The effect of sonicated *Cryptococcus neoformans & Salmonella typhimurium* Killed whole cell antigens on cellular immune response in rats

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Abstract:

The research was included to prepare the Sonicated *Cryptococcus neoformans* and killed whole cell of *Salmonella typhimurium* antigens and evaluate their effects on the cellular immune response by using DTH-skin test and Lymphocyte /Neutrophil ratio in rats. The rats were divided randomly into four groups (1st group: contain 20 rats were immunized S/C with KWCA-S($9x10^8$ cfu/ml) + KWCSA-C (1000μg/ml), 2nd group contain 20 rats were immunized S/C with KWCA-S ($9x10^8$ cfu/ml)+ KWCSA-C (500μg/ml), 3rd group immunized S/C with KWCA-S ($9x10^8$ cfu/ml) only, 4th group was inoculated S/C with PBS (pH 7.2) and considered as control group. There was significant differences (P<0.05) between 1st; 2nd and 3rd groups compared with control group in rat's footpad thickness. Also there was a significant differences (P<0.05) at day 50 for immunized groups compared with day 10 in Lymphocyte/Neutrophil ratio.

تأثير مستضدي المكورات الخبيئة المكسرة و الخلية الكاملة المقتولة لجرثومة السالمونيلا تايفيميوريم على الاستجابة المناعية الخلوية في الجرذان

الخلاصة:

البحث تضمن تحضير مستضدي المكورات الخبيئة المكسرة و الخلية الكاملة المقتولة لجرثومة السالمونيلا تايفيميوريم و تقييم تاثيرهما على الاستجابة المناعية الخلوية باستخدام فحص الحساسية المتأخر (فحص الجلد) و نسبة خلايا الدم اللمفية/العدلات في الجرذان.

قسمت الجرذان عشوائيا الى 4 مجاميع (20 حيوان لكل مجموعة): المجموعة الاولى منعت بمستضدي المكورات الخبيئة المكسرة (1000 مايكروغرام / مل) و الخلية الكاملة المقتولة لجرثومة السالمونيلا تايفيميوريم ($9x10^8$ cfu/ml) تحت الجلد، المجموعة الثانية منعت بمستضدي المكورات الخبيئة المكسرة (500 مايكروغرام / مل) و الخلية الكاملة المقتولة لجرثومة السالمونيلا تايفيميوريم ($9x10^8$ cfu/ml) تحت الجلد، المجموعة الثالثة منعت بمستضد الخلية الكاملة المقتولة لجرثومة السالمونيلا تايفيميوريم فقط ($9x10^8$ cfu/ml) تحت الجلد، المجموعة الرابعة (مجموعة سيطرة)حقنت تحت الجلد (S/C) بالمحلول الملحي الفسيولوجيPBS. المجموعة الاولى و الثانية و الثالثة لوحظ هناك وجود اختلاف مهم احصائيا على المستوى (P<0.05) في معدلات تثخن القدم مقارنة بمجموعة السيطرة. ويوجد اختلاف مهم احصائيا في نسب الخلايا اللمفية و العدلات بعد 50 يوم من تمنيع جرذان التجربة مقارنة بنسبة الخلايا في اليوم 10 من التمنيع بالمستضدات المحقونة.

Introduction:

Salmonella enterica serovar Typhimurium has over the years been

the most common serovar causing salmonellosis among humans and animals (1); ranging from systemic infection to gastroenteritis, depending on the particular bacterial serovar and the host species infected (2).

