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In vivo assessment of Immune Response to *Pseudomonas aeuroginosa* antigens

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Abstract

Intrduction: The study of immunogenicity of pseudomonal antigens is essential for both diagnosis and prevention for *Pseudomonas aeruginosa* infections.
Aims: This study is aimed to investigate the immunogenicity of Pseudomonal antigens in experimental animals.
Materials and methods: Outer membrane proteins (OMPs) and Lipopolysaccharaid (LPS) were used as antigens and administrated into lab animals via I.V. and I.P route. The immune response was assessed depending on the titer of anti-pseudomonal-antibody and phagocytic index.
Results: The mean of anti-LPS antibody titer injected via Intraperitoneally I.P and I.V intravenous route were 80 and160 respectively, while the mean of anti -OMP antibody titer injected via I.P and I.V route were 320 and 1280 respectively as compared with antibody titer for control (non-immunized rabbits) which was 20. Besides, it was found that OMPs stimulate cellular immune response, since there was significant increasing in phagocytic index($P< 0.05$) in rabbits that injected with OMPs via both I.P and I.V (12% and 13%) respectively as compared with control animals(9%), while there are no significant increase in phagocytic index ($P>0.05$) in rabbits that injected with LPS via both I.P and I.V (8%) and(6%) respectively as compared with control animals (9%).
Conclusion: OMPs were found to be good immunogen, since it was able to induce both types of immune response humoral and cellular, While Lipopolysaccharaid (LPS) induced only humoral immunity

الخلاصة

المقدمة: دراسة القابلية التمنيعية لمستضدات لبكتريا الزوائف الزنجارية ضروري لتشخيص ومنع الأصابة ببكتريا الزوائف الزنجارية.
هدف الدراسة: تهدف هذه الدراسة الى التحري عن القابلية التمنيعية لمستضدات الزوائف الزنجارية في الحيوانات المختبرية.
المواد وطرائق العمل: أستخدمت بروتينات الغشاء الخارجي و متعدد السكريد الشحمي كمستضدات و حقنت الحيوانات المختبرية عن طريق تحت الغشاء البريتوني و طريق الوريد. تم تقيم الأستجابة المناعية بالأعتماد على عيارية الضد للزوائف الزنجارية و الفعالية التمنيعية.
النتائج: وجد أن معدل عيارية الضد لمتعدد السكريد الشحمي المحقون عن طريق تحت الغشاء البريتوني وعن طريق الوريد هي 80 و160 على التوالي، بينما معدل عيارية الضد لبروتينات الغشاء الخارجي المحقونة عن طريق تحت الغشاء البريتوني وعن طريق الوريد هي 320 و 1280 على التوالي بالمقارنة مع عيارية الضد (20) لحيوانات السيطرة غير الممنعة. الى جانب ذلك، وجد أن بروتينات الغشاء الخارجي حفزت الأستجابة المناعية الخلوية . حيث كانت هنالك زيادة معنوية في الفعالية البلعمية في الارانب المحقونة ببروتينات الغشاء الخارجي بكلا الطريقين تحت الغشاء البريتوني والوريد (12٪ و13٪) بالمقارنة مع حيوانات السيطرة(9٪)، بينما لا توجد زيادة معنوية في الفعالية البلعمية للأرانب المحقونة بمتعدد السكريد الشحمي عن طريق تحت الغشاء البريتوني و الوريد(6٪ و 8٪) بالمقارنة مع حيوانات السيطرة(9٪) على التوالي.
الأستنتاج: وجد ان بروتينات الغشاء الخارجي ممنع جيد حفز كلا النوعين من الأستجابة المناعية (الخاطية و الخلوية) بينما متعدد السكريد الشحمي حفزالأستجابة المناعية الخلطية.

Introduction

Pseudomonas aeruginosa is gram negative bacteria colonizes eukaryotic hosts including plants, worms, animals, and humans[1]. It is highly versatile in virulence and it is known to possess intrinsic multi-drug resistance capabilities. *Pseudomonas aeruginosa* is the second most common bacteria cause nosocomial infections[2].

P.aeruginosa interacts with the external medium through its outer membrane (OM) The outer membrane composed of (Lipopolysaccharaid (LPS), proteins and phospholipid[3]. The major structural proteins of outer membrane represented by three types:porins, heatlabile

proteins lik OmpA and lipoproteins[4]. LPS is an important bacterial toxin that has been extensively studied as a mediator of gram-negative sepsis[5].

