Evaluation of interleukins (2, 6 and 8) in immunized white rats by *Salmonella enterica* subspecies *typhimurium* and *Cryptococcus neoformans* antigens

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Summary

This study was designed to evaluate the levels of interleukins (2, 6 and 8) in immunized white rats by killed whole cell antigens of *Salmonella enterica subspecies typhimurium* and sonicated *Cryptococcus neoformans*, and using ELISA in day 10, 20, 30, 40 and 50 after immunization one hundred white rats of both sexes divided into five groups (20 rats for each). The first group was immunized by killed whole cell antigens of *Salmonella enterica* subspecies *typhimurium* (9×10^8 CFU /ml) and sonicated *Cryptococcus neoformans* (1000 µg/ml), The second was immunized by killed whole cell antigens of *Salmonella enterica* subspecies *typhimurium* (9×10^8 CFU /ml) and sonicated *Cryptococcus neoformans* (500 µg/ml). The third was immunized by killed whole cell antigens of *Salmonella enterica* subspecies *typhimurium* (9×10^8 CFU /ml) as positive control group, The fourth was injected 1 ml of phosphate buffer saline (pH 7.2) as control negative group and fifth was immunized by sonicated antigens of *Cryptococcus neoformans* (1000 µg/ml). The results of IL-2 showed significant differences ($P<0.05$) between the 1st, 2nd and 3th groups compared with 4th and 5th group, while there was no significant difference ($P\geq0.05$) between 4th and 5th groups. Also IL-6 showed that there were significant differences ($P<0.05$) between the 1st, 2nd and 3rd groups in comparison with 4th group, while there was no significant difference ($P\geq0.05$) between the 1st, 2nd, 3rd groups and 5th group. In the IL-8 showed that there was a significant difference ($P<0.01$) between the1st and 2nd groups and between 3rd group and 1st and 2nd groups without significant difference ($P\geq0.05$), also between the 1st, 2nd and 3rd groups and 4th group significant difference ($P<0.05$) and with a significant difference ($P<0.01$) between 5th group and all other groups (1st, 2nd, 3rd and 4th).

Keywords: *Salmonella typhimurium*, *Cryptococcus neoformans*, IL2, IL6, IL8.

Introduction

Cytokines are soluble proteins and glycoproteins, and function as key modulators of the immune system (1). They are produced by a wide variety of cell types (e.g. haemopoietic and glial cells, hepatocytes, adipocytes, myocytes and may be neurons) (2), in order to bring about a change in the functions of the target cell. They are considered as the "hormones" of the immune and inflammatory response products of most cells (3). The main host defense against *Salmonella* species occurs through the neutrophils; followed by mononuclear cells. These inflammatory cells produce cytokines as TNF-α, IFN-Y, IL-1, IL-2, IL-6 and IL-8 (4), and the predominant protective immune response to *Cryptococcus neoformans* was cell – mediated immunity (CMI) by T-helper – 1(TH1)-type CD4T cells which responses via production of IL-2, TNF-α and IFN-Y. These cytokines induce lymphocyte and phagocyte recruitment and activation of anti *Cryptococcal* delayed–type hypersensitivity response, resulting in increased *Cryptococcal* uptake and killing by effectors phagocytes (5). Due to little information about the role of interleukins such as IL-2, IL-6 and IL-8 in the immune response of killed whole cell *Salmonella typhimurium* and sonicated *Cryptococcus neoformans* antigens in rats this study was conducted.

Materials and Methods

The Microorganisms isolates**,** *Salmonella enterica* subspecies *typhimurium* and *Cryptococcus neoformans*, were obtained from the Zoonosis Unit/ College of Veterinary Medicine/ Baghdad University by personal communication.

Killed whole cell antigen of *Salmonella enterica* subspecies *typhimurium* (KWCA-ST): KWCA-ST antigen was prepared according to (6) and estimated the immunized

dose according to McFarland tube (No3) to 9×10^8 CFU/ml.

Killed whole cell antigen of *Cryptococcus neoformans* (KWCA-CN), and *Cryptococcus neoformans* whole cell sonicated antigen (KWCSA-CN) was prepared according to (7), and the protein concentration of *Cryptococcus neoformans* was measured by using Biuret method according to (8).

