Immunogenic Activity of Lipopolysaccharide Isolated from *Salmonella enterica* serovar Typhimurium and Conjugated with Liposome in Albino Male Mice.

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Abstract

This study was designed to evaluate the role of *Salmonella enterica* serovar Typhimurium as a causative pathogen in hospitalized diarrhoeal patients younger than five years old, extract and purify endotoxin (lipopolysaccharide; LPS) from isolated and identified *S*. Typhimurium, and determine the immunogenic activity (humoral and cellular immune responses) of LPS-liposome conjugate in albino male mice. The patients (95 cases) were admitted to the Central Pediatric Hospital and Al-Kadhiymiah Pediatric Hospital in Baghdad during the period 24/10/2010 - 30/11/2010, because of severe diarrhoea and fever. Bacterial evaluation of stool samples revealed the identification of two (2.1%) *S*. Typhimurium isolates (S1 and S2). Antibiotic sensitivity test demonstrated that S1 isolate was more resistance than S2 isolate; therefore it was considered more virulent and subjected for extraction and purification of LPS. Chemical characterization of the extracted LPS revealed that the carbohydrate content was 2.34 mg/ml, while the protein concentration was very low (0.52 µg/ml). Partial purification of extracted LPS by using gel-filtration chromatography (Sephacryl S200) showed three peaks, and after determination of protein and carbohydrate concentrations for each peak, peak two was observed to have the highest carbohydrate content (25%) and the lowest contaminated protein (0.001%). The LPS of this peak was immunologically evaluated in mice at a concentration of 100 µg/ml, alone or in conjugation with a commercially available liposome (LIP). These evaluations revealed that LPS-LIP conjugate was able to modulate the humoral and cellular immune responses against live *S*. Typhimurium.

Keywords: Diarrhea, *Salmonella*, Lipopolysaccharide, Liposome.

الفعالية التمنيعية لمستضد متعدد السكريد الشحمي المعزول من بكتريا *Salmonella enterica* الضرب Typhimurium والمقترن مع الجسم الشحمي في ذكور الفأر الأبيض.

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الملخص

صممت الدراسة لتقييم دور بكتريا *Salmonella enterica* الضرب Typhimurium كممرض مسبب للإسهال في المرضى الراقدين في المستشفى وبعمر أقل من خمسة سنوات، واستخلاص وتنقية الذيفان الداخلي (متعدد السكريد الشحمي) المعزول والمنقى من بكتريا *S.* Typhimurium المشخصة، وتحديد الفعالية التمنيعية (الاستجابتين المناعيتين الخلوية والخلطية) للمقترن متعدد السكريد الشحمي–الجسم الشحمي في ذكور الفأر الأبيض. أدخل المرضى (95 حالة) إلى مستشفى الطفل المركزي ومستشفى الكاظمية للأطفال في بغداد خلال الفترة 2010/10/24–2010/11/30 بسبب الإسهال الحاد والحمى. وفي ضوء التقييم البكتيري لعينات الخروج، تم الحصول على عزلتين (2.1%) من بكتريا *S.* Typhimurium وهي S1 وS2، وكانت العزلة S1 أكثر مقاومة للمضادات الحيوية من العزلة S2 وبالتالي عدت أكثر ضراوة واستعملت في استخلاص وتنقية متعدد السكريد الشحمي. أظهر التوصيف الكيميائي لمتعدد السكريد الشحمي المستخلص بأن المحتوى الكاربوهيدراتي هو 2.34 ملغم/مل، في حين كان تركيز البروتين واطئ جدا (0.52 مايكروغرام/مل)، وعند التنقية الجزئية للمستخلص باستعمال تقنية الكروماتوغرافي على الهلام (Sephacryl S200)، ظهرت ثلاث قمم وبعد تحديد المحتوى الكاربوهيدراتي والبروتيني لها، انفردت القمة الثانية بأعلى محتوى كاربوهيدراتي (25%) وأقل تلوث بالبروتين (0.001%). أجري تقييم مناعي لمتعدد السكريد الشحمي لهذه القمة في الفئران بصورة مفردة وبتركيز 100 مايكروغرام/مل أو بصورة مقترنة مع الجسم الشحمي. أوضحت هذه التقييمات بامتلاك مقترن متعدد السكريد الشحمي–الجسم الشحمي القدرة على تعديل الاستجابتين المناعيتين الخلوية والخلطية ضد بكتريا *S.* Typhimurium الحية.

الكلمات المفتاحية: الاسهال، بكتريا السالمونيلا، متعدد السكريد الشحمي، الجسم الشحمي.

Introduction:

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is one of the non-typhoidal *Salmonella* serovars and being the most common serovar in the USA. Infection always occurs via ingestion of water or food contaminated with animal waste rather than human waste (17). The emergence of multidrug-resistant *S.* Typhimurium DT104 has been associated with outbreaks related to food contamination and resulted in increased rates of hospitalization (40). Therefore, *S.* Typhimurium continues to represent a major public health problem worldwide, and vaccine development has been an important target for researches in salmonellosis. The prevention of salmonellosis by vaccination has been the subject of many investigations, and despite this, the mechanism of protective immunity against *Salmonella* spp. infections remains a controversial subject (32).

