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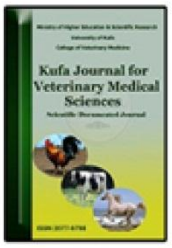

Study of cellular immune responses to the *Internalin B* protein extracted from *Listeria monocytogenes* in Mice

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Abstract:

This study was carried out to investigate the immunological activity of internalin B (InLB) protein which extracted from the cell wall of *Listeria monocytogenes.* Immunological activity was determined *in vitro* by the phagocytic activity assay and *in vivo* by bacterial clearance test in ninety six male white Swiss BALB/C mice were divided into five equal groups, G1 intrapertonailly injected with 0.5ml of InLB 50µg/ml;G2 intrapertonailly injected with 0.5ml of InLB 50µg/ml and inoculated with 1.2×10^{3}CFU/ml of *L. monocytogenes.*; G3 intrapertonailly injected with 0.5ml of InLB 50µg/ml followed by *L. monocytogenes* 24 hours later; G4 intrapertonailly injected with1.2×10^{3}CFU/ml *L. monocytogenes* a positive control; G5 intrapertonailly injected with 0.5ml of PBS a negative control, The results of the *in vitro* study showed the InLB at 50µg/ml induce the phagocytic activity by increase the Nitric oxide (NO) production as compared with Phytohemagglutinin-p (PHA); the results of the *in vivo* study was carried for 2weeks; at 4,8and 12days bacterial clearance assay; tissue of the internal organs (liver) of the G3 showed decrease in number of the bacteria at 12days (6×10^{-2} CFU) as compared to G2 and G4 (2×10^{-3}CFU and 1.8×10^{-4}CFU) respectively.The result presented in this study contribute for the first time in Iraq; that internalin B given intrapertonailly injection in mice for 12 days improves the immune responses by decreasing the bacterial number in liver tissue.

Keywords: Male mice, Phagocytic activity, Bacterial clearance.

دراسة الاستجابة المناعية الخلوية لبروتين *Internalin B* المستخلص من جرثومة *Listeria monocytogenes* في الفئران

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الخلاصة:

أجريت هذه الدراسة لمعرفة الفعالية المناعية لبروتين أنترنالين بي المستخلص من جدار الخلية لجرثومة *Listeria monocytogenes*.وأستخدم مقياس الفعالية البلعمية والطرح البكتيرية كمؤشر لفعاليته كمحفز مناعي.96 فأرة من الذكور نوع white Swiss BALB/C قسمت الى خمسة مجاميع متساوية،المجموعة الاولى أعطيت أنترنالين بي

بروتين،المجموعة الثانية أعطيت أنترنالين بي بروتين و*Listeria monocytogenes* ،المجموعة الثالثة أعطيت أنترنالين بي بروتين و*Listeria monocytogenes* بعد 24ساعة ،المجموعة الرابع أعطيت *Listeria monocytogenes* فقط والمجموعة الخامسة أعطيت محلول الملحي المتعادل. الدراسة خارج الجسم الحي (*invitro*) والتي تضمنت دراسة الفعالية البلعمية لبروتين internalin B (InLB) بأستخدام الـPhytohemagglutinin-p (PHA) ونترات الصوديوم القياسية وأظهرت النتائج زيادة في تركيز أوكسيد النترات المنتج عند تركيز(50مايكروغرام /مليلتر) من البروتين مقارنة مع (Phytohemagglutinin-p (PHA ونترات الصوديوم القياسية. إستمرت الدراسة على مدى 12 يوم. تم أخذ نماذج من الأعضاء الداخلية (الكبد) على مدى أربعة أيام ،ثمانية أيام وأثنتا عشر يوم لإجراء أختبار الطرح البكتيري أظهرت النتائج الطرح البكتيري من نسيج الكبد في المجموعة الثالثة عند يوم الاثنتا عشر حيث بلغ (6×10^{-2} CFU) بالمقارنة بالمجموعة الثانية والمجموعة الرابعة،حيث بلغ عدد الخلايا البكتيرية (2×10^{-3}CFU و1.8×10^{-4}CFU) على التوالي ،النتائج التي توصل اليها البحث ولأول مرة في العراق .أن حقن أنترنالين بي بروتين بالغشاء الخلبي في الفئران ولمدة 12يوم أدى الى تحسين الاستجابة المناعية من خلال أنخفاض العدد البكتيريا في نسيج الكبد.

