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.

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
(9x10⁸ 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 (9x10⁸ cfu/ml) of KWCA-Salmonella typhimurium subcutaneously. 3. The third group was immunized with 1 ml (9x10⁸ cfu/ml) of KWCA-Salmonella typhimurium subcutaneously. 4. The forth group (negative control group) was injected with 1 ml of PBS (pH 7.2) subcutaneously.  

- At day 14, the first, second, and third groups were given a booster dose of KWCA-Salmonella typhimurium 1 ml (9x10⁸ 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.
Table 1: The thickness of the right footpads-skin test in rats immunized by Salmonella typhimurium and Cryptococcus neoformans antigen:

<table>
<thead>
<tr>
<th>groups</th>
<th>Time</th>
<th>Mean ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>24 hr.</td>
</tr>
<tr>
<td>1\textsuperscript{st} group</td>
<td>KWCA-S+KWCSA-C (1000µg/ml)</td>
<td>3.68 ± 0.04 A c</td>
</tr>
<tr>
<td>2\textsuperscript{nd} group</td>
<td>KWCA-S+KWCSA-C (500µg/ml)</td>
<td>3.71 ± 0.03 A c</td>
</tr>
<tr>
<td>3\textsuperscript{rd} group</td>
<td>KWCA-S</td>
<td>3.83 ± 0.07 A d</td>
</tr>
<tr>
<td>4\textsuperscript{th} group</td>
<td>PBS</td>
<td>3.67 ± 0.05 A b</td>
</tr>
</tbody>
</table>

*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 secrete 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 quantities 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
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>
<thead>
<tr>
<th>Time</th>
<th>Groups</th>
<th>Mean ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 day</td>
<td>20 day</td>
</tr>
<tr>
<td>1st group: KWCA-S+KWCSA-C (1000μg/ml)</td>
<td>2.06 ± 0.07</td>
<td>2.71 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>B bc</td>
<td>A a</td>
</tr>
<tr>
<td>2nd group KWCA-S+KWCSA-C (500μg/ml)</td>
<td>1.94 ± 0.06</td>
<td>1.91 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>BC c</td>
<td>B c</td>
</tr>
<tr>
<td>3rd group KWCA-S</td>
<td>1.80 ± 0.03</td>
<td>1.90 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>C b</td>
<td>B b</td>
</tr>
<tr>
<td>4th group PBS</td>
<td>2.04 ± 0.11</td>
<td>2.10 ± 0.29</td>
</tr>
<tr>
<td></td>
<td>BC ab</td>
<td>B ab</td>
</tr>
</tbody>
</table>

*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:


