

# Efficiency evaluation of immunization protocol with *Echinococcus granulosus* antigen B (AgB)

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**Abstract** - Antigen B (AgB) , the major antigens of *Echinococcus granulosus*, is a polymeric thermostable lipoprotein that constitutes as much as 10% of hydatid fluid.

Antibody and cytokine response were both studied in rabbits immunized with *Echinococcus granulosus* antigen B (AgB) . This study aimed to assess the effect of antigen B (AgB) on immune response for this purpose hydatid cysts were collected from liver of infected sheep from slaughter house in Basra . AgB was purified from hydatid fluid by anion exchange chromatography.

For evaluation of AgB efficiency in immunization , 15 rabbits were enrolled in the study ,10 of them were immunized subcutaneously with AgB using Freund's complete adjuvant after two weeks three booster injections were repeated intramuscularly according to immunization schedule using Freund's incomplete adjuvant and 5 rabbits were treated as a control group. After six weeks , blood were collected from rabbits and the levels of IgG , IFN- $\gamma$  and IL-10 were measured by ELISA test.

Means of IgG of immunized rabbits were significantly ( $P < 0.05$ ) elevated (45.9) ng/ml in comparison with control rabbits (16.6) ng/ml. Cytokines levels of Th1 (IFN- $\gamma$ ) and Th2 (IL-10) were recorded a significant differences ( $P < 0.05$ ) between immunized and control groups , the mean concentrations of IFN- $\gamma$  in immunized rabbits (28.6 pg/ml) was lower than those of control group (83.6 pg/ml) while IL-10 mean concentrations in immunized rabbits (60.1) pg/ml was higher than those of control group (18.8) pg/ml .

The results indicated that AgB has the ability to modulate immune response toward Th2 bias in immunized rabbits as there was significant elevation in total IgG, IL-10 and significant decreasing IFN- $\gamma$  concentration.

**Keywords**-*Echinococcus granulosus*; antigen B ;interferon gamma; interleukin10;immunization.

## I.INTRODUCTION

Antigen B (AgB) is a 160-kDa polymeric thermostable lipoprotein that constitutes as much as 10% of hydatid fluid, it is the major immunodominant antigens and are thought to be responsible for the immunomodulatory activities of *E. granulosus*, promoting its survival within a mammalian host [1 , 2 ]. Immunohistological studies have shown that this antigen is located in the protoscolex tegument and germinal membrane of the metacestode and therefore secreted into the cyst fluid [3].

AgB can be measured in patient blood as circulating antigen [4], and it has been suggested that it plays an important role in the biology of the parasite and its relationship with the host [5,6]. AgB is a highly immunogenic molecule, a characteristic that underpins its value in serodiagnosis. It appears ladder-like under reduced condition on SDS-PAGE, with three bands with molecular sizes of approximately 8 or 12, 16, and 24 kDa, suggesting that it comprises polymers of 8-kDa subunits [7]. The smallest subunit has proved the most useful target in diagnostic studies [8]. Observing that the 12-kDa subunit of AgB is a protease inhibitor with the ability to inhibit neutrophil recruitment. Ref. [5] first suggested the role of this antigen in escape mechanisms from the early natural immunity.

Accordingly, AgB has received much attention due to its high immunogenicity and specificity [8]. Antigen B is encoded by a multigene family—EgAgB8/1, EgAgB8/2, EgAgB8/3, and EgAgB8/4, each of which codes for one subunit of 8 kDa [9,10]. *E. granulosus* enhances both humoral and cellular responses in its intermediate host. Humoral responses result in the production of immunoglobulins which are important for the diagnosis of disease. However, cellular responses also will take place in the infected host which are important criteria for the prevention of the disease. Prominent quantities of interferon gamma (IFN- $\gamma$ ), interleukin-4 (IL-4), IL-5, IL-6 and IL-10 observed in the majority of patients with hydatidosis supports Th1 and Th2 cell activation in CE patients. In particular, Th1 cell activation seems to be more related to protective immunity, while Th2 cell activation is associated with the susceptibility to the disease [8].

Genetic variant of *E. granulosus* could also exist in antigen coding gene. Comparison of excretory/secretory proteins from *E. granulosus* hydatid fluid from several hosts showed some different patterns. The analysis of sequences related to AgB showed variation between parasites with different hosts and geographical origin [9].

Aim of the study was testing the ability of purified antigen B in sensitization of humoral and cellular immune responses by rabbits immunization.

## II. MATERIAL AND METHODS

### A. Samples collection

Hydatid cysts were collected from liver and lung of infected sheep in the slaughter house in Basra (for a period extend from December/2011 to December /2012) and transferred to the Immunology laboratory, Science College, Basra University. The collected hydatid cysts were thoroughly washed in distilled water (DW) to remove the adhering dirt and clotted blood.

