The effect of *Brucella melitensis* (Rev-1)and sonicated antigen of *Salmonella typhimurium* on some interlukins (IL-4, IL-6) and IgM in rabbits

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Abstract

The research is designed to prepare antigens of *Brucella melitensis* and sonicated *Salmonella typhimurium* and their synergistic effect on some interlukines (IL-4,IL-6) and Immunoglobulin-M (IgM) which were evaluated by using enzyme linked immunosorbant assay (ELISA). For this purpose 25 Rabbits randomly divided into five groups each group contained five animals. The first group was immunized with *Brucella melitensis* (Rev-1) ($1\text{-}2^{x}10^{9}$) cfu/ml and sonicated *Salmonella typhimurium* 1000 µg/ml, Subcutaneously. The 2nd group was immunized with. *B. melitensis* (Rev-1) $1\text{-}2^{x}10^{9}$ cfu/ml and sonicated *S. typhimurium* 500 µg/ml Subcutaneously. The 3rd group was immunized with *B. melitensis* (Rev-1) ($1\text{-}2^{x}10^{9}$) cfu/ml. The 4th group was immunized with sonicated *S. typhimurium*1000 µg/ml only Subcutaneously as positive control. The 5th group was immunized by P.B.S.(pH7.2) as negative control group. The high concentration of IL-4,IL6 was recorded in the first group, It was (177.54±2.25 and 252.35±13.35), respectively and the lowest concentration was in the fourth group (144.32±4.85 and 119.78±3.34), respectively. Also the results of IgM showed that the highest concentration was 501.23±41.22 in the first group, while the lowest concentration was 63.99±8.76 in the fourth group. Apparent differences in the levels of IL-6, IL-4 and IgM-class antibody in the groups injected with Killed whole cell sonicated antigen of *Salmonella typhimurium* (KWCSA-ST1000 mg/ml) and *B. melitensis* (Rev-1) ($1\text{-}2^{x}10^{9}$) cfu/ml and the group injected with KWCSA-ST (500 mg/ml) and *B. melitensis* (Rev-1) ($1\text{-}2^{x}10^{9}$) cfu/ml as compared with the other groups which injected with KWCSA-ST(1000 mg/ml) only and *B. melitensis* (Rev-1) ($1\text{-}2^{x}10^{9}$) cfu/ml only.

تأثير لقاح البروسيلا Rev-1 ومستضدات السالمونيلا المكسرة على بعض الأنترلوكينات (انترلوكين-4 وانترلوكين-6) والكلوبيولين المناعي -M في الأرانب

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الخلاصة

صممت الدراسة لتحضير مستضدات البروسيلا المالطية (Rev-1) ومستضدات السالمونيلا الكاملة المقتولة المكسرة ومن ثم دراسة تأثيرها التآزري على بعض الأنترلوكينات (أنترلوكين-4 وأنترلوكين-6) والكلوبيولين المناعي M- والتي تم تقييمها بفحص المقايسة المناعية ELISA. واستعمل لهذا الغرض 25 أرنب قسمت عشوائيا إلى خمسة مجاميع كل مجموعة تضمنت خمسة أرانب، منعت المجموعة الأولى بمستضدي البروسيلا (Rev-1) (1- $2^{x}10^{9}$) cfu/ml والسالمونيلا الكاملة المقتولة المكسرة (1000 µg/ml) تحت الجلد، المجموعة الثانية منعت بمستضدي البروسيلا ($2^{x}10^{9}$ 2-1) cfu/ml والسالمونيلا الكاملة المقتولة المكسرة (500 µg/ml) تحت الجلد. المجموعة الثالثة منعت بمستضد البروسيلا (Rev-1) cfu/ml($2^{x}10^{9}$ 2-1) تحت الجلد. المجموعة الرابعة منعت بمستضد السالمونيلا الكاملة المقتولة المكسرة (1000 µg/ml) تحت الجلد. المجموعة الخامسة (مجموعة سيطرة) أعطيت المحلول الملحي الفسلجي (P.B.S.) امل تحت الجلد. سجلت اعلى تراكيز للانترلوكين الرابع في