Salmonella Typhimurium is classified as a facultative, intracellular bacterium by virtue of its ability to survive and multiply within specialized phagocytic cells of the host reticuloendothelial system (18). In their intracellular environment, the *Salmonella* spp. are provided with a considerable amount of protection from host defence mechanisms, and although both cell-mediated (CMI) and humoral immune responses are evoked in naturally occurring and in experimentally induced salmonellosis, the relative degree of protection afforded by each of the two arms of the specific immune response is still unclear (31). Thus, it has been observed that mice vaccinated with killed *Salmonella* or with soluble *Salmonella* antigens were not sufficiently protected from a lethal challenge inoculum despite the presence of a substantial amount of specific antibody. However, adoptive transfer experiments suggest that CMI is the overriding protective factor against *Salmonella* infections (16).

Several studies have demonstrated that lipopolysaccharide (LPS) of *S.* Typhimurium is a key component associated with bacterial virulence, but in terms of vaccine potential, the LPS itself is not very immunogenic, and as a result, attempts have been made to synthesize a vaccine which incorporates *Salmonella* LPS antigenic determinants, but is devoid of the toxic properties inherent in the lipid A moiety of the LPS, through a covalent attachment to carrier molecules, such as phospholipid bilayered vesicles (liposomes) (24). Liposomes have received a considerable amount of attention as carriers for the delivery of a wide variety of biologically active

substances to cells and tissues *in vitro* and *in vivo*, and they have been employed as immunological adjuvants for the enhancement or modulation of immune responses, especially CMI, to various antigens (9).

Accordingly, The present investigation was conducted first to assess the role of *S.* Typhimurium as a causative pathogen in hospitalized diarrhoeal patients younger than five years old, and then extract and purify endotoxin (LPS) from isolated and identified *S.* Typhimurium, and determine the immunogenic activity (humoral and cellular immune responses) of LPS-liposome conjugate in albino male mice.

Materials and Methods

Patients:

Patients of present study were hospitalized children and infants (95 cases: 60 males and 35 females) under the age of five years (40 days– 2.5 years). They were admitted to the Central Pediatric Hospital and Al-Kadhiymiah Pediatric Hospital in Baghdad during the period 24/10/2010 - 30/11/2010, because of severe diarrhoea and fever.

Stool Sample Collection:

A stool sample was taken from each pediatric patient upon admission to the hospital and before taking any medication using a disposable swab applicator, which was transferred to a test tube containing 10 ml sterilized peptone water.

Isolation and Identification of S. Typhimurium:

After 2-3 hours, the sample was incubated at 37°C with a total time of approximately 24 hours. After incubation, 5 ml of cultured stool sample were transferred to 250ml flask containing 50 ml tetra-thionate broth, and incubated at 37°C for 24 hours. After incubation a loopful from each flask was streaked on SS agar (selective medium) plates, and incubated at 37°C for 24 hours. The suspected colonies were subjected to further identifications, which included Gram stain, catalase test, indole test, methyl red test, Vogas-Proskauer test, citrate utilization test, Kligler iron test and glucose fermentation test (3). The *Salmonella* was further identified by APi 20E system, while *S.* Typhimurium was identified by serotyping using a commercially available kit (BioRad Company, USA). Antibiotic

sensitivity was also assessed for the isolated and identified *S.* Typhimurium.

Extraction and Purification of Lipopolysaccharide:

The LPS was extracted from the bacterial outer membrane of one isolate by a method given by (6). The extracted LPS was partially purified by gel filtration using Sephacryl S200 gel, which was washed and suspended in 0.025 M of PBS (pH 7.2), degassed by using vacuum pump and then poured with care to avoid bubbles into a column with a dimension of 75×2 cm. The final volume of the column was 235.5 cm^3. The column was equilibrated with 0.025 M of PBS (pH 7.2), and the flow rate was 75 ml/hour.

The recovery of LPS was according to (29), in which 5 ml of crude LPS was applied gently to the column, and flow rate was approximately 75 ml/hour. Five milliliters fractions were collected, and absorption was read at 280 nm for protein determination (5). Determination of carbohydrate content in the collected fractions was made by phenol-sulphuric acid method, by transferring 0.5 ml from each fraction in a sterile tube, then 0.5 ml of 5% phenol and 2.5 ml of sulphuric acid were added to each tube, and cooled in ice bath. Then, the absorbance was measured at 490 nm (14). Protein concentration was determined according to (4). The peak with the highest carbohydrate content and lowest contaminated protein was immunologically evaluated in albino male mice at a concentration of 100 µg/ml, alone or in conjugation with a commercially available liposome (LIP; Promega, USA).

Experimental Design:

The mice were distributed into eight groups (negative controls; NC, positive controls; PC, complete Freund's adjuvant; CFA, heat-killed bacteria; HKB, formalin-killed bacteria; FKB, LPS, LIP and LPS-LIP conjugate). Each mouse was injected intraperitoneally (IP) with 0.1 ml of the respective solution in day 1, and a further dose in day 8. These mice were considered as pre-challenged groups, and they were dissected for laboratory evaluations in day 15. Further similar groups were challenged with 0.1 ml live bacteria in day 15 (5×10^4 cell/ ml), and they were dissected for laboratory evaluations in day 29 (post-challenged groups) (13). The laboratory evaluations included total leukocyte count (TLC), absolute count of lymphocytes (ALC), neutrophils (ANC) and monocytes (AMC) (33), phagocytosis (peritoneal phagocytes) of heat killed yeast (*Saccharomyces cervisiae*)

(27), Arthus reaction (AR) and delayed-type hyper-sensitivity reaction (DTHR) (36).