### B. Crude hydatid fluid antigen preparation

Cysts were nicked and the crude sheep hydatid fluid (CSHF) was collected according to [11]. The fluid was aspirated slowly using a 20 ml syringe. A drop of hydatid fluid was examined for the presence of protoscolices and tested the fertility of the hydatid cysts. The

aspirated fluid was pooled together and kept in a glass beaker for settling of brood capsules, protoscolices and dead tissues.

The supernatant was collected and clarified by centrifugation at 10,000 rpm for 30 minutes to remove the sediments. The hydatid fluid was then poured into a 1000 Da cut off membrane (Sigma, USA) and dialysed against three changes of distilled water at 4 C°. The hydatid fluid was supplemented with 0.02 percent sodium azide, 5 mM EDTA and 0.5 M phenylmethylsulfonyl fluoride (PMSF). Aliquots of hydatid fluid were frozen at -20C° for further use .

### *C. Antigen B purification*

AgB was extracted from HF by anion exchange chromatography by using DEAE- sepharose fast flow (Sigma, USA) as the method described by [12] with minor modifications.

The DEAE-Sepharose must be activated before packed in the column, reactivation must be done after 2 run at least. The DEAE-Sepharose matrix was activated according to the method described previously by [13] with simple modifications.

### *D. Preparation and Regeneration of DEAE-Sepharose Column*

DEAE-Sepharose matrix was washed several times with distilled water to remove the ethanol and preserve materials by using a filtration unit and puncher funnel then treated with 750 ml of HCl (0.5M) for 15 min and washing process was repeated .DEAE-Sepharose was treated with 750 ml of NaOH (0.5M) for 15 min. and washing process was repeated. The pH of DEAE-Sepharose was adjusted to pH 7.2 with equilibrium buffer. Column (22 cm long and 2.5cm diameter) was packed with an affinity DEAE- Sepharose gel and equilibrated with 300 ml of equilibrium buffer.

### *E. Ion exchange chromatography*

Fast flow DEAE-sepharose (Sigma, USA) was slowly packed overnight to the column. The column was equilibrated with equilibrium buffer (Sodium phosphate 20Mm, pH 7.2). Flow rate was adjusted to 3 ml / minute and the frozen HF were thawed and loaded to a column. The column was washed with 5 column volumes of equilibrium buffer. The bound antigen fractions were eluted with elution buffer (NaCl ) . Linear gradients of elution buffer were prepared in 20mM phosphate buffer to reach to the best concentration which separate AgB.

### *F. Immunization schedule*

For evaluation of AgB efficiency in immunization , 15 young rabbits, each 2-2.5 kg, were enrolled in the study (10 of them were immunized and 5 were treated as control group ). AgB emulsified with an equal volume (v/v) of complete Freund's adjuvant and three booster injection performed for 10 rabbits as following :

First injection: 1 ml of AgB with a concentration of 500 µg / ml (500 µg of AgB dissolved in 1 ml of PBS) mixed with 1 ml of complete Freund's adjuvant was injected subcutaneously .

Second injection: Two weeks later, rabbits were injected intradermally in limbs with 1 ml of AgB with a concentration of 1000 µg / ml mixed with 1 ml of incomplete Freund's adjuvant.  
 Third injection: 10 days after the second injection, rabbits were injected intradermally in limbs with 1 ml of AgB with a concentration of 1000 µg / ml directly.  
 Fourth injection: 10 days after the third injection, rabbits were injected intradermally in limbs with 1 ml of AgB with a concentration of 500 µg / ml.

Control rabbits (5 rabbits) were injected with PBS only in same periods and volumes described in immunization schedule of immunized rabbits . Ten days after the last injection all rabbits were bled , blood were collected from heart and sera were stored at (-20c°) until used.

*G. Estimation of total IgG concentration , serum IFN-γ concentration and serum IL - 10 concentrations in immunized and control rabbits sera:*

Sera collected from immunized and control rabbits were tested for measuring total IgG , serum IFN-γ and serum IL - 10 concentrations after 6 weeks of immunization using ELISA kit (MyBioSource-USA)

*H. Statistical analysis*

Independent T test was used to compare the differences between immunized and control rabbits using SPSS program . Results were considered significant at  $p (\leq 0.05)$ .

**III.Result**

*A. Anion exchange chromatography*

The best NaCl concentration to separate AgB from HF was 0.45 M according to spectrophotometric reading which confirm to all separated elutions from EDEA- sepharose column for all using concentrations of NaCl (0, 0.15 , 0.25, 0.35,..... 1 M).