Phagocytic cells i.e., neutrophils and mononuclear phagocytes (circulating monocytes and tissue macrophages) are the body's primary defense against *P. aeruginosa.* Phagocytosis is enhanced in the presence of serum factors including IgG, and complement. Host defense against many invading gram-negative bacteria depends on innate immune recognition of endotoxin(LPS)[6]. Anti-Pseudomona vaccines that LPS based have been shown to provide limited serotype-specific protection. Also, the endotoxic properties of the LPS molecule have argued against the use of such vaccines in burn and in cystic fibrosis patients[7]. Therefore, OMPs have been used as major protective antigens in developing vaccines against infection with this pathogen. This Ag was shown to be highly immunogenic. OMPs as antigens have some advantages, the most important of which are their conservation among the 17 *P aeruginosa* serotypes and no cross reaction with other gram negative bacilli [8].

The aim of this study was to investigate the immunogenicity of LPS and OMPs extracted from clinical isolates of *P.aeruginosa* in experimental animals.

Materials and methods

Pseudomonas aeruginosa isolates

P.aeruginosa isolates were obtained from burn patients who admitted to Al-Hilla General Teaching Hospital. This organism was diagnosed and identified according to their characteristics diagnostic characteristics and then compared with their being reported in referential references [9] and [10].

Preparation of outer membrane proteins(OMPs)

Outer membrane proteins were prepared according to the procedure being recommended by[11].

Extraction of lipopolysaccharide(LPS)

LPS was extracted in this study using the procedure recommended by[12] modified by [13], then LPS was partial purified by procedure of [14], modified by [13].

Laboratory animals

Fifteen male rabbits apparently healthy New Zealand white *Oryctolagus Cuniculus* (weight 1.5-2 kg) used in this study which adapted to laboratory condition for 10 days under standard condition of eating and drinking [15].

Immunization program (suggested by this study)

Two methods were used for immunization of rabbits Intraperitoneally(I.P) injection and Intravenous injection(I.V). Three rabbits were used for each antigen and for each rout. Three rabbits don't injected with any immunogen which represent a control.

Protocol of immunization

1- Intraperitoneally(I.P) injection: The following protocol was employed for this experiment :

Table(1) Immunization protocol used in this study

Antigen injection	Outer membrane protein			LPS		
	Date	Dose	Rout of immunization	Date	Dose	Rout of immunization
Initial antigen injection	Day 0	0.25ml (10IU)	I.P	Day 0	0.1ml (10IU)	I.P
First antigen booster	Day 7	0.5ml (10IU)	I.P	Day 7	0.25ml (10IU)	I.P
Second antigen booster	Day 7	1 ml (10IU)	I.P	Day 7	0.5ml (10IU)	I.P
Third antigen booster	Day 7	1.5 ml (10IU)	I.P	Day 7	1ml (10IU)	I.P
Blood collection	Day 35			Day 35		

2-Intravenous injection: The same protocol mentioned above was also used in immunization of laboratory animals via intravenous rout.

Blood collection: Intracardiac puncture for large blood collection was performed under general anesthesia using Diethyl ether 98%. The injection area was cleaned with 70% alcohol. The blood was immediately put in sterile test tube to obtain serum and anticoagulant tube to obtain blood.

Assessment of immune response toward *P.aeruginosa* antigens

Cellular parameter: Phagocytic Index: Phagocytic activity was calculated according to the procedure of (Carlone *et.al.*,1986)[11]. Ingestion can be assessed by incubation of neutrophils with bacteria then intracellular bacteria can be seen microscopically[16]. Two hundreds PMNs were counted and the percentages of active phagocytic cells were determined.

Humoral parameters:Titration of anti-pseudomonal antibody

The titers of anti-lipopolysaccharaid antibody and anti - outer membrane antibody for *P .aeruginosa* antigens was calculated by tube agglutination test[17].

Statistical analysis

The mean, and SPSS of windows 1999 using t test for two variables at level of significant ($P< 0.5$) were calculated for analysis the results obtained in this study.

Result and discussion

The immunogenic ability of LPS and OMP was investigated by injection of these antigens (Ag) separately via two ways: intrapertoneal (I.P) and intravenous rout (I.V) in rabbits. The mean of anti-LPS antibody titer injected via I.P and I.V way were 80 and160 respectively, while the mean of anti - OMPs antibody titer injected via I.P and I.V way were 320 and 1280 respectively as compared with control non-immunized rabbits (table2). The presences of anti-pseudomonal antibody (titer 20) in serum of control animals may be related to cross-reaction with related bacterial species with a degree of Ag similarity, but the judgment for the indication of the presence of true infection titer-induced vaccination is the titer of Ab. Using of Ab titer as indicator for immunization or recent infection depends on the fact that an increasing in the host titer of two doubling titer is considered to be diagnostic of current natural infection or artificial immunization called a fourfold rise in titer[18].