Statistical Analysis:

They data were statistically analyzed using the statistical package SPSS (Statistical Package for Social Sciences) version 13.0. The investigated parameters were presented in as mean ± standard error (S.E.), and differences between means were assessed by ANOVA (analysis of variance), followed by LSD (least significant difference) or Duncan test. The difference was considered significant when the probability (P) value was ≤ 0.05, 0.01 or 0.001 (25).

Results and Discussion:

Frequency of *S*. Typhimurium in Stool Samples:

Out of 95 stool samples, *S*. Typhimurium was isolated and identified from two samples only (2.1%), which were belong to two children; the first was at age 25 months, while the second was 4 months older (S1 and S2 isolates). Both children had severe diarrhoea that was associated with fever.

Enteric infection with *Salmonella* spp. is an important cause of children diarrhoeal disease worldwide, but the frequency of children with the infection shows variations between studies. A study from Bolivia of 133 consecutive children less than 5 years old presenting with bloody diarrhoea revealed bacterial aetiology in 41%, and out of this percentage, *Salmonella* spp. accounted for 4% (35). However, a more recent study from Vietnam, no *Salmonella* infection was reported in 587 children with diarrhoea under the age 24 months, although other potential pathogens were identified in 67.3% of children with diarrhoea, including Gram negative bacteria (38). Accordingly, understanding the burden of pathogen specific diarrhoeal disease and the variation by region is important for planning effective control programmes for the overall reduction of diarrhoea disease among persons of all ages, especially in children under the age of 5 years.

Extraction and Partial Purification of Lipopolysaccharide:

Antibiotic sensitivity test demonstrated that S1 isolate was more resistance than S2 isolate; therefore it was considered more virulent and subjected for further manipulations, which included extraction of LPS from the bacterial outer membrane. Chemical

characterization of the extracted LPS revealed that the carbohydrate content was 2.34 mg/ml, while the protein concentration was very low (0.52 µg/ml). Partial purification of extracted LPS by using gel-filtration chromatography (Sephacryl S200) showed three peaks, and after determination of protein and carbohydrate concentrations for each peak, peak two was observed to have the highest carbohydrate content (25%) and the lowest contaminated protein (0.001%) (Figure 1 and Table 1).

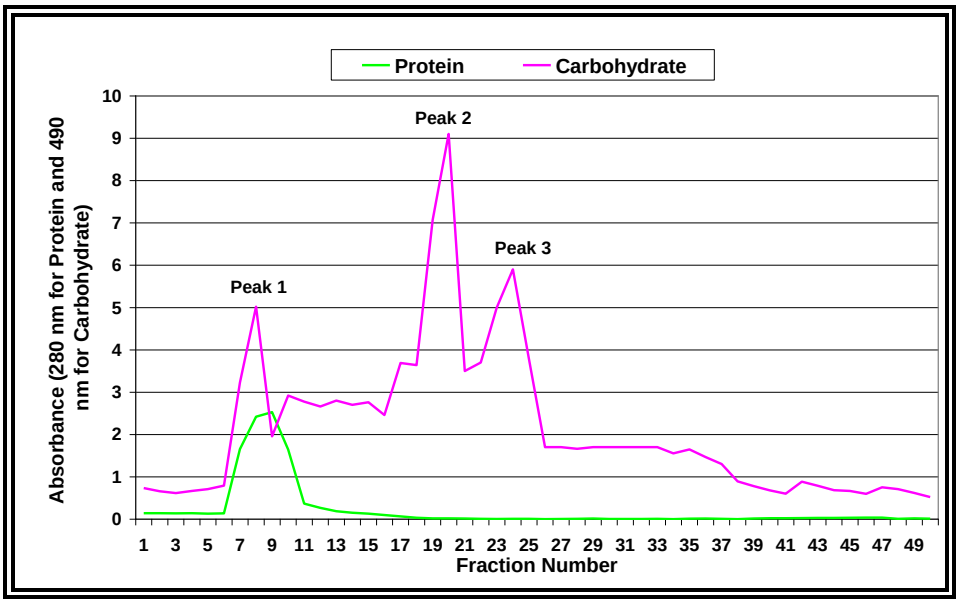

Figure (1): Gel-filtration chromatography for lipopolysaccharide partial purification from *S.* Typhimurium (S1 isolate) by using Sephacryl S200, 75×2 cm column equilibrated and eluted with 0.025 M PBS pH 7.2 with a flow rate of 75 ml/hour.

Table (1): Carbohydrate and protein contents of crude and partially purified lipopolysaccharide.

Lipopolysaccharide		Carbohydrate (%)	Protein (%)
Crude		17.2	6.3
Partially Purified	Peak 1	11.0	3.0
	Peak 2	25.0	0.001
	Peak 3	12.0	0.001

Most studies agree that percentage yield of carbohydrates may vary widely following endotoxin purification. The first group obtained 12-18% carbohydrate from partially purified endotoxin (20), while other investigators reported a less percentage range, which was 16.2-24.8% (39). In addition, 33.3% was obtained by a further group of investigators (37). These differences can be attributed to the types of bacterial species from which LPS was extracted, method of extraction and purification process.