Laboratory animals (rats) Immunization: One hundred (100) white rats of both sexes, aged 3-4 months, obtained from the College of Medicine/ University of Baghdad, were randomly divided into five equal groups as followed: The first group was immunized with 9×10^8 CFU/ml of KWCA-ST and 1000 µg/ml of KWCSA-CN subcutaneously. The second group was immunized with 9×10^8 CFU/ml) of KWCA-ST and 500 µg/ml of KWCSA-CN subcutaneously. The third group (Positive control) was immunized with (9×10^8) KWCA-ST subcutaneously. The forth group (negative control) was injected with phosphate buffer saline (PBS) (pH7.2)/ 1ml subcutaneously. And the fifth group was immunized with 1000 µg/ml of KWCSA-CN subcutaneously. The 1st, 2nd and 3rd groups were given a booster dose of 9×10^8 CFU/ml KWCA-ST subcutaneously at day 14 after immunization.

Blood samples (2 ml) were collected from heart puncture of all animals at days 10, 20, 30, 40 and 50 post immunization; the sera were separated and stored in a deep freeze (-20˚C) till used (9).

ELISA was done by using ELISA kits for IL-2, IL-6 and IL-8 (Cusabio-China) according to company procedure.

Results and Discussion

IL-2: In the first group reached 75.5±6.19; 67.25±9.12; 65±8.51 and 79.5±4.44 pg at 20, 30, 40 and 50 days respectively post immunization, in the second group, the concentration of rats' IL-2 was 48.75±7.98; 59.4 ±5.54; 64.75±6.28; 59.25±5.89 and 61.50 ±9.29 pg after 10, 20, 30, 40 and 50 days respectively post immunization of rats. There was a significant difference (P≤0.05) between 1st, 2nd and 3rd groups compared with control group and 5th group while there was no significant difference (P≥0.05) between control and 5th group (Table, 1).

Table, 1: IL-2 concentration (pg) in white rats immunized by whole cell *Salmonella enterica* subspecies *typhimurium* and sonicated whole cell *Cryptococcus neoformans* antigens.

Time Groups	Mean ± SE(pg)				
	10 day	20 day	30 day	40 day	50 day
1st group KWCSA-*S*+ KWCA-*C* (1000 µg/ml)	35.40 ± 11.27 AB b	75.50 ± 6.19 A a	67.25 ± 9.12 A a	65.00 ± 8.51 A a	79.50 ± 4.44 AB a
2nd group KWCSA-*S*+ KWCA-*C* (500 µg/ml)	48.75 ± 7.98 A a	59.40 ± 5.54 A a	64.75 ± 6.28 A a	59.25 ± 5.89 A a	61.50 ± 9.29 BC a
3th group KWCSA-*S*	55.40 ± 10.01 A b	69.50 ± 8.38 A ab	69.75 ± 3.77 A ab	68.00 ± 16.41 A ab	93.32 ± 8.71 A a
4th group PBS	20.75 ± 1.80 B c	29.52 ± 5.09 B bc	40.82 ± 9.63 B ab	30.40 ± 2.68 B bc	46.80 ± 2.64 CD a
5th group KWCA-*C* (1000µg/ml)	36.25 ± 3.63 AB a	30.32 ± 4.39 B ab	32.47 ± 1.34 B ab	26.25 ± 2.78 B b	28.37 ± 2.60 D ab

***P<0.05 KWCA – ST, KWCSA – CN PBS.**
***Different small and capital letters show significant differences (P<0.05) within (Horizontally) and between (Vertically) groups respectively.**

IL-6: The Concentration of IL-6 showed a significant difference (P≤0.05) at 10, 20, 40 and 50 days and a significant difference (P≤0.1) at 20 and 30 days post immunization in the first group .There was significant difference (P≤0.05) between 1st, 2nd and 3rd groups in compared with control group (4th), while there was a significant difference between 1st, 2nd, 3rd and 5th groups (Table, 2).

IL–8: The concentration of IL-8 showed significant differences (P≤0.01) between 1st and 2nd group and between 1st, 2nd, 3rd groups, with significant differences (P≤0.05) between 1st, 2nd, 3rd and 4th group. A significant difference (P≤0.01) between all groups with the 5th group (Table, 3).

Table, 2: IL-6 concentration (pg) in white rats immunized by killed whole cell Salmonella enterica subspecies typhimurium and sonicated whole cell Cryptococcus neoformans antigens.