Salmonella infection can trigger both the innate and adaptive host immune system (3); the host defense against intracellular pathogens such as Salmonella typhimurium, is mainly cell mediated and requires the cooperation of various effector cells, such as T lymphocytes, granulocytes, and monocytes / macrophages. The functional activities of these cells are regulated by a complex network of cytokines (4). Protective response against infection with such Salmonella serotypes as S.typhimurium and S.enteritidis is largely serogroup specific (5). Cryptococcus neoformans is consist of different antigens in their structure such as capsule which performed of mucopolysaccharide that consisted of Glucouronoxylomannas (GXM) and mannoprotein (MP), that diffused in host body fluid after infection which have the important role in stimulating the immune response (6); that released extracellularly; induces a number of deleterious effects to the host, such as phagocytosis, inhibition of leukocyte migration to infected tissues, and modulation of cytokine production (7); There are no literature before mentioned the synergistic correlation between these two antigens that has affected the immune response in rats so the aim of this research was to evaluate the cellular immune response of synergistic effect;

sonicated Cryptococcus neoformans and Salmonella typhimurium killed whole cell antigens in rats.

Materials and methods:

Salmonella typhimurium and Cryptococcus neoformans which were obtained from Zoonotic Diseases Unit / College of Veterinary Medicine / Baghdad University; were used to prepare:

- Killed whole cell antigen of Salmonella typhimurium (KWCA-S); was prepared according to (8); and the immunized dose was $9x10^8$ cfu/ml according to (9).

-Killed whole cell sonicated antigen of Salmonella typhimurium (KWCSA-S); Prepared according to (8) procedure, which used for delayed type hypersensitivity (skin test) in rats.

-Killed whole cell sonicated antigen of Cryptococcus neoformans (KWCSA-C); was prepared according (10) and used for rats immunization.

The protein concentration of Salmonella typhimurium and Cryptococcus neoformans were measured by using Biuret method according to (11).

Laboratory animals:

Eighty (80) rats of both genders with age range between 3-4 months which obtained from the College of Medicine / Baghdad University; divided into 4 groups as follows:

1. The first group was immunized with 1 ml (1000µg/ml) of KWCSA-Cryptococcus neoformans and 1ml

$(9x10^8$ cfu/ml) according to of KWCA-*Salmonella typhimurium* subcutaneously.

2. The second group was immunized with 1ml (500μg/ml) of KWCSA-*Cryptococcus neoformans* and 1 ml $(9x10^8$ cfu/ml) of KWCA-*Salmonella typhimurium* subcutaneously. 3. The third group was immunized with 1 ml $(9x10^8$ cfu/ml) of KWCA-*Salmonella typhimurium* subcutaneously.

4. The forth group (negative control group) was injected with 1 ml of PBS ($_p$H 7.2) subcutaneously.

- At day 14, the first, second, and third groups were given a booster dose of KWCA-*Salmonella typhimurium* 1 ml $(9x10^8$ cfu/ml) subcutaneously.

- At day10, 20, 30, 40, 50, blood samples (1ml) were collected from all animal groups for Lymphocyte/Neutrophil ratio.

- At day 18, skin test was done for all treated animals (Delayed type hypersensitivity test).

Differential leukocytes count;this test was done according to (12)

Delayed type hypersensitivity test (DTH) skin test;this test was done according to (13)

Results and Discussions:

Delayed type hypersensitivity (DTH) skin test:

The result of delayed type hypersensitivity have showed increases in the thickness of the right footpads of the rats of all groups and the highest mean of the thickness was after 72 hours post immunization then declined after passage of 48 hours and the normal thickness after 24 hours post injection of sonicated antigen of *S.typhimurium* as showed in the table (1)

In 1st group,the mean of footpad thickness (measured by mm unit) after 24 hours was 4.06±0.01 and after 48 hours was 4.16±0.05 then after 72 hours reach 4.46±0.01. In 2nd group, the mean of footpad thickness after 24 hours was 4.09±0.03 and after 48 hours was 4.3±0.03 then after 72 hours reach 4.42±0.13. In 3rd group, the mean of footpad thickness after 24 hours was 4.07±0.01 and after 48 hours was 4.26±0.03 then after 72 hours reach 4.5±0.06. In control group (PBS), the mean of footpad thickness after 24 hours was (3.72±0.06) and after 48 hours was 4.0±0.11 then after 72 hours reach 4.06±0.08. There is significant differences ($P<0.05$) between 1st; 2nd and 3rd groups compared with control group.

