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STUDY OF *CANDIDA ALBICANS* MANNOPROTIEN IMMUNOMODULATORY EFFECT ON IMMUNE RESPONSE IN MICE VACCINATED WITH HBs VACCINE

Hanan H. A.
Vet. Med. College / Al-Slymania University/Iraq

ABSTRACT
A study was conducted to determine the effect of the *Candida albicans* mannoprotein on Phagocytic activity, antibody production, serum gammaglobuline percentage and lymphocyte transformation index after vaccination with hepatitis B surface (HBs) antigen. It can be concluded that Mannoprotein isolated from *Candida albicans* cell wall are an important immunomodulatorts in the development of immune response against *HBs* antigen vaccine. The results demonstrated a clear immunomodulatory effect of the mannoproteins of *Candida albicans* cell wall (improvement of non-specific, cellular and humoral immune response) of the treated mice which Compared to the first three groups of mice(negative and positive controls), others groups that were immunized with combination of vaccines and *candida albicans* mannoprotein revealed higher serum anti-HBs level and a significance increased in Phagocytic activity and lymphocyte proliferation percentage.

KEYWORDS: HBs antigens vaccine, Candida albicans cell wall mannoprotein, NBT, MTT and serum anti HBs antibodies level.

INTRODUCTION

Hepatitis B virus (HBV) is a serious public health problem and major cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma. It was estimated that approximately 2 billion people have serological evidence of past or present HBV infection and there are 350 million carriers of virus, worldwide (Wright, 2006). The World Health Organization (WHO) strategy for effective control of HBV infection and its sequel is mass vaccination of neonates and children within the framework of Expanded Program on Immunization (EPI) and recommended that hepatitis B vaccination should be included in national Immunization system in all countries by (Hou, 2005). In a series of studies it has been demonstrated that 90-99% of healthy neonates, children, adolescents and adults developed protective levels of anti-HBs antibody following a standard vaccination course with hepatitis B vaccine (Shokri and Jafarzadeh 2001; Jafarzadeh and Sajjadi, 2005). The effectiveness of routine infant hepatitis B immunization in significantly reducing the prevalence of chronic HBV infection has been demonstrated in a variety of countries (Wright, 2006). Accordingly, some investigators have suggested the need for a booster dose after 5-15 years (Fitzsimons *et al*., 2005, Williams *et al*., 2003). Hepatitis B virus (HBV) is an envelope, double-stranded DNA virus belonging to the Hepadnaviridae family and is recognized as the major cause of blood transmitted hepatitis together with hepatitis C virus HCV (Keating and Noble 2003). Hepatitis B surface antigen or HBsAg, previously described as Australia antigen, is the most important protein of the envelope of Hepatitis B Virus. The surface antigen contains the determinant “a”, common to all known viral subtypes and immunologically distinguished in two distinct subgroups (ay and ad). HBV has 10 major serotypes and four HBsAg subtypes have been recognized (adw, ady, ayw, and Ayr) (Hipgrave, 2006). The serological detection of HBsAg is a powerful method for the diagnosis and prevention of HBV infection and ELISA has become an extensively used analytical system for screening of blood donors and clinical diagnosis of HBV in infected individuals (Agladioglu *et al*., 2010). Polysaccharide immunomodulator were first discovered over 40 years ago. Mannan and mannoprotein fractions derived from digested surface cell walls of *C. albicans*, and their role in the immunization was determined (*Shigetoshi et al.*, 2000). These polymers can influence innate and cell-mediated immunity through interactions with T cells, monocytes, macrophages, and polymorphonuclear lymphocytes. The ability to modulate the immune response in an appropriate way can enhance the host's immune response to certain infections (Tzianabos, 2000). Immunization with *C. albicans* mannoproteins (MAN) in mice showed immunopotentiator effects on the three cell types (antigen presenting cells, T cells, and B *Cells*) that are involved in immune responses (Nossal, 2003; Al-jindeel, 2011) .

