INNATE AND CELLULAR IMMUNITY STUDY ON SOME IMMUNOLOGIC PARAMETERS IN MICE VACCINATED WITH COMBINATION OF CANDIDA ALBICANS CELL WALL MANNOPROTEIN AND BRUCELLA REV.-1 VACCINE

Hanan Hassan Ali1 & Tareq Jaafar Al-Jindeel2
1Department of Microbiology, Veterinary Medicine College, Sulaimania University, Iraq; 2Dean of the Veterinary Medicine College, Al-Muthana University/Iraq

ABSTRACT
A study was carried out to investigate the immunomodulatory effect of the Mannoproteins of Candida albicans cell wall on the immune response of mice vaccinated with Brucella Rev-1 vaccine and considered as biological immunomodulators. The study included eight groups; the first four subgroups were I: treated with distilled water. II: injected subcutaneously with a dose of (300 µg /ml) cell wall mannoprotein only, III: injected subcutaneously with a dose of (200 µg /ml) cell wall mannoprotein only, IV: mice treated with brucella-Rev-1 vaccine only. The V, VI were treated with combination of Brucella Rev-1 vaccine and cell wall mannoprotein while VII and VIII groups were injected with the immunosuppressive drug prednisolone prior to the forthcoming treatment 5 days. All these treatments were carried out on day 1, and then the mice were sacrificed on day 8 to estimate serum phagocytic activity by Eliza assay and on day 14 for estimation of lymphocyte transformation by MTT assay. The results demonstrated a clear immunomodulatory effect of the mannoproteins of Candida albicans cell wall (improvement of non-specific, and cellular immune response) of the treated mice vaccinated with Brucella-Rev-1. The phagocytic activity (showed a significant increased (P≤0.05 and P≤0.01) in serum level in Mannoproteins-treated vaccinated mice in comparison with negative and positive controls groups, and group VI showed a highest increased. In lymphocyte, transformation by MTT assay, an increased index was significantly increased (P≤0.05 and P≤0.01) in Mannoproteins-treated vaccinated mice in comparison with negative and positive groups.

KEYWORDS: Mannoproteins, Brucella Rev -1, NTB, MTT assay, Mice.

INTRODUCTION
Brucellosis is one of the most important zoonetic diseases worldwide, resulting in serious economic losses and public health issues. It is caused by intracellular Gram-negative bacteria of the genus Brucella, which are responsible for a debilitating disease in humans and a chronic infection in domestic animals (1). In this regard, biological immunomodulator are materials that mediate the effectors mechanisms of the immune system through immune stimulation to a given antigen or potentiate the effectiveness of a vaccine (2). Recent advances in immunology have led to design vaccines to maximize activation of the humoral or cell-mediated branches of the immune system (3). Candida albicans has been one of the fungal species that share the interest of investigators in the field of immune modulation. Mannoproteins purified from C. albicans and administered to mice before or during immunization with viable C. albicans developed a significant increased in both humoral and cellular immune response (4). Furthermore, (5) have demonstrated that immunization with mannan (a mannoproteins fraction) and mannoproteins derived from digested cell walls of C. albicans induced resistance to a systemic candidiasis. (6)

Were purified cell wall mannoproteins of intact yeast using a simple treatment of yeast with mercaptoethanol and sodium dodecyl sulfate followed by Concanavalin A chromatography. They are found that Cell wall mannoproteins to be the main cause of adherence of C. albicans to epithelial cells in the first step of an infection process.(7) was study the immunomodulatory effect of the Mannoproteins of Candida albicans cell wall on the immune response of mice vaccinated with BrucellaRB51 vaccine and considered as biological immunomodulators. The results demonstrated a clear immunomodulatory effect of the mannoproteins of Candida albicans cell wall (improvement of non-specific, and cellular immune response) of the treated mice vaccinated with Brucella-RB51.

MATERIAL AND METHODS
There were eight groups in this experiment, which was designed to evaluate the immunomodulator potential of C. albicans cell wall mannoproteins in mice vaccinated with Brucella Rev-1 vaccine. The total number of animals in these groups was 200 mice (25 mice in each group).