Introduction:

Listeria monocytogenes is small short gram-positive, non-spore forming Coccobacilli high-virulence pathogens causing septicemia, abortion, and central nervous system (CNS) infection in a wide range of animal species including humans (1). Spread in nature where, exists largely in decaying vegetation, soil, animal feces, feed and water as make it one of the major pollutants of food and play essential role in the transfer of the infection occurring between humans and animals (2,3).

L. monocytogenes possesses several virulence factors, such as Internalin B (InIB) protein of the important factors in the bacterium, where there are two forms, one form associated with the cell wall of the bacteria its need to complete the invasion of many animal cells such as Epithelial cells, Hepatocyte and Endothelial cells (4), The other form is the form that liberates the bacteria into the center of their existence to act as a growth (Growth factor), InlB binds to c-Met, a receptor tyrosine kinase (RTK) and Hepatocyte Growth Factor (HGF) (5).

InlB is found at the bacterial surface and to some extent in bacterial culture supernatants. InlB is also able, when externally added, to associate with *L. monocytogenes* and several other Gram-positive bacteria. These suggest not only that InlB may interact with the cell wall after secretion or release from the bacterial surface but also that this interaction could contribute to invasion (6). Macrophages are important for uptake of *Listeria* into both liver and spleen, but by their antimicrobial action, they are also key effectors of the innate immune response and can present antigens to develop the adaptive response. Also InlB promote phagocytic events, it also activates signaling pathways, such as the NF-kB and the Ras-MAP kinase pathways that are themselves linked to phagocytosis (7).

Materials and Methods:

Strain of *Listeria Monocytogenes*

The *Listeria monocytogenes* isolate was obtained from the unit of Zoonotic diseases in the College of Veterinary Medicine, it was injected into a group of mice and then isolated from the internal organs of these mice and then confirmed by culturing on selective culture media and the biochemical tests were carried on used the API LISTERIA SYSTEM to be sure that it was *Listeria Monocytogenes*.

Internalin B

The internalin B protein used in this study was extracted from *Listeria monocytogenes* according to the method published by (8).the total concentration of protein in the extract was determined according to the method (9)and the value was 3.232 mg /ml.

Animals

Ninety six male white Swiss BALB/C mice, aged 6-8 weeks and weight range (20-25g), were used in this study. They were housed and maintained in a

conventional animal facility, with controlled conditions of temperature (20 ± 5ºC) and (10 and 14 hours of light and dark respectively). The animals were fed on special formula feed pellets and given water *ad libitum*. Throughout the experiments, each ten mice were housed in a plastic cage containing hard-wood chip as bedding. The bedding was changed weekly to ensure a clean environment.

Experimental Design

This experiment was designed to determine the effect of InlB on the phagocytic activity (*in vitro*) and bacterial clearance (*in vivo*). Ninety six male white Swiss BALB/C mice were divided into five group (12-24) mice and treated as follow for 12 days:

G1: The 12 mice were i.P. injected with 0.5ml of inlB 50µg/ml.

G2: The 24 mice were i.P. injected with 0.5ml of inlB 50µg/ml and inoculated 1.2×10^3CFU/ml of *L. monocytogenes*.

G3: The 24 mice were i.P. injected with 0.5ml of inlB 50µg/ml and inoculated 1.2×10^3CFU/ml of *L. monocytogenes* after 24 hours.

G4: The 24 mice were i.P. injected with 0.5ml of 1.2×10^3CFU/ml *L. monocytogenes*.