MATERIALS & METHODS

Eight groups of BALB/c mice, the first three groups were inoculated with PBS, moderate and high dose mannoprotein as negative and positive controls while group four immunized with a HBs antigen vaccine only, groups five and six immunized with combination of HBs vaccine and cell wall

mannoprotein the last two group inoculated with dose of prednisone prior to immunized with combination of HBs vaccine and cell wall mannoprotein. Blood samples were collected 10 days for phagocytic activity measured by NBT test reading by Eliza, 2 weeks for lymphocyte transformation measured by MTT test and 3, 4 weeks post-vaccination and anti-HBs antibodies in the serum were measured by indirect immunoflouresnt. *Candida albicans* was isolated, cultured, and maintained from women with vaginitis. The isolated strain was identified by using Candida check (Achkar and Fries, 2010).

Identification of *Candida albicans* was performed according to the method of (Ha *et al*., 2011) by conducting biochemical test (germ tube) which is considered as specific test for identification the *Candida albicans* microscopically and crossly. Microbiological observations of pseudohyphae, hyphae and chlamydospores were made on cornmeal tween 80 agar incubated at 35°C for 3 days. Culture medium GYEP containing 2% glucose, 0.3% yeast extract and 0.1% peptone (supplemented with penicillin 100 IU/mL and streptomycin 100 µg/mL) were used for *C. albicans* Identification (Ha *et al*., 2011). To Prepared Candida *albicans* Cell Wall Mannoproteins, Candida colonies were harvested in 2 liters of culture medium, and then they were subjected to a series of laboratory manipulations including ultra-centrifugation to prepare mannoproteins, which had a final weight of 2.8 grams. Estimation of total protein in the prepared solution of mannoproteins revealed that it was 82 mg/ml, while glucose content was 78 mg/m estimated by UV spectrophotometer. We have shown that the extraction of mannoprotein from the intact cell wall of yeast using chemical method. Using this procedure, it was expected that purified antigens should retain main epitope features and conformational characteristics such that they may be successfully used as immunogenic and antigen base for assay development (Farahnejad *et al*., 2005). The following products of Difco Company (U.S.A.) which prepared the culture media were used in the experiments of this study, and they were Agar agar, Sabouraud dextrose agar, Sabouraud dextrose broth, Tryptase soya agar, Tryptase soya broth. The following kits were used in the experiments of the study FITC-Rabbit Anti-Mouse IgG (H+L), Trypan blue stain (The Institute of Sera and Vaccines, Baghdad, Iraq), Hellabio agarose gels (Hellabio, Spain), Nitro blue tetrazolium (Sigma, USA), MTT (Sigma, USA).

There were eight sub-groups in this experiment, which was designed to evaluate the immunomodulator potential of *C. albicans* cell wall mannoproteins in mice vaccinated with *HBs* vaccine. The total number of mice in these groups was 200 mice (25 mice in each group).

Group I: mice were injected subcutaneously with a single dose (0. 2ml) of deionized distilled water at 1st day, Group II: mice were injected subcutaneously with a high dose (200 ug/ml) of mannoproteins in a total volume (0.2 ml) at 1st day, Group III: mice were injected subcutaneously with a moderate dose (300 ug /ml) of mannoproteins in a total volume (0.2 ml) at 1st day, Group IV: mice were injected subcutaneously with a single dose *HBs* vaccine at 1st day, Group V and VI: mice were injected subcutaneously with a single dose of *combination of HBs vaccine* vaccinated moderate and high dose respectively at 1st day, Group VII and VIII: mice were injected subcutaneously with a single dose of prednisone 5 days prior to the combination of the *HBs* vaccine and moderate and high dose of mannoprotein at 1st day. Laboratory Method used in the present study were Nitro blue Tetrazolium (NBT) index, the assay was carried out on peripheral blood of immunized mice according to a method presented by (Zakaria *et al.*, 2011). The procedure of (Farid *et al*., 2003) was followed to prepare lymphocytes from immunized mice blood. The procedure of MTT assay (3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) measured by Elisa to assess the lymphocytes transformation percentage after an *in vitro* stimulation with specific antigen (Zakaria *et al.*, 2011). The IFAT was used to assess anti-*HBs* antibody titer in the sera of mice that were immunized with *HBs* vaccine in different treatment regimens. The procedure of WHO (1997) was adopted to determine such titer, Serum electrophoresis was carried out using a commercially available kit (Hellabio, Spain). Statically analysis the values of the investigated parameters were given in terms of means ± standard errors (S.E.), and differences between means were assessed by conducting analysis of variance (ANOVA), least significant difference (LSD) and Duncan test, using the computer programmer SPSS (Statistical Package of Social Sciences) version 7.5. The difference was considered significant when the probability value was equal or less than 0.05.