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Table 1: The thickness of the right footpads-skin test in rats immunized by _Salmonella typhimurium_ and _Cryptococcus neoformans_ antigen:

Time / groups	Mean ± SE			
	0	24 hr.	48 hr.	72 hr.
1st group KWCA-_S_+ KWCSA-_C_ (1000μg/ml)	3.68 ± 0.04 A c	4.06 ± 0.01 A b	4.16 ± 0.05 AB b	4.46 ± 0.01 A a
2nd group KWCA-_S_+ KWCSA-_C_ (500μg/ml)	3.71 ± 0.03 A c	4.09 ± 0.03 A b	4.30 ± 0.03 A ab	4.42 ± 0.13 A a
3th group KWCA-_S_	3.83 ± 0.07 A d	4.07 ± 0.01 A c	4.26 ± 0.03 A b	4.50 ± 0.06 A a
4th group PBS	3.67 ± 0.05 A b	3.72 ± 0.06 B b	4.00 ± 0.11 B a	4.06 ± 0.08 B a

*P<0.05

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

The DTH responses usually eliminate the intracellular pathogens, but in some individuals the pathogen is not eliminated in spite of DTH responses. Consequently more macrophages accumulate around the site of microbial presence. That adhere to each other, the activated macrophages in turn secrete a number of cytokines and biologically active substance that cause inflammation and destruction of microbe; the T helper cells secretes many cytokines such as (MIF, INF, GM-CsFs, TNF, LIF, IL-8, and IL-2) in turn activate the nearby lymphocytes and macrophages (14). The DTH response has been used as an indicator of cell-mediated immune status and is dependent upon both T helper 1(Th1)-driven responses as well as cell recruitment and chemotaxis to a local site. As a result, the DTH functional response may be influenced by disruption of Th1-driven, antigen-dependent T cell development or mobilization of sensitized T cells to a local site (15). Delayed type hypersensitivity reaction (DTH) consists of a sequential cascade of steps depending on different types of T cells, as well as mast cells, endothelial cells and macrophages (16).When small quentities of antigen are injected dermally, a hallmark response is elicited which includes induration, swelling and monocytic infiltration into the site of the lesion within 24 to 72 hours (17). It has been shown that CD4+ TH1 lymphocytes ("inflammatory type") play a central role in DTH reaction (14).This reaction has been shown to be absolutely dependent on the presence of memory T cells. Both the CD4+ and CD+ fractions of cells have been shown to modulate response. Contemporary debate regarding the reaction is focused on the role of the Th1 and Th2 cells originally discovered by Mosmann. It has been

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postulated that the Th1 cell is the "inducer" of a DTH response since it secretes interferon gamma (IFNγ), a potent stimulator of macrophages and induces a cell mediated immune response (18) while the Th2 cells secrete cytokines such as IL-4, IL-5, and IL-6, which activate B cells and induce humoral immunity. Induction of the Th1 or Th2 phenotype is due to the antigen presenting cells secreting IL-12 which induces Th1 cells or secreting IL-10 which induces Th2 cells. Mosmann and others originally proposed that the Th1 cell that secretes IFNγ is the only T cell capable of inducing a DTH reaction (19). The kinetics of the DTH responses are slightly different, with Th2 DTH lasting only 48 hours as opposed to 72 hours for Th1 DTH, but this is a relatively minor difference (20).

- **Lymphocyte/Neutrophil ratio:**

L/N ratio in the first group, as shown in table (2), it was reached 2.06 ± 0.07 at day 10 post immunization, then elevated to reach 2.71 ± 0.17, 2.25 ± 0.23, 1.9 ± 0.05, and 2.59 ± 0.26 at 20, 30, 40, 50 days respectively post immunization; with significant differences ($P<0.05$) at day 10 with day 50. In the second group the L/N ratio was 1.94 ± 0.06, 1.91 ± 0.12, 2.1 ± 0.06, 2.37 ± 0.14, and 3.17 ± 0.19 after 10,20,30,40,and 50 days respectively post immunization of rats, these results showed significant differences ($P<0.01$) at day 50. The third group showed that L/N ratio represent the highest value (2.43 ± 0.18) and was showed significant differences ($P<0.05$) at day 50. And the control group (group four), L/N ratio was 2.04 ± 0.11, 2.1 ± 0.29, 2.05 ± 0.16, 1.89 ± 0.13, and 2.55 ± 0.24 at 10,20,30,40, and 50 day post immunization and showed no significant differences ($P<0.05$).