Time groups	Mean ± SE				
	10 day	20 day	30 day	40 day	50 day
1st group KWCA-*S* + KWCSA-*C* (1000 µg/ml)	124.87 ±4.75 C b	132.60 ±5.78 CD b	207.50 ±32.3 A a	225.42 ±21.43 A a	242.25 ±19.85 A a
2nd group KWCA-*S* + KWCSA-*C* (500 µg/ml)	137.25 ±9.69 BC b	176.15 ±9.87 AB ab	188.72 ±10.07 AB a	201.17 ±19.78 AB a	187.97 ±25.46 B a
3th group KWCA-*S*	175.42 ±11.05 A a	185.27 ±2.53 A a	207.00 ±25.93 A a	175.50 ±13.07 B a	188.50 ±7.67 B a
4th group PBS	135.87 ±9.38 BC a	128.50 ±12.37 D ab	112.75 ±4.42 C b	111.75 ±0.85 C b	127.50 ±5.56 C ab
5th group KWCSA-*C* (1000 µg/ml)	150.50 ±3.75 B abc	154.00 ±7.62 BC ab	143.25 ±8.29 BC bc	168.00 ±2.58 B a	132.50 ±7.93 C c

***P<0.05, Different small and capital letters showed significant differences (P<0.05) within (Horizontally) and between (Vertically) groups respectively.**

Table, 3: IL-8 concentration (Pg) in white rats immunized by killed whole cell *Salmonella enterica* subspecies *typhimurium* and sonicated whole cell *Cryptococcus neoformans* antigens.

Time groups	Mean ± SE				
	10 day	20 day	30 day	40 day	50 day
1st group KWCA-*S* + KWCSA-*C* (1000µg/ml)	143.32 ±30.53 B a	153.42 ±32.98 B a	148.00 ±21.9 B a	142.10 ±25.9 C a	154.92 ±24.46 C a
2nd group KWCA-*S* + KWCSA-*C* (500µg/ml)	226.00 ±2.04 A a	234.00 ±12.74 A a	228.25 ±5.28 A a	248.75 ±9.72 B a	233.50 ±12.38 B a
3th group KWCSA-*S*	109.75 ±2.95 BC d	283.00 ±15.07 A c	244.50 ±9.16 A c	571.00 ±16.34 A a	370.50 ±26.43 A b
4th group PBS	71.42 ±20.63 CD b	111.85 ±8.29 BC a	129.75 ±7.98 B a	114.20 ±9.18 CD a	131.00 ±5.80 CD a
5th group KWCSA-*C* (1000µg/ml)	58.75 ±2.83 D b	57.25 ±15.00 C b	77.00 ±3.87 C ab	71.75 ±1.54 D ab	85.00 ±1.47 D a

***P<0.05, Different small and capital letters showed significant differences (P<0.05) within (Horizontally) and between (Vertically) groups respectively.**

The results suggest that immunization with KWCA – *Salmonella enterica* subspecies *typhimurium* elicit Th type– 1T–cell response, characterized by predominance of IL-2 production in the groups immunized by KWCA-STT with KWCA-C 1000 and 500 µg/ ml and control group. These results agreed with (10) who observed that, LPS – activated macrophage to secrete inflammatory mediators like IL-2 and (11) who showed that *Salmonella enterica* subspecies *typhimurium* was capable of eliciting significant level s of IL-2 production in immunized mice, whereas no significant levels of IL-2 production were induced by porin of *Salmonella enteritidis* or *Escherichia coli*; *Cryptococcus neoformans* capsular polysaccharide is prominent virulence factor because it is antiphagocytic and interferes with antigen presentation by non professional antigen – presenting cell (APC) (12) leading to inhibit T-cell activation when monocytes exposed to *Cryptococcus neoformans*, act as APC (13). This inhibition was due to reduced capability of T-cell to produce interleukin-2 (IL-2), in contrast, the same T-cell population produced more interferon-Ɣ (11).

The level of IL-6 was increased in the KWCA–ST group than KWCA-CN. The level of 1st and 2nd group declined at day 10 and 20 then increased in day 30, 40 and 50 which may be due to act as anti- inflammatory mediators in these groups while in 3rd group the same level may be due to IL-6 as proinflammatory mediators according to (14). Also the roles of IL-1β and IL-6 in protecting against *Cryptococcus neoformans* have not been defined and probably the lack of these two cytokines could compromise the protective responses of the host (15). Also Interleukin 6 play a crucial role in B-cell terminal differentiation and development of secretary IgA responses at mucosa (16), 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 hosts response to infection by different Salmonella species (17).