المجموعة الأولى والتي كانت 177.54±2.25 و252.35±13.35 أما اقل تراكيز كانت في المجموعة الرابعة 144.32±4.85 و119.78±3.34 على التوالي، كما أظهرت نتائج الكلوبيولين المناعي –M اعلى مستوياتها في المجموعة الأولى والتي كانت 501.23±41.22 أما اقل تركيز فقد سجل في المجموعة الرابعة والذي كان 63.99±8.76.

Introduction

Brucellosis is a zoonotic disease in human and animals caused by *Brucella* species which are gram- negative, non motelic facultative intracellular pathogens (1). Brucellosis caused a significant economic loss to owners of domesticated animals due to decrease in milk yield, loss of progeny and infertility, but human rarely die from this infection (1, 2). Cell-mediated immune response play an essential role in the resistance of intracellular pathogens such as *Brucella*e species. This type of immunity involves activation of the bactericidal mechanisms of antigen presenting cells such as macrophages and dendritic cells and subsequent expansion of CD4+ and CD8+ T cells clones. *Brucella* antigen activate the production of T helper Type 1 (Th 1) cytokines, and an adequate Th 1 immune response is critical for the clearance of *Brucella* infection (4). Antibodies have been shown to have limited role in *Brucella* immune response (5). A panel of monoclonal antibodies (M- Abs) to *Brucella* outer membrane lipoproteins (OMPS) which share antigenic determinants of this family are used to determine the presence of common (OMPS) epitops in *Brucella* (6); To provide immune potentiation of live brucellosis vaccine we had to interact some biological adjuvants (7). The development of protective immunity against *Salmonella typhimurium* infection depend on cross-talk between humoral and cellular branches of immune system (8). *Salmonella typhimurium* serotypes contained antigenic determinants similar to but not identical with antigenic structures shared by smooth *Brucella*e (spp) (9); IL-4 was initially described as an immune response protein that is a growth factor for activated B-cells and resting T-cells and mast cells. Also called B-cell stimulating factor-1 (10); It is exhibit potent antitumor ability, tumors genetically modified to produce IL-4 were rejected, while parental tumors grew progressively (11). and its levels in serum were significantly increased during acute cases of brucellosis infection (12). The pro-inflammatory IL-6 and TNF-α and anti-inflammatory IL-10 cytokines are released during intracellular *Brucella* infection (13). The aim of this study was to provide the host immunity with effective *Brucella* antigens with intact some biological adjuvant such as *Salmonella typhimurium* sonicated antigen and also to study the synergestic effect of these antigens on some interleukins (IL-4, IL-6)and IgM.

Materials and Methods

- **Bacterial isolate:** *Salmonella typhimurium* which was obtained from pathology Unit/ College of Veterinary Medicine/ Baghdad University and the diagnosis were confirmed in central public health labrotory according to API-20E 3.2.2.1system.
- **Enzyme-linked immunosorbent assay (ELISA):** ELISA kit Rabbit IL-4, IL6, IgM (interleukin-4,6,and IgM), Mybiosourse. USA.
- **Antigen Preparation:** *Brucella melitensis* (Rev-1) ($1\text{-}2^{x}10^{9}$) cfu/ml. Killed whole cell sonicated antigen of *Salmonella typhimurium (KWCSA-S)*: Prepared according to Motive (1992).
- **Detection of protein concentration:** The protein concentration of *Salmonella typhimurium* was measured by using Biuret method according to Henry et al.,(1974).
- **Laboratory animal (rabbits) immunization:** Twenty five rabbits of both sexes were used which were randomly divided into five equal groups (5 animals for each group), as follows: The first group was immunized with 1 ml (1000µg/ml) of