Table(2)The titers of anti-lipopolysaccharaid antibody and anti - outer membrane antibody for *P .aeruginosa* antigens

Antigens	rout of immunization	titer
Lipopolysaccharied	I.P	80
	I.V	160
Outer membrane proteins	I.P	320
	I.V	1280
Control	No injection of any immunogen	20

There is an increasing in antibody titer in rabbit being immunized with LPS antigens by both routs as compared with control, Ab titer increase in rabbits injected via I.P with LPS antigens 4 times (titer 80) than control (20) and Ab titer increase in rabbits injected with LPS via I.V antigens 8 times (titer 160).Al-Muslemawi, (2007) reported that immunized rabbit with LPS of *C.freundii* led to induce high antibody titer[19]. Jashau and Fred, (1997) mentioned that injection of rats with LPS of *E.coli* (serotype O18) lead to stimulate B-cells for production of IgG[20]. Antibodies against LPS mediate antibacterial and endotoxin-neutralizing functions[21]. Furthermore ,LPS are T-cell-independent antigens ,and antibodies induced in response to them are mostly of the IgM isotype[22]. LPS stimulates B-cell production in germinal centers, while its effect on T-cells in which its act as adjuvant and also acts as immunomodulater, in which phagocytosis cells taken from mice immunized with LPS secrete IL-1 and the later increase the effect of LPS on others phagocytosis cells and lead to increase stimulation of T-cells that secrete IL-2 and B-cells growth factors and then increase B-cells numbers and increase antibody formation[3].

In the present study high antibody titer was seen after immunization of rabbits with OMPs via I.V and I.P as compared with control. This results confirmed results being reported by Lee *et.al*., (2000) who noted that immunization with OMPs of *P.aeruginosa* induced high titers of serum antibody both in rabbits and humans[23]. Sharifi-Yyazdi *et.al*., (2007) reported that the crude OMPs extracted from *P. aeruginosa* induced a significant protection in mice against *Pseudomonas* infections and could be used as a vaccine candidate. *P.aeruginosa* can be added to growing list of organisms for which the potential of OMPs as vaccine has been investigated[24]. These organisms include *Nesseria meningitides*, *N.gonorrhoeae*, *S. yphimurium*, *Shigella* species. *H.influenza* type b, *E.coli*, *V.cholerae* and *P.mirabilis*[25].

Antigen immunized via I.V route has high antibody titer than Ag immunized via I.P route, especially seen with OMPs in which high titer of antibody yield by I.V rout compared with I.P rout, antibody titer for (I.V) injection of LPS was twice the titer of antibody obtained via (I.P) injection. This may due to the abundance of immune cells in blood compared to other host parts.

Results of this study showed that high antibody titer was seen after immunization with OMPs than LPS immunization. This may due to the high protein content in OMPs than LPS. Proteins as immunogens induce strong immune response characterized by T-cell dependent pathway resulting in immunological memory which is the corner stone of vaccination[26].

The phagocytic activity of rabbit immunized with LPS and OMPs was studies. Results expressed in table(3) show significant increasing in phagocytic index($P < 0.05$) in rabbits injected with OMPs via both I.P and I.V (12% and 13%) respectively as compared to control(9%), while there is no significant increase in phagocytic index ($P>0.05$) in rabbits injected with LPS via both I.P and I.V (8% and 6%) respectively as compared to control (9%). Mishra *et.al*.,(2012) stated that polysaccharide inhibit efficient opsonization by complement, resulting in reduced neutrophile reactive oxygen species(ROS) production and decreased killing by phagocytes. This provides a survival advantage *in vivo*[27].

Table(3) Phagocytic activity on *P.aeruginosa*

Antigens	rout of immunization	Phagocytic activty
Lipopolysaccharied	I.P	6%
	I.V	8%
Outer membrane proteins	I.P	12%
	I.V	13%
Control	0	9%

There is an increasing in phagocytic activity of neutrophil for animals immunized with OMPs as compared with control, this result similar to that results reported by Abdurrahman, (2002) in which he observed that immunized with of *P.aeruginosa* OMPs causes increase in phagocytic activity[28]. Wood and Moller,(1985) extracted two types of glygoproteins from outer membrane of *K.pneumoniae* and these act as immunodulators[29]. Later, studies showed that this type of protein has immune stimulating activity and precipitates in humoral immunity and increase the level of antibody production and phagocytosis activity[30]. Jebur, (2010) showed that phagocytic index was increased in rabbits immunized with whole pseudomonal antigens[31]. Phagocytosis of bacteria by neutrophils provides an important first line of defense against bacterial infections[32].

Thus from the results expressed above, OMPs induce high level of humoral and cellular immune response when injected I.V. as compared to LPS. .

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