The results showed that IL-8 levels in 3rd group increased significantly as compared with 1st, 2nd and control groups; this was in agreement with (18) who demonstrated that serotype *Salmonella enterica* subspecies *typhimurium* causes a neutrophils influx in the

intestinal mucosa because its PAMPs (flagella and LPS) activate TLR signaling pathways in host cells (epithelial cell and macrophages) which results in the release of neutrophils chemo attractants (IL-8). The polysaccharide capsule of *Cryptococcus neoformans* was believed to contribute to virulence by being antiphagocytic which has been associated with a variety of deleterious effects that can affect the host immune response (19).

The main host defense against *Salmonella* species occurs through the neutrophils, followed by mononuclear cells. These inflammatory cells produce cytokines such as TN F-α, IFN-Y, IL-1, IL-2, IL-6 and IL-8. The inflammatory micro movement is completed by chemokines that are capable of stimulatory leucocytes motility (Chemokines) and (Chemotaxis) of neutrophils and mononuclear cells. Chemokines bind to CC and CXC receptors in the surface of inflammatory cells. They help the blood leucocytes migration directly to host cells infected by bacteria (4). It concluded that the immunization by killed whole cell *Salmonella enterica* subspecies *typhimurium* with sonicated whole cell *Cryptococcus neoformans* lead to change in the levels of interleukins 2, 6 and 8 in white rats. Our conclusion that immunization by *Salmonella* and *Cryptococcus* antigens leading to change in the levels of interleukins 2, 6 and 8 in white rats and there was a marked significant decrease in the level of IL8 when immunized by *Cryptococcus* antigen while there was no change in the level of IL2 when immunized by *Cryptococcus* compared with *Salmonella* antigens.

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تقييم مستويات المدورات الخلوية (٢ و٦ و٨) في الجرذان البيضاء الممنعـــــة بمستضـدي *Cryptococcus neoformans* و*Salmonella enterica* subspecies *typhimurium*

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الخلاصة

صُمّمت هذه الدراسة لمعرفة مستويات المدورات الخلوية (٢ و٦ و٨) في الجرذان البيضاء الممنعة بمستضدي السالمونيلا الكامل المقتول وخميرة الخبيئة المكورة المكسرة، باستعمال فحص المقايسة المناعية الألايزا في الأيام ١٠ و٢٠ و٣٠ و٤٠ و٥٠ بعد التمنيع، باستعمال مئة جرذ ابيض، قسمت عشوائياً الى خمس مجاميع، منعت المجموعة الأولـى بمستضدي السالمونيلا الكامل المقتول $10^8\times$٩ وحدة تكوين مستعمرة/مل + خميرة الخبيئة المكورة المكسرة (1000 مايكروغم/مل)، المجموعة الثانية منعت بمستضدي السالمونيلا الكامل المقتول $10^8\times$٩ وحدة تكوين مستعمرة/مل + خميرة الخبيئة المكورة المكسرة (500 مايكروغم/مل)، المجموعة الثالثة منعت بمستضد السالمونيلا الكامل المقتول $10^8\times$٩ وحدة تكوين مستعمرة/مل المجموعة الرابعة (مجموعة سيطرة) أعطيت المحلول الفسلجي الملحي والمجموعة الخامسة منعت بمستضد خميرة الخبيئة المكورة المكسرة (1000 مايكروغم/مل). اظهرت النتائج وجود فرق معنوي ($P\leq0.05$) في مستوى المدور الخلوي 2 بين المجاميع 1 و2 و3 مقارنة بالمجموعتين 4 و 5، في حين لم يسجل فرق معنوي ($P\geq0.05$) بين المجموعة 4 (مجموعة السيطرة السالبة) والمجموعة 5 ، كذلك وجد فرق معنوي ($P\leq0.05$) في مستوى المدور الخلوي 6 بين المجاميع 1 و 2 و 3 ومجموعة السيطرة السالبة، في حين لم يسجل فرق معنوي ($P\geq0.05$) بين المجاميع 1 و 2 و 3 والمجموعة 5. وكان هنالك فرق معنوي ($P\leq0.01$) في مستوى المدور الخلوي 8 بين المجموعتين 1 و2 وبين المجاميع 1 و2 و3 بمستوى معنـــــوي ($P\leq0.05$)، وكذلك بين المجاميع 1و 2 و3 والمجموعة 4 وبمستوى معنوي ($P\leq0.01$) بين المجموعة 5 وبقية المجاميع (1 و2 و3 و4).

الكلمات المفتاحية: السالمونيلا تايفيميوريم، خميرة الخبيئة المكورة المكسرة، المدورات الخلوية ٢ و٦ و٨.