- Group I: mice were injected subcutaneously with a single dose (0.2 ml) of deionized distilled water in day 1.
- Group II: mice were injected subcutaneously with a high dose (300 µg /ml) of mannoproteins in a total volume (0.2 ml) day 1.
- Group III: mice were injected subcutaneously with a moderate dose (200 µg/ml) of mannoproteins in a total volume (0.2 ml) day 1.
Immunologic parameters in mice vaccinated with combination of candida albicans and brucella rev.-1 vaccine

- Group IV: mice were injected subcutaneously with a single dose Brucella Rev-1 vaccine in day 1.
- Group V and VI: mice were injected subcutaneously with a single dose of combination of Brucella Rev-1 vaccinated moderate and high dose respectively in day 1.
- Group VII and VIII: mice were injected subcutaneously with a single dose of prednisone 5 days prior to the combination of the Brucella Rev-1 vaccine and moderate and high dose of mannoprotein in day 1.

Nitro blue tetrazolium test was carried out for evaluation of phagocytic activity percentage according to a method presented by (8) for all the mice. The method is outlined in a microtiter plate of 96 wells, a peripheral Blood Leucocytes 1×10⁶ cell/ well (100 ul Blood, with 175 ul MEM media) was isolated from peripheral blood and add to each well, NBT 1 g / liter was added as indicator for oxygen-dependant phagocytosis by peripheral leucocytes. ELIZA evaluated results at 650-wave length. The phagocytic activity percentage, which was calculated according to the following equation: (9).

\[
\text{Phagocytic activity percentage} \times 100 = \left( \frac{A - B}{B} \right) \times 100
\]

A = Treated groups; B = Negative control group.

The procedure of (8) was followed to assess the lymphocyte transformation activity percentage after an in vitro stimulation specific antigen (brucelline) by Lymphocyte Transformation test (MTT). The procedure of MTT assay (3-(4, 5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) measured by Elisa at 490-wave length.

1. The Lymphocyte transformation percentage which was calculated according to the following equation: (9).

\[
\text{Phagocytic activity percentage} \times 100 = \left( \frac{A - B}{B} \right) \times 100
\]

A = Treated groups; B = Negative control group.

RESULTS AND DISCUSSION
The results of NBT index were given in table 1, while the treatment efficiency for each group of treated mice was presented in figure 2. All groups of mice showed different significant increases in the NBT index which represent the phagocytic activity% (195.2%, 103%, 149%, 144%, 295%, 183% and 187%, respectively) as compared to group I (0%), which was injected with distilled water (control group). The best treatment efficiency was recorded in group VI (295%), which included mice that were treated with combination of 300 µg/ kg of Candida albicansmannoproteins and Brucella Rev-1 vaccine while lowest treatment efficiency was recorded in group III (103%) included mice that were treated with 200 µg/ kg of Candida albicansmannoproteins only.

TABLE 1: Nitro blue tetrazolium (NBT) index in mice vaccinated with Brucella Rev-1 vaccine and treated with C. albicans cell wall mannoproteins

<table>
<thead>
<tr>
<th>Groups</th>
<th>NBT OD (mean ± S.E. %)*</th>
<th>Phagocytic activity%</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1.01±0.15 c</td>
<td>0%</td>
</tr>
<tr>
<td>II</td>
<td>2.96±1.03 b</td>
<td>195.2%</td>
</tr>
<tr>
<td>III</td>
<td>2.05±0.98b</td>
<td>103%</td>
</tr>
<tr>
<td>IV</td>
<td>2.52±0.84 b</td>
<td>149%</td>
</tr>
<tr>
<td>V</td>
<td>2.47±0.81 b</td>
<td>144%</td>
</tr>
<tr>
<td>VI</td>
<td>4.00 a</td>
<td>295%</td>
</tr>
<tr>
<td>VII</td>
<td>2.88±1.11 b</td>
<td>183%</td>
</tr>
<tr>
<td>VIII</td>
<td>2.90±1.09 b</td>
<td>187%</td>
</tr>
</tbody>
</table>

*Different letters: Significant difference (P<0.05) between means of the same column.

**The comparison is between means of the two columns (horizontal comparison).
Results of NBT index, which showed a significantly increased percentage in treated mice, are also in favour of such agreement, and IFN-γ can activate murine macrophages to destroy intracellular bacteria. Nitro blue tetrazolium reduction by polymorphonuclear cells may require oxidative metabolism by the hexose monophosphate shunt, and is impermeable to cell membrane, but it enters the cell during the process of phagocytosis, and it is reduced by diaphorase activity within phagosome (10,11).