The present finding (carbohydrate yield of 25%) differs from that recorded by (2), who demonstrated that the carbohydrate percentage in partially purified LPS of local *P. aeruginosa* isolate was 15%, and a further contradicting finding was also recorded by (20), in which 12-18% range was observed. However, an agreement was reached with further studies. The first demonstrated a yield of 16-24% (7), and the same finding was reported by (39), in which the carbohydrate percentage in purified LPS was 16.2-24.8%.

In table 1, we can notice that the protein percentage in partially purified LPS was 3% in peak 1 and 0.001% in peaks 2 and 3. The percentage of peak 1 differs from the percentage recorded by (2), who recorded that the protein percentage in partially purified LPS from *P. aeruginosa* was 2%. It also differs from the percentage recorded by (30), who stated that the percentage of protein bound to the purified LPS was 4.3%. However, an agreement with (11) is reached, because they found that the percentage was less than 0.1%. Furthermore, (39) recorded that the percentage of contaminated protein was very little, so they neglected such finding.

The differences in protein and carbohydrate percentages in the purified LPS may be related to differences between the bacterial strains and their content of LPS, differences in the methods used for extraction and purification of LPS and experiment circumstances. The results also showed that there were no nucleic acids in the partially purified LPS.

It was also observed that the carbohydrate percentage in the partial purified LPS (25% in peak 2) was higher than that of the crude LPS (17.2%), and a similar observation was made for the protein (3.0 and 0.001 *vs*. 6.3%). Both observations suggest the efficiency of the applied method of purification by gel filtration.

Total and Absolute Counts of Leukocytes:

The TLC in all groups (pre- and post-challenged) was almost approximated the normal leukocyte count range in mice, but mice vaccinated with HKB or FKB (11250 and 12083 cells/cu. mm blood, respectively) showed the highest count in pre-challenged animals (Table, 2), and such increase also contributed to increased counts of lymphocytes, neutrophils and monocytes in the two groups of mice (Tables 3, 4 and 5). However, mice vaccinated with LPS showed a significant increased ALC in post-challenged animals as compared with pre-challenged animals (4970 *vs*. 3527 cells/cu. mm blood) (Table, 3).

Table (2): Total leukocyte count in groups of mice pre- and post-challenged with *S*. Typhimurium after different vaccination protocols.

Groups	**Mean ± S.E. (Total Leukocyte Count: cells/cu. mm blood)**		**P ≤**
	Pre-Challenge	Post-Challenge	
Negative Control	7966 ± 380C	10400 ± 400A	0.01
Positive Control	5200 ± 171D	4800 ± 115D	N.S.
Complete Freund's Adjuvant	5466 ± 133D	6633 ± 270C	N.S.
Heat-Killed Bacteria	11250 ± 1295A	4700 ± 480D	0.001
Formalin-Killed Bacteria	12083 ± 1719A	4466 ± 480D	0.001
Lipopolysaccharide	6500 ± 152D	7733 ± 197C	N.S.
Liposome	6333 ± 168D	6766 ± 238C	N.S.
Lipopolysaccharide+Liposome	9366 ± 95B	8533 ± 240B	N.S.

Different letters: Significant difference ($P \leq 0.05$) between means of columns.

Table (3): Absolute lymphocyte count in groups of mice pre- and post-challenged with *S.* Typhimurium after different vaccination protocols.

Groups	Mean ± S.E. (Absolute Lymphocyte Count: cells/cu. mm blood)		P ≤
	Pre-Challenge	Post-Challenge	
Negative Control	5020 ± 149A	6198 ± 355A	0.05
Positive Control	2711 ± 97B	2993 ± 30C	N.S.
Complete Freund's Adjuvant	2688 ± 95B	3447 ± 190C	N.S.
Heat-Killed Bacteria	5833 ± 763A	2761 ± 313C	0.001
Formalin-Killed Bacteria	5075 ± 1222A	2469 ± 297C	0.001
Lipopolysaccharide	3527 ± 149B	4970 ± 118AB	0.01
Liposome	3343 ± 108B	3483 ± 175C	N.S.
Lipopolysaccharide+Liposome	5731 ± 116A	4389 ± 108B	0.05

Different letters: Significant difference (P ≤ 0.05) between means of columns.

Table (4): Absolute neurophil count in groups of mice pre- and post-challenged with *S.* Typhimurium after different vaccination protocols.

Groups	Mean ± S.E. (Absolute Neutrophil Count: cells/cu. mm blood)		P ≤
	Pre-Challenge	Post-Challenge	
Negative Control	2098 ± 63C	3016 ± 134B	0.01
Positive Control	1872 ± 106C	1538 ± 66D	N.S.
Complete Freund's Adjuvant	2136 ± 27C	2596 ± 103B	N.S.
Heat-Killed Bacteria	4355 ± 441A	1318 ± 109D	0.001
Formalin-Killed Bacteria	4623 ± 618A	1471 ± 159D	0.001
Lipopolysaccharide	2240 ± 67C	2168 ± 99C	N.S.
Liposome	2483 ± 60C	2656 ± 91B	N.S.
Lipopolysaccharide+Liposome	3637 ± 154B	3930 ± 163A	N.S.

Different letters: Significant difference (P ≤ 0.05) between means of columns.

Table (5): Absolute monocyte count in groups of mice pre- and post-challenged with *S*. Typhimurium after different vaccination protocols.