RESULTS & DISCUSSION

After the tabulated procedures and calculations the LD_{50} of *C. albicans* mannoproteins range a widely between (from 100 - 600 ug /mouse) the result were given in (table 1). Based on these findings, dose of 200 ug /mouse was considered as the moderate dose and 300 ug / mouse as the high dose in the present study, which was subjected to investigations Dixon (1980). 36 mice were injected with Mannoprotein and no mortality rate.

TABLE 1: Doses of *C. albicans* cell wall mannoproteins that were used in the assessment of LD50.

C. albicans	Dose/mouse	Dose/Kg	Number of Animals	Mortality Rate (%)
Cell Wall Mannoproteins	100 µg	4 mg	6	0.0
	200 µg	8 mg	6	0.0
	300 µg	12 mg	6	0.0
	400 µg	16 mg	6	0.0
	500 µg	20 mg	6	0.0
	600 µg	24 mg	6	0.0

Safety assessments of mice treated with different materials (mannoproteins, prednisolone and HBs vaccine) in the present study revealed no major alternations in the general activities of the animals. Their weights showed no significant changes between pre- and post-treatments and their food consumption were normal. Furthermore, there were no clinical signs, which may reflect a deleterious effect of the treatment. The results of NBT index were given in table 2. All groups of mice showed different significant increases in the NBT index which represented the phagocytic activity% as compared to group I (0%), which was injected with deionized water (control negative group). The best NBT index was recorded in group VI (268%), which included mice that were treated with combination of 300 µg/ kg of *Candida albicans* mannoproteins and *HBs* vaccine while lowest NBT index was recorded in group II (105.8%) included mice that were treated with 200 µg/ kg of *Candida albicans* mannoproteins only.

TABLE 2: Nitro blue tetrazolium (NBT) index in treated mice

Groups	NBT OD (mean ± S.E.)*	phagocytic activity%
I	1.03±0.15 [c]	0%
II	2.12±0.84 [b]	105.8 %
III	2.36±1.03 [b]	129.13 %
IV	3.02±1.02 [b]	1.93.2 %
V	3 .35±1.02 [a]	225.25 %
VI	3 .80±1.04 [a]	268.93%
VII	2.88±1.11 [b]	276.69 %
VIII	2.90±1.09 [b]	181.55 %

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

Results of NBT index, which showed a significantly increased percentage in immunized mice, are also in favour of such agreement. Phagocytic activity by reduction of nitro blue tetrazolium (NBT) to insoluble blue Formozan granules occurred 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 phagocytosis by neutrophils (Kumar *et al*., 2003). Normal neutrophils showed 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 incorporated 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 coloured diformazan (Sanja *et al*., 2009). Although macrophages and monocyte possess killing mechanisms in the resting state, these mechanisms can be enhanced, and new mechanisms can be expressed when they are activated. Activation can occur through exposure to microbial products (*i.e. C. albicans* cell wall mannoproteins and HBs antigen).Such immunomodulators can cause a direct activation of phagocytes, or indirect activation through triggering cytokine release from them to induce macrophage for killing intracellular bacteria (Balwinder *et al*., 2005). 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) (Savolainen, and Johannes. 2006). Oxygen-independent killing mechanisms are also a further pathway, which may be more important than was previously thought, because many organisms can be killed by cells from patients who cannot produce ROIs (Shigetoshi *et al*., 2000). It is expected that the isolated mannoproteins are effective immunomodulators. These are in agreement with this conclusion; several researchers suggested the potentianal use of *C. albicans* cell wall mannoproteins in this line of experimental immunology by using different laboratory approaches and animals (23; 24; 25 and 13).