Phagocytic activity by reduction of nitro blue tetrazolium (NBT) to insoluble blue Formozan granules occurs during the stimulus-induced respiratory burst of mature granulocytes. Nitro blue tetrazolium (NBT) test addition of the yellow NBT dye to plasma results in the formation of a NBT–heparin or NBT–fibrinogen complex, which may be phagocytosed by neutrophils (12). Cell wall components from Candida albicans were compared to intact cells for their ability to induce natural cytotoxic immuneeffectors in the peritoneal cavity of mice, a soluble mannoprotein extract (MP) stimulated the generation of peritoneal; the NK and macrophage effectors generated by these materials had similar functional and phenotypic properties(13). Intracellular conversion of nitro blue tetrazolium (NBT) toformazan by immunomodulator was used to measure thegeneration of reactive oxygen species and the amount of formazanformed was measured (14).Normal neutrophils show little incorporation of the complex unless they are ‘stimulated’ to phagocytic activity, e.g. by the addition of endotoxin. This technique was used to measure the degree of ‘stimulation’ of untreated cells or their capacity for phagocytosis after stimulation. Stimulated neutrophils incorporate the dye complex into phagosome and, after lysosomal fusion, intracellular reduction results in the formation of blue insoluble crystals of formazan. The percentage of phagocytic cells may be determined using a light microscope or, as described below, the total dye reduction may be quantified spectrophotometrically after alkaline DMSO, which reacts with NBT to produce colored diformazan, formed dioxin extraction Super oxide free radical. The methanolic extract of Portulacaoleraceaescavenges superoxide radical and thus inhibits formazan formation (15). Attachment of the phagocyte to the organism is an important interaction, which may determine whether the uptake is subsequently occurred or not, and whether the killing mechanisms are triggered or not. The attachment can be mediated by two entities; lectins on the organism and lectins on the phagocyte. Of a particular interest in this respect are the complement receptors CR3, P150, and the related molecule LFA-1, which have multiple binding sites specific for different carbohydrate moieties (16). They can bind to B-glucans and to the lipopolysaccharide endotoxin of Gram-negative bacteria and this can lead to complement deposition via the alternative or classical pathways (17). Although macrophages and monocyte possess killing mechanisms in the resting state, these mechanisms can be enhanced, and new mechanisms can be expressed when they were activated. Activation can occur through exposure to microbial products (i.e. C. albicans cell wall mannoeproteins) and/or materials extracted from plants (i.e. ethanol and aqueous extracts of neem seeds). Such picture is enhanced by the findings of the present study and confirmed by other investigators (18,19). Such immunomodulators can cause a direct activation of phagocytes, or indirect activation through triggering cytokine release from them. Once the organism is internalized, it is exposed to an array of killing mechanisms; oxygen-dependent killing mechanisms (this pathway is also called reactive oxygen intermediates; ROIs) and reactive nitrogen intermediates (RNI) (16). The latter pathway has recently been discovered, and it may be particularly important, because it results in the formation of nitric oxide (NO), which is toxic for bacteria (19). For optimal expression of this mechanism, macrophages need both activation by IFN-γ and triggering by TNF-alpha, and this mechanism enables murine macrophages to kill mycobacterium and probably other intracellular bacteria like Brucella (17). Oxygen-independent killing mechanisms are also a further pathway, which may be
Immunologic parameters in mice vaccinated with combination of candida albicans and brucella rev.-1 vaccine

more important than was previously thought, because many organisms can be killed by cells from patients who cannot produce ROIs (20). It is expected that the isolated mannoproteins are effective immunomodulators. These are in agreement with this conclusion, several researchers suggested the potential use of C. albicans cell wall mannoproteins in this line of experimental immunology by using different laboratory approaches and animals (10, 21, 22, 24, 25, 27). The results of lymphocyte transformation index were given in table 2, while the treatment efficiency for each group of treated mice were presented in figure 2. mice showed different significant increases in the MTT index which represent the lymphocyte transformation index % (122%, 100%, 133%, 144%, 255%, 122% and 122%, respectively) as compared to group I (0%), which was injected with distilled water (control group). The best treatment efficiency was recorded in-group VI (255%), which included mice that were treated with 300 µg/kg of Candida albicansmannoproteins prior to the vaccination withBrucella Rev-1 vaccine while lowest treatment efficiency was recorded in-group III (100%) included mice that were treated with 200 µg/kg of Candida albicansmannoproteins only.