Groups	Mean ± S.E. (Absolute Monocyte Count: cells/cu. mm blood)		P ≤
	Pre-Challenge	Post-Challenge	
Negative Control	566 ± 194B	946 ± 51A	0.01
Positive Control	535 ± 44B	329 ± 31BC	N.S.
Complete Freund's Adjuvant	583 ± 49B	628 ± 39B	N.S.
Heat-Killed Bacteria	1010 ± 157A	549 ± 61B	0.001
Formalin-Killed Bacteria	1205 ± 242A	401 ± 50BC	0.001
Lipopolysaccharide	691 ± 45B	595 ± 39B	N.S.
Liposome	585 ± 59B	627 ± 62B	N.S.
Lipopolysaccharide+Liposome	49 ± 31C	213 ± 76C	N.S.

Different letters: Significant difference (P ≤ 0.05) between means of columns.

Leukocytes are considered as the active cells in carrying out the functions of the immune system, both non-specifically and specifically, and their count may give a general picture about the function of the immune system (22). These cells are originated in the bone marrow through two cell lineages; myeloid progenitor and lymphoid progenitor, which give rise to the five types of leukocytes (neutrophils, lymphocytes, monocytes, eosinophils and basophils). Each type of these cells is commenced to carry out an immune function. The neutrophils are mainly involved in the innate immune system to carry out phagocytosis, while lymphocytes represent the humoral and cellular arms of specific immunity. Monocytes are involved in carrying out phagocytosis, but they are also professional antigen presenting cells. Eosinophils are involved in allergic and inflammatory reactions, as well as, parasitic infections. Basophils release histamine, heparin and some pharmacological mediators of immunological reactions (21). Due to these diverse immunological functions, the normal counts of leucocytes (total and absolute) can be deviated by infections (1). In agreement with such theme, both counts of leukocytes were deviated in the present study, but the deviation was subjected to the group investigated and whether pre- or post-challenged. In general, mice vaccinated with HKB or FKB showed the highest count of leukocytes in pre-challenged animals, and such

increase also contributed to increased counts of lymphocytes, neutrophils and monocytes. These counts were almost approximated the counts of the other investigated post-challenged groups. However, it is worth to mention that TLC in all groups (pre- and post-challenged) approximated the normal leukocyte count range in mice, which was depicted by (15) as 2000-10000 cells/cu.mm.blood, and in a more recent estimation, the range was 5000-12000 cells/cu.mm.blood (26). Therefore, it is not possible to draw a clear effect of the present vaccination protocols on total and absolute counts of leukocytes. An exception was observed in mice vaccinated with LPS, in which the ALC was significantly increased in post-challenged animals as compared with pre-challenged mice; an observation that may suggest that LPS may able to enhance the adaptive immunity against *S.* Typhimurium. Such suggestion has some support from a study carried out by (23), in which they demonstrated that the wild-derived inbred mouse strain SPRET/Ei is resistant to LPS, although an enhanced immunity was observed. In addition, it has been demonstrated that such resistance in SPRET/Ei mice to *S.* Typhimurium infection is associated with increased leukocyte counts reaching the upper limit of the range in the circulation and enhanced neutrophil influx into the peritoneum during the course of infection. However, when these results were compared with results in a further inbred mouse (C3H/HeN), some variations were observed, and they suggested that genetic differences account for the variation in leukocytes of mice (12).

Phagocytosis:

The phagocytic index (PI) was significantly ($P \leq 0.001$) increased in post-challenged NC (64.83 *vs*. 39.00%) and CFA (62.33 *vs*. 55.16%) mice as compared with the corresponding mice in pre-challenged groups, while an opposite observation was made in post-challenged HKB (61.16 *vs*. 66.50%) and FKB (62.13 *vs*. 66.66 %) groups. However, pre- and post-challenged LPS+LIP mice demonstrated the highest PI (73.66 and 72.50%, respectively), and without significant difference between their means (Table, 6).

Table (6): Phagocytic index in groups of mice pre- and post-challenged with *S*. Typhimurium after different vaccination protocols.

Groups	Mean ± S.E. (Phagocytic Index: %)		P ≤
	Pre-Challenge	Post-Challenge	
Negative Control	39.00 ± 1.31D	64.83 ± 1.30B	0.001
Positive Control	67.50 ± 1.40B	63.50 ± 1.72B	N.S.
Complete Freund's Adjuvant	55.16 ± 1.07C	62.33 ± 0.88B	0.001
Heat-Killed Bacteria	66.50 ± 1.94B	61.16 ± 1.57B	0.01
Formalin-Killed Bacteria	66.66 ± 2.15B	62.13 ± 1.04B	0.01
Lipopolysaccharide	69.50 ± 1.17AB	68.50 ± 0.76A	N.S.
Liposome	63.50 ± 0.76B	60.83 ± 1.35B	N.S.
Lipopolysaccharide+Liposome	73.66 ± 1.14A	72.50 ± 1.40A	N.S.

Different letters: Significant difference ($P \leq 0.05$) between means of columns.