G5: The 12 mice were i.P. injected with 0.5ml of phosphate buffer saline as control.

Phagocytic activity

The Phytohemagglutinin-p (PHA) and internalinB protein was incubated (100µL/well) in 24 multiwell plate in different concentration (50,100,200,300µg/mL) with macrophages for four h in RPMI medium without serum at 37 °C. Non-adherent cells were removed by washing the monolayers with RPMI medium. Infected macrophages were maintained in RPMI 1640 with 5% fetal calf serum at 37 ºC in a 5% CO2 incubator for 20 h. The absorbance was determined in a spectrophotometer at 600nm, as described (10).

Bacterial Clearance

Liver were taken from mice on days 4,8,12, and 10-fold serial diluted in saline and homogenized Serial dilutions of the homogenates were plated on Brain Heart Infusion Agar plates. The plates were incubated aerobically at 37°C overnight, and then count the bacterial colonies, as described (11).

Results and Discussion

***In vitro* study:**

Determination of phagocytic activity:

The phagocytic activity assay was done with the different concentration of InLB of *L. monocytogenes* as shown in the figure (1).

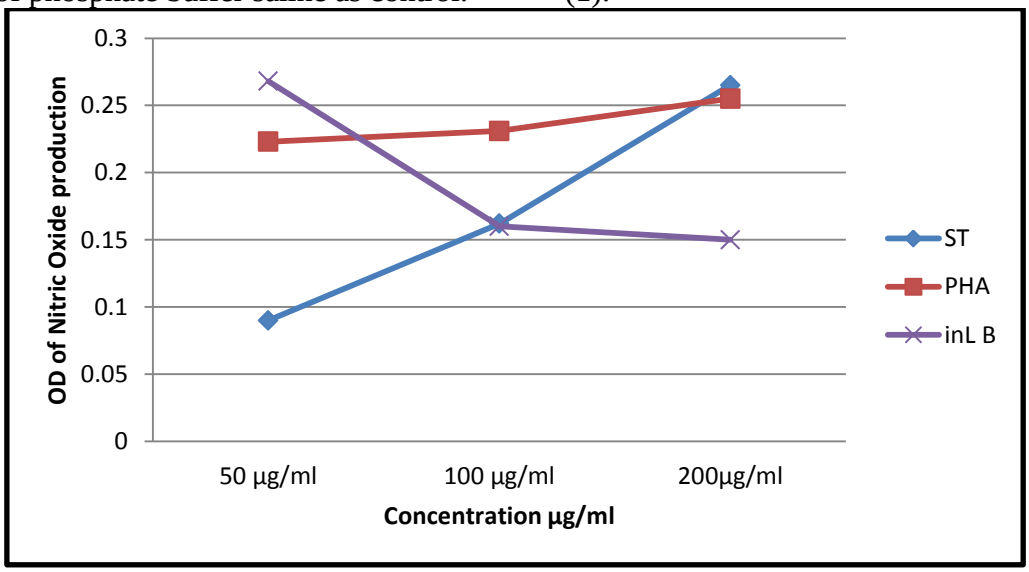

Figure (1): The percentage of Nitric oxide (NO) production by Internalin B protein at the 50µg/ml compared with PHA according to the standard of sodium nitrite.

The result showed increase in the concentration of Nitric oxide (NO) production by InLB at the 50µg/ml compared with Phytohemagglutinin-p (PHA) according to the standard of sodium nitrite.

***In vivo* study**

Bacterial clearance:

The experiment was carried out on the groups (G2, G3, and G4) as shown in table (1). The G3 that was treated with InLB and *L. monocytogenes* after 24 hours, shown a high increase in the bacterial clearance of the liver tissue at 8days (1×10^{-3} CFU) and 12 days (6×10^{-2} CFU) as compared to G2 and G4.The G2 that was treated with internalin B protein and *L. monocytogenes*, shown increase in the bacterial clearance of the liver tissue at 12 days (2×10^{-3}CFU).While the G4 that was treated with *L. monocytogenes* only, shown increase in the bacterial clearance of the liver tissue at 12days (1.8×10^{-4}CFU).