Table 2: L/N ratio (Mean$\pm S_E$) of rats immunized by *Salmonella typhimurium* and *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)	2.06 ± 0.07 B bc	2.71 ± 0.17 A a	2.25 ± 0.23 B abc	1.90 ± 0.05 C c	2.59 ± 0.26 BC ab
2nd group KWCA-*S*+ KWCSA-*C* (500µg/ml)	1.94 ± 0.06 BC c	1.91 ± 0.12 B c	2.10 ± 0.06 B bc	2.37 ± 0.14 B b	3.17 ± 0.19 AB a
3th group KWCA-*S*	1.80 ± 0.03 C b	1.90 ± 0.08 B b	1.86 ± 0.20 B b	1.95 ± 0.06 C b	2.43 ± 0.18 C a
4th group PBS	2.04 ± 0.11 BC ab	2.10 ± 0.29 B ab	2.05 ± 0.16 B ab	1.89 ± 0.13 C b	2.55 ± 0.24 BC a

*P<0.05

- Different small and capital letters showed significant differences (P<0.05) within and   between   groups   respectively.

One of the first lines of the innate immune response is the recruitment of neutrophils. These are phagocytic cells whose main function is to eliminate pathogens (21). Neutrophils and macrophages are the main cell types that harbor *Salmonella* during infection in mouse models (22). After recruitment to infected organs these cells amplify the inflammatory response initiated by resident cells by producing inflammatory mediators such as cytokines and chemokines. They also exert the important function of phagocytosing and killing *Salmonella.* Even though *Salmonella* has evolved a number of mechanisms to evade killing, the ability of phagocytes to reduce the growth of bacteria through exhibit potent microbicidal activity mediated by antimicrobial peptides, lysosomal enzymes, and reactive oxygen and nitrogen species (23). Conlan, (24) found that neutropenic mice have increased susceptibility to several facultative intracellular pathogens including *Listeria, Francisella,* and *Salmonella.* Hassan & Curtiss, (25) found that Lymphocyte depletion and immunosuppression were associated with prolonged fecal shedding of *S. typhimurium* in chickens.

References:

Lan, R., Davison A. M., Reeves P. R., Ward, L. R. (2003). AFLP analysis of *Salmonella enterica* serovar Typhimurium isolates of phage types DT 9 and DT 135: diversity within phage types and its epidemiological significance. Microbes and Infection 5:841–850**.**

Majowicz SE, Musto J, Scallan E, Angulo FJ, Kirk M, O'Brien SJ, Jones TF, Fazil A, Hoekstra RM (2010) The global burden of nontyphoidal Salmonella gastroenteritis. Clin Infect Dis 50(6):882–889.

Dougan G., John V., Palmer S., Mastroeni P.(2011). Immunity to salmonellosis. Immunol. Rev.; 240:196–210.

Langermans, J.A.M. & Van Furth, R. (1994). Cytokines and the host defense against *Listeria monocytogenes* and *Salmonella typhimurium. Biotherapy, 7*, 169–178**.**

Barrow, P.A. (2007). Salmonella infections: Immune and non-immune protection with vaccines. Avian Pathology, 36(1):1-13.

Reiss, E.; Cherniak, R.; Eby, R.; and Kaufman, L. (1984). Enzyme immunoassay detection of IgM to galactoxylomannan of *Cryptococcus neoformans*. Diagn. Immunol., 2:109-115.