As presented, the highest percentage of phagocytosis was observed in mice vaccinated with LPS+LIP in pre- and post-challenged groups. Such finding highlights the importance of conjugation between LPS and LIP in enhancing the function of phagocytic cells. The adjuvant action of LIP in the stimulation of phagocytosis may be reasoned by the fact that LIP possesses many of the characteristics associated with inducers of cell-mediated responses. It is well established that the majority of LIP are taken up by phagocytic cells of the lymphoreticular system. Thus, the association of antigens with LIP provides a means of targeted delivery of the associated antigens directly to antigen-processing cells of the reticuloendothelial system (10). In addition, the incorporation of LPS into LIP renders the soluble LPS molecule particulate and much more hydrophobic. Furthermore, it has been shown that incorporation of LPS into phospholipids bilayers markedly reduced the adverse biological effects of lipid A. Additionally, since LIP are rapidly removed from the circulation by the reticuloendothelial system, the half-life in the blood of liposome-associated LPS is very short. Therefore, it is likely that the LPS-LIP complex is considerably less toxic than the equivalent amount of free LPS (8). After endocytosis of the LPS-LIP complex, it is assumed that disruption of the LIP bilayer must occur within the phagolysosomes to expose free LPS molecules for processing. This, along with the fact that nearly the entire antigenic mass is concentrated in the macrophage population of the

reticuloendothelial system, may result in a longer retention time of the LPS and therefore prolonged antigenic stimulation (19).

Arthus and Delayed-Type Hypersensitivity Reactions:

The most significant increase in AR index was observed in post-challenged mice of LPS (1.350 *vs*. 0.466 mm; $P \leq 0.001$) groups, followed by LIP (1.150 *vs*. 0.566 mm; $P \leq 0.01$) and NC (1.050 *vs*. 0.450; $P \leq 0.01$) group, as compared with the corresponding pre-challenged groups, while an opposite observation was made in FKB group (1.019 *vs*. 2.216 mm; $P \leq 0.001$). However, pre- and post-challenged LPS+LIP mice were among the highest AR index values (1.683 and 1.533 mm, respectively), and without significant difference between their means (Table, 7).

Table (7): Arthus reaction index in groups of mice pre- and post-challenged with *S*. Typhimurium after different vaccination protocols.

Groups	Mean ± S.E. (Arthus Reaction: mm)		P ≤
	Pre-Challenge	Post-Challenge	
Negative Control	0.450 ± 0.092C	1.050 ± 0.180B	0.01
Positive Control	1.680 ± 0.337B	1.300 ± 0.096B	N.S.
Complete Freund's Adjuvant	0.433 ± 0.098C	1.000 ± 0.112B	0.01
Heat-Killed Bacteria	1.516 ± 0.231B	1.116 ± 0.142B	N.S.
Formalin-Killed Bacteria	2.216 ± 0.157A	1.019 ± 0.134B	0.001
Lipopolysaccharide	0.466 ± 0.055C	1.350 ± 0.095B	0.001
Liposome	0.566 ± 0.084C	1.150 ± 0.085B	0.01
Lipopolysaccharide+Liposome	1.683 ± 0.166B	1.533 ± 0.098A	N.S.

Different letters: Significant difference ($P \leq 0.05$) between means of columns.

The DTHR index was significantly ($P \leq 0.001$) increased in post-challenged NC (1.933 *vs*. 0.400 mm) and CFA (1.450 *vs*. 0.500 mm) groups as compared with the corresponding pre-challenged groups, while an opposite observation ($P \leq 0.05$) was made in FKB (1.883 *vs*. 2.350 mm) and LPS+LIP (1.932 *vs*. 2.466 mm) mice. However, the post-challenged groups showed no significant variation between their means, while in pre-challenged groups, PC, HKB, FKB, LPS and LPS+LIP mice (2.166, 1.700, 2.350, 2.050 and 2.466 mm, respectively) demonstrated the highest significant means (Table, 8).

Table (8): Delayed type hypersensitivity reaction index in groups of mice pre- and post-challenged with *S*. Typhimurium after different vaccination protocols.

Groups	Mean ± S.E. (Delayed Type Hypersensitivity Reaction: mm)		P ≤
	Pre-Challenge	Post-Challenge	
Negative Control	0.400 ± 0.063C	1.933 ± 0.066A	0.001
Positive Control	2.166 ± 0.168A	1.966 ± 0.142A	N.S.
Complete Freund's Adjuvant	0.500 ± 0.036C	1.450 ± 0.156A	0.001
Heat-Killed Bacteria	1.700 ± 0.222A	1.783 ± 0.218A	N.S.
Formalin-Killed Bacteria	2.350 ± 0.330A	1.883 ± 0.288A	0.05
Lipopolysaccharide	2.050 ± 0.088A	1.666 ± 0.135A	N.S.
Liposome	1.100 ± 0.139B	1.500 ± 0.112A	N.S.
Lipopolysaccharide+Liposome	2.466 ± 0.142A	1.945 ± 0.241A	0.05

Different letters: Significant difference ($P \leq 0.05$) between means of columns.

In most types of post-challenged vaccinated groups, AR and DTHR responses were increased, while pre-challenged groups showed some variations, but mice vaccinated with LPS+LIP conjugate were almost recorded the highest results. Arthus reaction is a type III hypersensitivity reaction, which is mediated by immune complex formation in a second challenge with the same antigen. The immune complexes are formed between antibodies and the challenged antigen in the area of injection. Such formation leads to the activation of the classical pathway of complement system, which in turn leads to the generation of chemo-attractants factors (C3a and C5a) that enhance the migration of neutrophils to the area of injection. As a consequence, a local inflammatory response is generated with the manifestation of a local erythema and oedema after 3-4 hours of the injection (34).