Lymphocyte transformation index by MTT assay

The results of lymphocyte transformation index can be found in table 3. Mice showed different significant increases in the MTT index which represent the lymphocyte transformation index % as compared to group I(control negative group) (0%), which was injected with deionized water (control group). The best MTT index was recorded in-group VI (271.79%), which included mice that were immunized with combination of 300 µg/ kg *Candida albicans* mannoproteins with *HBs* vaccine while the lowest index was recorded in-group II (117.9 %) included mice that were injected with 200 µg/ kg of *Candida albicans* mannoproteins only .

TABLE 3: Lymphocyte transformation index in treated

Groups	Lymphocyte Transformation OD (mean ± S.E.)*	Lymphocyte Transformation activity index %
I	0.078 ± 0.02 [f]	0 %
II	0.17 ± 0.02[e]	117.9 %
III	0.19 ± 0.01[d]	143.6 %
IV	0.28 ± 0.01 [a]	258.97 %
V	0.18 ± 0.01[d]	130.76 %
VI	0.29 ± 0.01 [a]	271.79 %
VII	0.19 ± 0.01[d]	143.59 %
VIII	0. 20 ±0.1[c]	156.41 %

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

TABLE 4: Anti-*HBs* antibody titer in sera of treated mice after 21 days.

Groups	AntiHBs antibodies Titer after 21 days								
	16	32	64	128	256	512	1024	2048	4096
I	0	0	0	0	0	0	0	0	0
II	16	32	0	0	0	0	0	0	0
III	0	0	0	0	0	0	0	0	0
IV	16	32	64	0	0	0	0	0	0
V	16	32	64	128	0	0	0	0	0
VI	16	32	64	128	256	512	0	0	0
VII	16	32	64	0	0	0	0	0	
VIII	16	32	64	0	0	0	0	0	

TABLE 5: Anti-*HBs* antibody titer in sera of treated mice after 28 days.

Groups	AntiHBs antibodies Titer after 28								
	16	32	64	128	256	512	1024	2048	4096
I	0	0	0	0	0	0	0	0	0
II	16	32	64	0	0	0	0	0	0
III	0	0	0	0	0	0	0	0	0
IV	16	32	64	128	0	0	0	0	0
V	16	32	64	128	256	0	0	0	0
VI	16	32	64	128	256	512	0	0	0
VII	16	32	64	128	0	0	0	0	0
VIII	16	32	64	128	0	0	0	0	0

The lymphocyte transformation test (LTT) has been an in vitro test the 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 MTT (3-[4, 5-dimethyl-2-thiazolyl] -2, 5-diphenyl -2H- tetrazolium bromide)-reduction method measured by ELIZA. The test has the advantage over skin tests of avoiding re-exposure of individuals (Khosravi *et al*., 2007). However, the LTT measures only the sensitization of lymphocytes, but not the effecter 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 based on the fact that lymphocytes, which have been sensitized by a certain antigen (memory cells), transform into blasts and proliferate when they are again exposed to this antigen, (Moragues *et al.,* 2003). The MTT [3-(4, 5-dimethyl-2-thiazolyl) -2, 5-diphenyl -2H- tetrazolium bromide] were based on the capacity of viable cells to reduce MTT to formazan that was assayed by spectrophotometric quantitation of optical density (OD) after its extraction with acid-propanol, with the OD taken as a measure of the metabolic status and the total, viable mass of the *Candida* cells. Development of the protective immune response to HBsAg is T-cell dependent and is associated with the production of specific neutralizing antibodies. The immunobiology mechanism may be due to increase of T-lymphocytes CD receptors; MHC 1 and enhance cytokines production result in stimulates TH1 cells and macrophages, and then causes an elevation of both immunoreactive and bioactive TNF-alpha and gamma interferon in serum and mesenteric lymph nodes (Arseculeratne *et al*., 2007).

Indirect Fluorescent Antibody Test (IFAT)

The sera of mice in groups II, III, and I showed no anti-*HBs* antibodies at the start titer 1:16 after 21 days off vaccination, while the other groups showed some variations. All mice of

group VI showed a higher positive immunoflourescent reaction at the titer 1:512, while the other groups IV and VIII showed a positive reaction which was observed at the titer 1:64. After 28 days the sera of mice in groups II, III, and I showed no anti-*HBs* antibodies at the start titer 1:16, while the highest anti HBs antibodies titer was recorded in mice of group VI at the titer I: 512. After that positive Immunoflourescent reaction at the titer 1:128 was observed in mice of groups V after 28 day of vaccination. These results were given in (table 4 and 5).