TABLE 2: Lymphocyte transformation index in mice vaccinated with Brucella Rev-1 vaccine and treated with C. albicans cell wall mannoprotein.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Lymphocyte Transformation Index (mean ± S.E.; %)*</th>
<th>Lymphocyte Transformation activity%</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0.09±0.02c</td>
<td>0%</td>
</tr>
<tr>
<td>II</td>
<td>0.20±0.01d</td>
<td>122%</td>
</tr>
<tr>
<td>III</td>
<td>0.18±0.02d</td>
<td>100%</td>
</tr>
<tr>
<td>IV</td>
<td>0.21±0.007c</td>
<td>133%</td>
</tr>
<tr>
<td>V</td>
<td>0.22±0.01b</td>
<td>144%</td>
</tr>
<tr>
<td>VI</td>
<td>0.32±0.01a</td>
<td>255%</td>
</tr>
<tr>
<td>VII</td>
<td>0.20±0.01d</td>
<td>122%</td>
</tr>
<tr>
<td>VIII</td>
<td>0.20±0.10d</td>
<td>122%</td>
</tr>
</tbody>
</table>

* Different letters: Significant difference (P≤0.05) between means of the same column.
** The comparison is between means of the two columns (horizontal comparison).

FIGURE 2: Lymphocyte transformation index in mice vaccinated with Brucella Rev-1 vaccine and treated with C. albicans cell wall mannoprotein.

The lymphocyte transformation test (LTT) has been proven useful especially in the diagnosis of drug-induced allergic disorders. An in vitro test is because lymphocytes, which have been sensitized by a certain antigen, transform into blasts and proliferate when they are again exposed to this antigen. This proliferation is determined by measurement of the incorporation of [3H]-thymidine or bromodeoxyuridine into replicating DNA. The test has the advantage over skin tests of avoiding re-exposure of individuals, and it was, therefore, hoped that it might also help to diagnose metal allergies and especially sensitization toward beryllium (26). However, the LTT measures only the sensitization of lymphocytes, but not the effector reaction, i.e., there may be positive results in exposed individuals even in the absence of clinical symptoms. Different research groups for the evaluation of various cell-mediated immune reactions have applied the test. The principle of the LTT is because lymphocytes,
which have been sensitized by a certain antigen “memory cells”, transform into blasts and proliferate when they are again exposed to this antigen. According to the different biological mechanisms occurring in a transforming cell, there are several chemical and physical methods to measure this transformation into blasts, as, for instance, determination of metabolic processes, biochemical alterations such as protein biosynthesis, or the synthesis of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA). The LTT, which measures the replication of DNA (27).

The MTT [3-(4, 5-dimethyl-2-thiazolyl) -2, 5-diphenyl -2H- tetrazolium bromide] test has been used earlier on Candida albicans after treatment with heat, formaldehyde, hypochlorous acid or amphoterin B (28) and to determine the sensitivity of Candida albicans and Aspergillus spp. to therapeutic anti-fungal agents. (29) MTT-reduction was demonstrated to correlate with the viability of the antifungal agent-treated Candida cells on culture. (29, 28) also used the MTT-reduction to investigate the effects of anti-Candida monoclonal antibodies in vitro; that appears to be the only report based on the use of MTT for this purpose. That specific antibody mediates anti-Candida immunity in vivo by inhibition of adherence to host cells or surfaces and by the promotion of phagocytosis and intra-phagocytic killing. The MTT (3-[4, 5-dimethyl-2-thiazolyl] -2, 5-diphenyl -2H- tetrazolium bromide)-reduction method as a test of the viability of fungi was used to investigate the effect of complement, normal serum and immune serum on these two species of Candida that are of increasing importance as opportunistic pathogens. We report that normal rabbit serum or strain-specific, polyclonal anti-Candida rabbit antibody, with or without guinea pig complement, did not cause the reduction of total cell mass or of the viability of either C. albicans or C. krusei, in vitro as determined by the MTT-reduction test. Complement alone without specific antibody, also, had no such effect on these two Candida species (The investigations described here with C. albicans and C. krusei and their strain-specific antibodies, were done to investigate the effect of sera containing strain-specific anti-Candida antibodies on the viability of C. albicans and C. krusei, in vitro). The tests were based on the capacity of viable cells to reduce MTT to formazan that was assayed by spectrophotometric quantization of optical density (OD) after its extraction with acid-propionate, with the OD taken as a measure of the metabolic status and the total, viable mass of the Candida cells. (30).

REFERENCES
Immunologic parameters in mice vaccinated with combination of candida albicans and brucella rev.-1 vaccine