Delayed type hypersensitivity reaction represents the fourth type of hypersensitivity reaction (Type IV), which differs from AR in the immunological constituents that participate in its generation. It is cell-mediated reaction, in which a specific T-helper lymphocyte, called T_{DTH}, plays a major role in its initiation together with macrophages. It occurs locally after 24-48 hours of a second challenge with the same antigen. Such time is required to activate the T_{DTH} by the antigen that is presented by macrophages; a process that requires production of cytokines (IL-2, IFN-γ and tumour necrosis factor-β). These cytokines stimulate the migration of more macrophages to the area of injection, which in turn produce extracellular lysozymes that are responsible for the inflammatory reaction in the area of injection (28).

Conclusion:

It is possible to suggest that LPS+LIP conjugate enhanced both immune responses; humoral and cellular. These findings can be justified by increased internalization of hydrophobic lipid antigens by macrophages that ultimately improved antigen presentation to cells. These macrophages function as antigen-presenting cells which take up antigen, catabolize them, and express the antigenic determinants in an energy requiring process before being presented to antigen specific T-cells (8). It is also possible that these LPS+LIP activated macrophages contribute towards positive regulating effects on the induction of specific immune response. Thus, the results of the present study elucidate that incorporation of *S.* Typhimurium LPS into LIP not only makes it possible to achieve a considerable immune response to lipid A-based immunogens by circumventing toxicity associated with lipid

A, but also to convert polysaccharide antigens into thymus-dependent antigens (19).

References

1. Ad'hiah, A. H.; Al-Kashaly, S. S. and Abbas, T. A. A. (2002). Group A streptococcus (*Streptococcus pygoenes*) and the mitotic activity of lymphoid organs in albino mice. The Eight Scientific Conferences of the Technical Education Committee. 302-208.
2. Al-azzawi, R. H. M. (1998). Extraction and partial purification of the local *Pseudomonas aeruginosa* lipopolysaccharide. M.Sc. Thesis. College of Science/University of Baghdad.
3. Atlas, R. M.; Paks, L. C. and Brown, A. E. (1995). Laboratory Manual of Experimental Microbiology. 1st ed., Mosby- Year Book, Inc., U.S.A.
4. Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Annu. Biochem. 72: 248-254.
5. Bruck, C.; Portetelle, D.; Glineur, C. and Bollen, A. (1982). One-step purification of mouse monoclonal antibodies from ascitic fluid by DEAE affinity gel blue chromatography. J. Immunol. Methods. 53: 313-319.
6. Chandan, V. and Fraser, A. D. (1994). Simple extraction of *Campylobacter* lipopolysaccharide and protein antigens and production of their antibodies in egg yolk. Inter. J. Food Micriobiol. 22: 189-200.
7. Chester, I. R. and Meadow, P. M. (1975). Heterogenicity of the lipopolysaccharide from *Pseudomonas aeruginosa*. Eur. J. Biochem. 58: 273-282.
8. Chhibber, S.; Wadhwa, S. and Yadav, V. (2004). Protective role of liposome incorporated lipopolysaccharide antigen of *Klebsiella pneumoniae* in a rat model of lobar pneumonia. Jpn. J. Infect. 57: 150-155.
9. Christensen, D.; Korsholm, K. S.; Andersen, P. and Agger, E. M. (2011). Cationic liposomes as vaccine adjuvants. Expert. Rev. Vaccines. 10: 513-521.
10. Cluff, C. W. and Zigler, K. K. (1987). An early response to lipopolysaccharide in the elicitation of macrophages specialized for antigen degradation with negative regulatory effects on the

induction of specific immune response. Infect. Immun. 55: 1346-1354.

11. Darveau, R. P. and Hancock, R. E. W. (1983). Procedure for isolation of bacterial lipopolysaccharides from smooth and rough *Pseudomonas aeruginosa* and *Salmonella typhimurium* strains. J. Bacteriol. 155: 831-838.

12. Dejager, L.; Pinheiro, I.; Bogaert, P.; Huys, L. and Libert, C. (2010). Role for neutrophils in host immune responses and genetic factors that modulate resistance to *Salmonella enterica* serovar Typhimurium in the inbred mouse strain SPRET/Ei. Infec. Immun. 78: 3884-3860.

13. Desiderio, J. V. and Campbell, S. G. (1983). Immunization against experimental murine salmonellosis with liposome – associated O-antigen. Infect. Immun. 48: 658-663.

14. Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A. and Smith, F. (1956). Determination of sugars and related substances. Anal. Chem. 28: 350-356.

15. Everds, N. E. (2007). Hematology of the Laboratory Mouse, 2nd ed., Elsevier Inc., U.S.A. Chapter five.

16. Girard, M. P.; Steele, D.; Chaignat, C. L. and Kieny, M. P. (2006). A review of vaccine research and development: human enteric infections. Vaccine. 24: 2732-2750.

17. Gray, J. T. and Fedorka-Cray, P. J. (2002). *Salmonella*. In: Cliver, D.O. and Riemann, H.P. (Editors). Foodborne Diseases. San Diego: Academic Press. 55-68.

18. Groisman, E. A. and Mouslim, C. (2000). Molecular mechanisms of *Salmonella* pathogenesis. Curr. Opin. Infect. Dis. 13: 519-522.