KWCSA-ST and 1ml ($2x10^9$cfu/ml) of *B. melitensis* (Rev-1) antigen subcutaneously. The second group was immunized with 1ml (500µg/ml) of KWCSA-*ST and* 1ml ($2x10^9$cfu/ml) of *B. melitensis* (Rev-1) antigen subcutaneously. The third group was immunized with 1 ml ($2x10^9$cfu/ml) of *B. mellitensis* (Rev-1) antigen subcutaneously. The forth group (positive control group) was injected with 1 ml of (1000µg/ml) of KWCSA-ST subcutaneously. And the fifth group (negative control group) was immunized with 1ml PBS (PH 7.2) subcutaneously. At day 10 of immunization blood samples were collected from the direct puncture of the heart by sterile syringes for blood picture and sera were separated for estimate interleukins (IL-4, IL-6) and IgM concentration by ELISA kits. At day 14, the first, second, and third groups were given a booster dose of 1 ml ($2x10^9$cfu/ml) *B. melitensis* (Rev-1) antigen vaccine subcutaneously. At day 20, 40, 60, blood samples (3ml) were collected from all animal groups to estimate the interleukins (IL-4, IL-6) and IgG concentration by ELISA and Lymphocyte/ Neutrophil ratio.

- **Blood samples:** Blood samples (3 ml) were collected from the heart puncture of all animals at day 10, 20, 30, 40, 50, post immunization. and the serum stored in a deep freeze (-20 $^{\circ}$C) according to Weiss and Wardrop (2010).

Results

Immunological test:

Enzyme linked immunosorbant assay (ELISA)

- **IL-4 concentration:** The IL-4 concentration of in the first group, reached 165.02±1.56 at 10 day post immunization, then elevated to 177.54±2.25 at 20 day; subsequently decline to 166.58±3.71, 160.98±0.35 at 40, 60 day post immunization respectively, the results showed significant differences (P>0.05) in their means at 20 day as compared with 10, 40 and 60 day. In the second group the concentration of Rabbit IL-4 was 160.70±5.41, 168.93±2.06, 159.74±8.57 and 156.37±4.82 at 10, 20, 40 and 60 days respectively post immunization of rabbit these results showed significant differences (P< 0.05) in their means at (20, 40) day as compared with 10 and 60 day. There were significant differences (P<0.05) between the 1st group as compared with other groups.

Table (1) IL-4 concentration in the immunized rabbits

Time / Groups	Means+ S.E			
	10 day	**20 day**	**40 day**	**60day**
1st group WCSA-ST (1000µg/ml)	165.02±1.56 B a	177.54±2.25 A a	166.58±3.71 B a	160.98±0.35 B a
2nd group WCSA-ST (500µg/ml)	160.70±5.41 B a	168.93±2.06 B b	159.74±8.57 C b	156.37±4.82 C a
3rd group Rev-1 2x109cfu/ml	154.56±4.47 A b	156.86±5.02 A c	147.11±2.33 B c	145.89±4.60 B b
4th group KWCSA-ST 1000 µg/ml (positive control)	154.00±5.26 A b	157.79±6.04 A c	146.29±9.26 B c	144.32±4.85 B b
5th group PBS.(pH7.2) (negative control)	142.54±2.92 A c	142.15±5.83 A d	140.08±2.02 A d	141.25±1.88 A b

***p<0.05**

Means with different small letters in the same column differ significantly (P<0.05).

Means with different capital letters in the same row differ significantly (P<0.05).

- **IL-6 concentration:** The concentration of IL-6 in the first group, as shown in table (2), reached 128.83±3.89; 134.82±4.86 at day 10, 20, respectively after immunization, then elevated to reach 231.25±11.53 and 252.35±13.35 at, 40, 60 days, respectively after immunization. Results showed significant differences (P<0.05) at 10, 20 day and 40, 50 days. A significant differences (P<0.1) at 20 and 30 day post immunization. The second group showed that the concentration of rabbits IL-6 was 140.35±7.36; 179.83±8.16; 205.31±14.15 and 181.75±18.14 after 10, 20, 40, and 60 days respectively post immunization of rabbits, these results showed significant differences (P<0.05) at day 10 as compared with 20, 40 and 60 there were significant differences (P<0.05) between the 1st and 2nd groups as compared with other groups.