Monari, C., F. Bistoni, and A. Vecchiarelli. 2006. Glucuronoxylomannan exhibits potent immunosuppressive properties. FEMS Yeast Res. 6**:**537-542.

Motive, I.; Denchen, V. and Linde, K. (1992). Humoral and cell mediated immunity in mice after immunization with live oral vaccines of Salmonella typhimurium: auxotrophic mutants with two attenuating markers Vacc. 10: 61-66.

Baron, E.T.; Peterson, L.R. and Fingold, S.M. (1994). Baily and Scotts Diagnostic Microbiology, 9th ed., Mosby. St. Louis. Pp. 761-766.

Mark, A.D.; and Lorraine, F. (1978). Pathogenesis, Lethality, and immunizing effect of experimental cutaneous Cryptococcosis.

Henry, R.J.; Cannon, D.C. and Winkelman, J.W. (1974). Clinical chemistry, Principles and techniques. 2nd eds. Harber and Row Company. England.

Weiss,D.J. and Wardrop, K.J. (2010). Schalm's Veterinary Hematology. 6th ed. Wiley-Blackwell. USA.

Hudson, L. and Hay, F.C (1980). Practical immunology. 3rd ed. Blackwell Scientific Publications, Oxford. London.

Kogut, M.H.; Rothwell, L.and Kaiser, P. (2003) Differential regulation of cytokine gene expression by avian heterophils during receptor-mediated phagocytosis of opsonized and nonopsonized Salmonella enteritidis. J. Interferon Cytokine Res.;23:319-327.

Dietert RR, Bunn TL, Lee JE. (2010).The delayed type hypersensitivity assay using protein and xenogeneic cell antigens. Methods Mol Biol. 598:185-94.

Marcinkiewicz J.(1990). Cell-mediated immunity: role of IL-3 and IL-6 in the regulation of contact sensitivity reaction. Folia Histochem Cytobiol. 28(3):107-19.

Black, C.A.(1997). Delayed type hypersensitivity: current theories with an historic perspective. Department of Obstetrics, Gynecology and Reproductive Sciences Magee-Womens Research Institute Pittsburgh, USA. 5 (1):7.

Black, C. A., (1999) "Delayed Type Hypersensitivity: Current Theories with an Historic Perspective," Dermatology Online Journal, 5(1):7.

Mosmann, T.R., and K.W. Moore. (1991). The role of IL-10 in crossregulation of TH1 and TH2 responses. Immunol Today 12:A49-53.

Müller, K., F. Jaunin, I. Masouyé, J. Saurat, and C. Hauser.(1993). Th2 cells mediate IL-4 dependent local tissue inflammation. J Immunol. 150:5572-84.

Gill N, Ferreira RBR, Antunes LCM, Willing BP, Sekirov, Fatimah Al-Zahrani, Martin Hartmann, B. Brett Finlay.(2012) Neutrophil Elastase Alters the Murine Gut Microbiota Resulting in Enhanced Salmonella Colonization. PLoS ONE 7(11): e49646. doi:10.1371/journal.pone.0049646.

Yrlid, U., Svensson, M., Hakansson, A., Chambers, B. J., Ljunggren, H. G., and Wick. M. J., (2001). In vivo activation of dendritic cells and T cells during *Salmonella enterica serovar Typhimurium* infection. *Infect. Immun.* 69:5726-5735.

Milling, S. W., Cousins, L., and MacPherson, G. G. (2005). How do DCs interact with intestinal antigens? *Trends Immunol* 26:349-352.

Conlan JW.(1997). Critical roles of neutrophils in host defense against experimental systemic infections of mice by *Listeria monocytogenes, Salmonella typhimurium,* and *Yersinia enterocolitica*. Infect Immun. 65(2):630-5.

Hassan, J.O. and Curtiss R., (1994). Virulent *Salmonella typhimurium*-Induced Lymphocyte Depletion and Immunosuppression in Chickens. Infection and Immunity. 62(5): 2027-2036.