19. Hashioka, S., Han, Y.H., Fujii, S., Kato, T., Monji, A., Utsumi, H., Sawada, M., Nakanishi, H. and Kanba, S. (2007). Phosphatidyl serine and phosphatidyl choline-containing liposomes inhibit amyloid beta and interferon-gamma-induced microglial activation. Free Radic. Biol. Med. 42: 945-954.

20. Horton, D.; Rodemeyer, G. and Haskell, T. H. (1977). Analytical characterization of lipopolysaccharide antigens from seven strains of *Pseudomonas aeruginosa.* Carb. Res. 55: 35-47.

21. Kramer, R. J. (2003). Complete Blood Count. Internet: www.jci.org.

22. Lydyard, P. and Grossi, C. (1998). Cells involved in the immune response. In: Immunology, 5th ed., Edited by I. Roitt, J. Biostoff and D. Male. Mosby International Ltd.UK. 14-30.

23. Mahieu, T.; Park, J. M.; Reverts, H.; Pasche, B.; Lengerling, A.; Staelense, J.; Wullaert, A.; Vanlaere, I.; Hochepied, J.; van Roy, F.; Karin, M. and Libert, C. (2006). The wild-derived inbred mouse strain SPRET/Ei is resistant to LPS and defective in IFN-beta production. Proc. Natl. Acad. Sci. 103: 2292-2297.

24. Masoud, H. (2007). LPS-based conjugate vaccines composed of saccharide antigens of smooth-type *Salmonella enteritidis* and rough-type *S. gallinarum* 9R bound to bovine serum albumin. Scand. J. Infect. Dis. 39: 315-322.

25. McDonald, J. H. (2009). Handbook of Biological Statistics. 2nd ed., Sparky House Publishing, Baltimore, Maryland.

26. McGarry, M. P.; Protheroe, C. A. and Lee, J. (2010). Cell differential assessment to peripheral blood films. Mouse Hematology: a Laboratory Manual, Cold Spring Harbor Laboratory Press, U.S.A. 39-42.

27. 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.

28. Moore, A. R.; Gilroy, D. W.; Colville-Nash, P. R.; Greenslade, K.; Asculai, S. and Willoughby, D. A. (1999). Effects of hyluronan on models of immediate and delayed hypersensitivity in the rat. Int. J. Immunopharm. 21: 195-203.

29. Morrison, D. C. and Leive, L. (1975). Fractions of lipopolysaccaride from *Escherichia coli* O111:B4 prepared by two extraction procedures. J. Biol. Chem. 250: 2911-2919.

30. Pier, G. B.; Sidberry, H. F.; Zolyomi, S. and Sadoff, J. C. (1978). Isolation and characterization of a high molecular weight polysaccharide from the slime of *Pseudomonas aeruginosa.* Infect. Immun. 22: 908-918.

31. Sa´nchez-Vargas, F. M.; Abu-El-Haija, M. A. and Go´mez-Duarte, O. G. (2011). *Salmonella* infections: An update on epidemiology, management, and prevention. Travel Med. Infect. Dis. 9: 263-277.

32. Singh, B. R. (2009). *Salmonella* vaccines for animals and birds and their future perspective. The Open Vaccine Journal, 2: 100-112.

33. Sood, R. (1986). Hematology for Students and Practitioners. Jaypee Brothers, New Delhi, India.

34. Szalai, A. J.; Digerness, S. B.; Agrawal, A.; Kearney, J. F.; Bucy, R. P.; Niwas, S. and Kilpatrik, J. M. (2000). The Arthus reaction

in rodents species requirement of complement. J. Immu. 164: 463-468.

35. Townes, J. M.; Quick, R.; Gonzales, O. Y.; Linares, M.; Damiant, E.; Bopp, C. A.; Wahlquist, S. P.; Hutwagner, L. C.; Hanover, E.; Mintz, E. D. and Tauxe, R. V. (1997). Etiology of bloody diarrhea in Bolivian children: implication for empiric therapy. J. Inf. Dis. 175: 1527-1530.

36. Triolo, A. J.; Osteholm, L. and Kratky, M. T. (1989). Enhancement of the Arthus reaction and suppression of delayed type hypersensitivity (DTH) by pluronic F-68, a detergent frequently used to prepare perfluorocarbon emulsions. Int. J. Immunopharm. 11: 241-248.

37. Vinh, T.; Adler, B. and Faine, S. (1986). Ultrastructure and chemical composition of lipopolysaccharide extracted from *Leptospira interrogans* serovar Copenhageni. J. General Microbiol. 123: 103-109.

38. Vu Nguyen, T.; Le Van, P.; Le Huy, C.; Nguyen Gia, K. and Weintraub, A. (2006). Etiology and epidemiology of diarrhea in children in Hanio, Vietnam. Int. J. Inf. Dis. 10: 298-308.

39. Wilkinson, S. G. and Galbraith, L. (1975). Studies of lipopolysaccharides from *Pseudomonas aeruginosa*. Eur. J. Biochem. 52: 331-343.

40. Yousef, A. E. and Carlstrom, C. (2003). *Salmonella*. In: Yousef, A.E. and Carstrom, C. (Editors). Food Microbiology: A Laboratory Manual. New Jersey: John Wiley and Sons, Inc. 167-205.