Primed	5	<i>S.typhi</i>	-	0/4	90 %
Non- -LPS primed	5	<i>S.typhi</i>	Fever and death	5/5	0 %
Control	1	Saline	-	-	100 %

The findings of evolution of the LPS-immuno protection revealed the similarity between the

systemic humoral immune response in pre and post challenge. The antibody titer in serum ranged between (160-320)..However, at the cellular level, *S. typhi* induced cellular immunity, since the LIF value ranged between 0.40_ 0.41at systemic immunity in pre and post challenge (Table - 5).

Table (5) Immunologic evaluation of *S. typhi* challenger rabbit primed with LPS- vaccine

seq.	Pre-challenge				Post-challenge			
	Vaccine titers				Vaccine titers			
	STO	STH		LIF	STO	STH		LIF
1	320	160		0.35	320	320		0.35
2	320	320		0.30	320	320		0.30
3	160	320		0.45	160	320		0.45
4	160	160		0.5	160	320		0.45
5	320	320		0.35	320	320		0.35
6.control	80	80		0.90	80	80		0.90
Rang	160- 320	160- 320		40%	160- 320	320		41%

***LPS -Immunology**

The results of post vaccine and post challenge immune state evaluations , death rate and protective rate indicate that LPS antigen were immunoprotective against live - *S. typhi* challenging in a lapin model (Kenneth ,2002).Immunity to *S. typhi* requires both cell mediated and humoral immune responses and is achievable by vaccination (Forrest *et al.*, 1991) . *S. typhi*. LPS-Antigen used as oral vaccine elicits immune response (Table-4) and the protection rate of LPS - vaccine was 90 % , while the death rate was 1/5 . The results demonstrated that the oral immunization with LPS-antigen elicits strong systemic cell mediated immunity to live infection bacteria .(Michetti *et al*,1993 Copra *etal*, 1994).

The data presented in(Table-5) showed that the titer of antiserum to LPS- Antigen was the highest in both pre and post challenge with -*S- typhi* . Then inducing an antibody and cellular immune response, significant LIF were 40 % and the mean value were 40 for both challenge . Very effective protection was observed when LPS vaccine administered within 24 hr after challenge . This effect could be increased with repeated injections .The result showed that the LPS-antigen with IFA, could be more effective than LPS-antigen alone . This finding agrees with (Robbins *et al* ., 1994; Sharn , 2004) . Vaccine studies indicate that LPS- Lipopolysaccharide antigen should be an important

immune target , since parental immunization with this antigen has lead to increased protection against *S-typhi* (Schumonn,1990; Schiff *et al*, 1998).

***Cellular immunology**

The cell produces and releases a variety of effectors molecules called lymphokines (Weinberg ,1984 ; Weir *et al*,1994).There are biochemical mediators of a number of widely studied cases in vitro phenomena , but it is believed that a similar activity is responsible for the immune response seen in tissues , the best characterized lymphokin is LIF the subject of present assay (Table 5). Upon release from lymphocytes , LIF can be identified by this ability to trap macrophage and inhibiter their migration . The macrophage is the target cell responding to the product LIF of lymphocyte which is the effectors cell (Adoose , 2005) .

*** LIF test**

The data presented in (Table 5) indicate that *S. typhi* induce specific cellular immunity when culture filtrate were used as a sensitizer , the mean values of LIF were 40% and 41% at systemic level, pre and post challenge respectively .The results showed that the LIF formation increased peripheral blood leukocyte . (Weinberg ,1984 ; Weir *et al* ,1994; Adoose , 2005).

*** References**

- * Adoos , S.A., (2005). The correlation between mucosal and systemic immune response to antigen of bacteria associated with chronic ear discharge .PH.D .Thesis, College of Science. Babylon University.
- *Blanden R.V ;Mackaness M.B and Collins F.M (1966) Mechanism of acquired resistance in mouse typhoid .J. Exp .Med 124:585 .
- *Chopra .A.K ;Peterson J.W; Chary P.(1994). Molecular characterization of in enterotoxin from *S Typhimurium* . Microb pathogen.16:85.
- * Collee , J. ; Barrie , P. ; Marmion, A. ; Fraser ,G. and Simmons ,A. I (1996) . Practical Medical Microbiology . 4th.ed. , New York .
- * Garvey, J.S; Cremer, N.E and Sussdrof, D.H., (1977) Methods in Immunology 3th ed , pp 53-267. Addition- Wesley Publishing Company Inc, Reading .
- * Forrest, B. D ; Labrooy ,J.T;Breyer ,L. and others (1991) . The human humoral immune response to *S. typhi* Ty21. J. Infect. Dis.,163,P: (3) 6- 45.
- * Giannella,A. (2001) *Salmonella*. Med. Micro .,Chapter (21):1-10
- * Goldsby, R. A.; Kindt, T.J. and Osborne, B.A., (2000) Kuby Immunology, 4th ed .:95-101. W.H. Freeman and Company, New York .

- *Kenneth.T. (2002) . Mechanisms of Bacterial Pathogenicity : Bacterial Endotoxin . University of Wisconsin .Mid Micro–P:237-245.
- * Macfaddin , J.F(2000). Biochemical tests for identification for medical bacteria 3ed. Lippincott Williams and Wilkins.
- * Michetti , P. ; Mahan , M.J ; Slauch , J.M ; and others (1992) . Monoclonal secretory immunoglobulin A protects mice against oral challenge with the invasive pathogen *Salmonella typhimurium* . Infect. Immune., 1 (60): 178-179 vaccine J.Immunol., 169,:2196-2203.
- *Robbins, J. B; Schneerson, R. and Szu, S.C.(1994).Perspective hypothesis: Serum IgG antibody is sufficient to confer protection against infectious disease by inactivating the inoculum. J. Infect .Dis.,171:1387-1398.
- * Schiff, D.E.K ; Soldaa ,K.J.; Lee,J.; puglin ,K.D and Tobia,P.S., ,(1998).Phagocytosis of gram negative bacteria by a unique CD14 dependent mechanism. J. Biol., 62,P :786.
- * Schumann R. R; leong G.W ; Flaggs P.W ;Gray S.D and Wright J.C(1990) .Structure and functions of LPS binding protein, Science, 249-1429.
- * Sharn H.,(2004)Vi of *S. typhi* target , the protection family of molecule is intestinal and suppression of inflammation response , Published .Mid Micro, J pp: 123 129.
- * Shnawa, I.M.S. and Thwaini, Q.N. (2002). Lapin mucosal humoral versus systemic humoral and cellular immune response post to intracellular administration of heat killed *C . Feutus* J. Baby. Univ. 7 (3): 538-543 .
- * Soberg , M. (1968) . Invitro Migration inhibition of peripheral blood leucocytes in delayed type hypersensitivity . Acta Medica. Secand. 184, : 13-25 .
- * Weinborg , E.D.,(1984) . Iron withholding : a defense against infection and neoplasia . Physiol , Rev., 64(2) PP : 65 .
- * Weir , C.R. ; Richardson , J. ; Prabhala , R. (1994) . Endocrine regulation of mucosal immunity . Handbook of Mucosal Immunology . San Diego : Academic Press .: 705-716 .
- Westphal's O. ; Luderitz O.;Mayer H. and Galanos C. (1952) The story of bacteria endotoxin. Adv .Immunopharma col J,pp: 341-348.