Table (2) IL-6 concentration in the immunized rabbits

Time / Groups	Means+ S.E			
	10 day	**20 day**	**40 day**	**60day**
1st group WCSA-ST (1000µg/ml)	128.83±3.89 C d	134.82±4.86 C d	231.25±11.53 B a	252.35±13.35 A a
2nd group WCSA-ST (500µg/ml)	140.35±7.36 C c	179.83±8.16 B b	205.31±14.15 A b	181.75±18.14 B b
3rd group Rev-1 2x109cfu/ml	179.43±12.12 B a	189.34±5.12 A a	179.18±11.08 B c	189.25±15.34 A b
4th group KWCSA-ST 1000 µg/ml (positive control)	144.00±8.47 A c	130.74±10.76 B d	119.78±3.34 C d	131.98±6.44 Ac
5th group PBS.(pH7.2) (negative control)	153.60±4.54 B b	158.71±8.48 B c	171.45±3.45 A c	137.56±7.93 C c

*** p<0.05**
Means with different small letters in the same column differ significantly (P<0.05)
Means with different capital letters in the same row differ significantly (P<0.05).

- **IgM concentration:** The concentration of IgM in the first group, was shown in table (7), reached 501.23±41.22; 266.54±12.55; 177.65±8.59 and 98.63±7.88 at 10, 20, 40, and 60 days post immunization, respectively. The concentration of rat IgM in the second group was 311.44±33.87; 144.76±14.12; 91.77±9.43 and 68.37±8.43 after 10, 20, 40, and 60 days, post immunization respectively.

Table (3) IgM concentration in the immunized rabbits

Time / Groups	**Means+ S.E - concentration**			
	10 day	**20 day**	**40 day**	**60 day**
1st group WCSA-ST (1000µg/ml)	501.23±41.22 A a	266.54±12.55 B a	177.65±8.59 C a	98.63±7.88 D a
2nd group WCSA-ST (500µg/ml)	311.44±33.87 A b	144.76±14.12 B b	91.77±9.43 C b	68.37±8.43 D b
3rd group Rev-1 2x109cfu/ml	254.75±21.54 A c	126.87±16.43 B c	81.87±11.22 C b	66.44±7.98 C b
4th group KWCSA-ST 1000 µg/ml (positive control)	140.35±14.29 A d	100.23±12.14 B d	77.67±12.12 C b	63.99±8.76 C b
5th group PBS.(pH7.2) (negative control)	24.56±13.12 A e	28.21±9.90 A e	21.76±7.88 A c	18.54±8.65 A c

Means with different small letters in the same column differ significantly (P<0.05)
Means with different capital letters in the same row differ significantly (P <0.05).