Table (1): The effect of internalin B protein on the Bacterial clearance.

Days / Groups	Colony forming unit/organ (liver)		
	4days	**8days**	**12days**
G2	**3.5×10^{-4}**	**4.2×10^{-3}**	**2×10^{-3}**
G3	**1.8×10^{-4}**	**1×10^{-3}**	**6×10^{-2}**
G4	**2×10^{-5}**	**4×10^{-4}**	**1.8×10^{-4}**

G2: i.p. injected with inlB+ *L. monocytogenes*
G3: i.p. injected with inlB and after24 *L. monocytogenes*.
G4: i.p. injected with *L. monocytogenes*.

In the current study the results of phagocytic activity showed that InLB has the ability to induce phagocytosis; the process is initiated by intimate contact between bacterial invasion proteins with the host cell receptors and is followed by the progressive engulfment of the bacterial body into the target cell, a so-called "zipper-like" mechanism, InLB involved in a regulatory step of the entry process. (12). InLB has the ability to stimulate many of different enzymes important in Cytoskeleton rearrangement to coated *L. monocytogenes* , that's made it one of the important proteins has an essential role in increase ability of bacteria to invasion and proliferation in phagocytic cells and spread to other tissue of the host body (13). The first step to phagocytosis is the reaction that occurred between invader and membranes of phagocytic cells that contain many surface receptors react with some of bacterial surface proteins such as InLB protein of *L. monocytogenes* (14,15). Jonquieres., 2001; was confirmed that InLB has ability for aggregation in a high concentration at the specific sites on the surface of phagocyte cells in from a large number of bacteria get entry inside the cells (16).

The present result obtained was in agreement with different studies demonstrated and explain the role of InLB in phagocytic activity, the ability of the innate immune system to quickly recognize and respond to an invading pathogen is essential for controlling the infection by triggering effective immune responses including phagocytosis and proinflammatory factor production, leading to the elimination of infectious agents (14, 17). The increase in the total and

differential white blood cells count as a first step in innate immune response such as Neutrophil and Monocyte in the blood stream to the *L. monocytogenes* and its secreted proteins such as InL B protein that was produced it in high quantity when the bacterium get entry to the host body to work as a growth factor for the bacterium from one hand, and to invade the host body cells from the other hand (18). Also InL B has ability to stimulate many of phagocytic cells after binding to its specific receptors to secrete several cytokines that's played an important role in attracting many of different phagocytic cells at the site of the protein, in addition increase the production of these cells from the bone marrow (19, 20).

Macrophages have been the focus of innate immunity during L. monocytogenes infection since replication occurs primarily within them and they are also an essential cell subset in mediating clearance of bacteria. Resident macrophages, especially Kuffer cells in the liver, are responsible for the initial killing of the majority of the injected bacteria. In response to infection, macrophages secrete TNFα and IL-12, these two cytokines drive natural killer (NK) cells to produce IFNγ, which in turn leads to activation of the macrophages and increases their bactericidal activity (23).

Many researchers were confirmed the ability of InL B protein to stimulate these cells to secretion of TNF that's can stimulate other immune system component such as B-lymphocytes to production of antibody and increase in monocytes through its ability to stimulation and regulation of hematopoiesis from steam cells in the bone marrow that have important role in phagocytosis and introduced antigen to B-lymphocytes, thus, eliminate the infectious agent (21, 22).

Conclusions:

From this study we can conclude the following points:-

1- Extraction of Internalin B protein from *L. monocytogenes* by using Tris-hydrochloric acid 1M PH =7.5, and purification by using of Ion Exchange Chromatography, the concentration of protein was 3.232 mg /ml.

2- Internalin B at concentration of 50µg/ml increases the phagocytic activity response in *vitro.*

3-Internalin B showed increase in bacterial clearance from internal organs (spleen and liver).

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