Immunofluorescence is the visualization of antigens within cells using antibodies as fluorescent probes. Anti-HBs antibodies showed an increased titer in all immunized groups treated with the immunomodulators used in the study, especially groups V1 and IV as compared to the control group that received vaccine only. Such observation suggests that the immunomodulation also involved the humoral immune response, although the pathway may be through the modulation of macrophages and T lymphocytes as both types of cells are required to enhance the B-lymphocytes to produce immunoglobulin (Shigetoshi *et al*., 2000). Development of the protective immune response to HBsAg is T-cell dependent and is associated with the production of specific neutralizing antibodies. Previous studies in nonresponsive but otherwise healthy people did not find defects in antigen uptake or processing by antigen-presenting cells. (McMahon *et al*., 2005). However, the different cell surface glycoproteins responsible for presenting protein antigens to $CD4^+$ T cells, largely contributes to the human antibody response to HBV vaccine (Agladioglu *et al*., 2010).These are in agreement with this conclusion; several researchers suggested the potentianal use of *C. albicans* cell wall mannoproteins in this line of experimental immunology by using different laboratory approaches and animals (Farahnejad *et al*., 2005 and Donatella *et al*., 2008).

Gamma Globulin Serum Fraction

The results of gamma globulin fraction are shown given in (table 6). The highest significant increase in the percentage of gamma globulin fraction was observed in groups VI and V (46.53%) and (34.54 %) as compared to group I (24.06%) at 21 days after vaccination, while the highest percentage of gamma globulin fraction was observed in VI and IV groups (35.50 % and 32.55%) after 28 days of vaccination of mice. The lowest percentage of gamma globulin fraction was showed in-group II (24.24 %) after 28 days of vaccination.

TABLE 6: Gamma globulin serum fraction in treated mice

Groups	Gamma Globulin Serum Fraction (mean ± S.E.) %)*		Probability**
	After 21 days	After 28 days	≤
I	24.06 ± 0.15 ^{d}	22.63 ± 0.12 ^{e}	0.05
II	25.23 ± 0.23 ^{c}	24.24 ± 0.12 ^{d}	0.05
III	27.23 ± 0.23 ^{c}	26.34 ± 0.12 ^{c}	0.05
IV	33.86 ± 0.25 ^{b}	32.50 ± 0.06 ^{b}	0.05
V	34.53 ± 0.12 ^{b}	29.55 ± 0.06 ^{c}	0.01
VI	46.84 ± 0.35 ^{a}	35.37 ± 0.09 ^{b}	0.01
VII	30.36 ± 0.25 ^{b}	29.16 ± 0.25 ^{c}	0.05
VIII	31.36 ± 0.25 ^{b}	30.26 ± 0.25 ^{b}	0.05

** The comparison is between means of the two columns (horizontal comparison).

Serum electrophoresis was carried out using a commercially available kit (Hellabio, Spain). The Hellabio Agarose Gels for protein electrophoresis are intended to be used for *in vitro* diagnosis, and they enable quantitative and qualitative estimation of proteins in serum and other biological materials. After serum gel electrophoresis, five fractions (albumin, α1, α2, β and γ globulin) were recognized, which were given as percentages of the total. These result was supported the effectiveness role of *Candida albicans* cell wall mannoprotein on humoral immune response in mice vaccinated with HBs vaccine. The evaluation of *C. albicans* cell wall mannoproteins LD_{50} demonstrated a dose of a wide range safety (100 to 600 µg/kg), also was effective in terms of toxicity and immunomodulatory backgrounds. The *C. albicans* cell wall is essential to nearly every aspect of the microorganism biology and pathogenicity, because it contains materials that are able to mediate interactions with the host immune response (11; 13). These contents are mainly polysaccharides in addition to proteins and minor amounts of lipids (24 and 25). Therefore, it was expected that the isolated mannoproteins are effective immunomodulators. In agreement with this conclusion, several researchers enhanced the potentional use of *C. albicans* cell wall mannoproteins in this line of experimental immunology using different laboratory approaches and animals (11; 12; 13; 22; 23; 24 and 25).

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