Discussion

Interleukin 4 (IL4) is a cytokine that induces differentiation of naive helper T cells (Th0 cells) to Th2 cells. Upon activation by IL-4, Th2 cells subsequently produce additional IL-4 in a positive feedback loop. The cells that initially produces IL-4, thus has not been identified, but studies suggest that basophils may be the effector cell (15), It has many biological roles, including the stimulation of activated B-cell and T-cell proliferation, and the differentiation of B cells into plasma cells. It is a key regulator in humoral and adaptive immunity. It is also induces B-cell class switching to IgE, and up-regulates MHC class II production and decreasing the production of Th1 cells, macrophages, IFN-gamma, and dendritic cell IL-12 (10). Naive CD4 T cells differentiate into at least two different sets of effector cells called Th1 and Th2 (16). Th1 cells produce IFN-γ and lymphotoxin α (TNFβ), confer cell-mediated immunity against intracellular pathogens, and cause autoimmunity. Th2 cells produce IL-4, IL-5, IL-10, and IL-13 and are involved in allergy, humoral immunity, and immunity to parasites. Of the many conditions that regulate T helper cell differentiation, the cytokine environment is the most critical determinant of differentiation into Th1 or Th2. In the presence of antigen, IL-12 drives differentiation of CD4 T cells to Th1 effector cells, while IL-4 drives naive T cells to become Th2 effectors. Regulation of IL-4 gene expression is critically important for the induction of Th2 cell differentiation and, therefore, Th2 immune responses (16, 17). So far, studies of tissue-specific IL-4 gene regulation have mainly focused on the proximal IL-4 promoter region, and detailed information has been accumulated on this region (17). The result of this study showed that excessive increases in IL-6, to levels that were observed in serum post bacterial infection, (20), this high levels due to enhancement in IL-6 gene expression in monocyte which stimulated by *S. typhimurium* proteins (21). IL-6 plays a crucial role in B-cell terminal differentiation and development of secretory IgA responses at mucosae (22). In addition to playing a role in acute phase reactions, mammalian IL-6 not only is involved in the proliferation and differentiation of T cells and mucosal B cells but also is an important component of the host's response to infection with different *Salmonella typhimurium* species (23). Interleukin-6 (IL-6) is a proinflammatory cytokine that is normally tightly regulated and expressed at low levels, except during infection, trauma, or other stress. Among several factors that down-regulate IL-6 gene expression are estrogen and testosterone. After menopause or and ropause, IL-6 levels are elevated, even in the absence of infection, trauma, or stress. IL-6 is a potent mediator of inflammatory processes, and it has been proposed that the age-associated increase in IL-6 accounts for certain of the phenotypic changes of advanced age, particularly those that resemble chronic inflammatory disease. Furthermore, the age-associated rise in IL-6 has been linked to lymphoproliferative disorders, multiple myeloma, osteoporosis, and Alzheimer's disease, and the relation ship of IL-6 level to age-associated diseases and to frailty. Like the syndrome of inappropriate antidiuretic hormone, it is possible that certain clinically important late-life changes are due to an inappropriate presence of IL-6 (24). IgM is the first immunoglobulin expressed by mature B cells. It is also the first immunoglobulin expressed in the fetus (around 20 weeks) and phylogenetically the earliest antibody to develop IgM antibodies appear early in the course of an infection and usually reappear, to a lesser extent, after further exposure; Those antibodies have been known for decades to enhance humoral immune responses in an antigen-specific fashion (25). During a bacterial infection IgM is the first line of defense The high IgM concentration which recorded in the first immunized group, but after a few days other antibodies and in particular IgG are produced. Though it takes the immune system longer to start producing IgG, once produced it is more effective at killing bacteria than

IgM. IgG also is important for the immune system's 'memory'. If on another date the same bacteria tries to cause a second infection, then this time IgG can be produced from the very beginning and prevent infection (26). bacterial infection elicited a relatively large population of IgM memory B cells, T cell-dependent IgM memory B cells can be elicited at high frequency and can play an important role in maintaining long-term immunity during bacterial infection (27). It was conduced from this study, that the high levels of many cytokines such as IL-6, IL-4 in the first group which was immunized with *Brucella melitensis* (Rev-1) ($1\text{-}2\times10^9$) cfu/ml and sonicated *Salmonella typhimurium* 1000 µg/ml, Subcutaneously. And the 2nd group which was immunized with. *B. melitensis* (Rev-1) $1\text{-}2\times10^9$ cfu/ml and sonicated *S. typhimurium* 500 µg/ml Subcutaneously. Have been an indication for the cellular resistance to facultative intracellular *B. melitensis* and *Salmonella typhimurium* infection.; Also rising levels of specific IgM-class antibody in serum specimens of those two groups can be regarded as serological evidence of recent infection, although IgM-class antibodies may persist for months following acute disease.

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