*B. Efficiency evaluation of AgB in immunization*

In this study , rabbits were immunized with AgB to examined the ability of this antigen in inducing antibodies production and determining any type of the immune response which will be induced. After 6 weeks of immunization with AgB , the examined sera of (10) immunized rabbits showed a significant ( $P < 0.05$ ) elevated levels (45.9) ng/ml of IgG in comparison with control rabbits (16.6) ng/ml. Table (1).

Table (1): IgG levels in immunized and control rabbits.

Groups	Number	Range	mean	SD±	SE
Immunized rabbits	10	38-55	45.9 ng/ml	5.466	1.728
Control rabbits	5	13-20	16.6 ng/ml	3.049	1.363

Cytokines levels of Th1 (IFN- $\gamma$ ) and Th2 (IL-10) after 8 weeks of immunization were measured in all experimental groups. Data analysis revealed that the mean concentrations of IFN- $\gamma$  in immunized rabbits (28.6 pg/ml) was significantly ( $P < 0.05$ ) lower than those of control group (83.6 pg/ml), table (2).

Table (2): Serum IFN- $\gamma$  levels in immunized and control rabbits.

Groups	Number	Range	mean	SD $\pm$	SE
Immunized rabbits	10	20-37	28.6 pg/ml	6.736	2.130
Control rabbits	5	77-95	83.6 pg/ml	6.985	3.124

Conversely, in the case of IL-10 mean concentrations in immunized rabbits (60.1) pg/ml was significantly ( $P < 0.05$ ) higher than those of control group (18.8) pg/ml, table (3).

Table (3): Serum IL-10 levels in immunized and control rabbits.

Groups	Number	Range	mean	SD $\pm$	SE
Immunized rabbits	10	25-90	60.1 pg/ml	25.886	8.185
Control rabbits	5	3-40	18.8 pg/ml	13.442	6.011

#### ***IV. Discussion***

CE is in fact a chronic disease and presence of cysts for several months or years may have a major role in modulating cytokine production via prolonged stimulation by cyst fluid antigen. Humoral and cellular responses characterized by elevation in some serum antibodies and cytokines [14,15].

Present data indicated that immunized rabbits showed elevated IgG and IL-10 level and decreased IFN- $\gamma$  level in comparing with control group after two months of immunization with AgB. This results provide an evidence that purified AgB is an immunogen has the ability to produce polyclonal antibodies.

Although a high level of total IgG was produced following the immunization of the mice with different antigens, such immune responses were more prominent in mice which were immunized with the crude antigens [16]. Ref. [17] found that the highest level of antibody produced in mice immunized with crude antigen comparing with other purified antigens. Ref. [18] found that immunization of mice with HF induced the elevation of IFN- $\gamma$ , and low levels of IL-4, but still higher than those of control groups. This result may be due to the presence of several antigens with AgB in HF. Ref. [19] when inoculated mice with HF and AgB found that these antigens unable to induce IL-10 secretion this result differ from that obtained in recent study which recorded an elevation of IL-10 in comparison with control group.

Most studies on CE cytokines are mainly based on *in vitro* experiments, determination of cytokine production following stimulation of peripheral blood mononuclear cell or T helper

cells of patients and healthy with crude and B antigens [20,6]. These studies demonstrated an increasing in the production of some cytokines such as IFN- $\gamma$ , IL-4, and IL-5. Others demonstrated concurrent intervention of the Th1 or Th2 cells [21,22].

Ref. [17] found that the proliferation assay showed a statistically significant production of cytokines in the mice immunized with crude antigens. The highest levels of IFN- $\gamma$ , IL12 and IL-4 were produced in mice immunized with crude antigen and E/S antigen of the *in vitro* reared *E.granulosus* adult worms followed by antigens this result disagree with present study where IFN- $\gamma$  decreased in immunized rabbits this might attributed to crude and E/S antigens contain other antigen which induce different immune response.

Like the recorded result in present study which related with elevated levels of IL-10 in immunized than control rabbits , the same elevation of IL-10 was also documented in studies were done by [23,24] on human patients with hydatidosis. Ref. [25] reported that IFN- $\gamma$  and IL-10 were elevated in the first three months of infection .

Simultaneous elevated levels of IFN- $\gamma$ , IL-4, IL-5, IL-6 and IL-10 were detected in the majority of CE patients is an indication for the activation of Th1 and Th2 cells in CE [16] .This result disagree with present result when IFN- $\gamma$  decreasing in immunized rabbits indicating the activation of Th 1 and agree in increasing IL-10. There is difference in immune response in human infection , experimental infection with crude antigen and experimental infection with purified antigen due to diversity of antigens found in human infection and experimental infection with crude antigen comparing with purified antigen. In particular, Th1 cell activation seems to be more related to protective immunity, whereas Th2 cell activation is related to the susceptibility to disease [8].

Ref. [26] were implicated that in the different stages of hydatid disease, Th2 cells express IL-4, IL-5, IL-6 and IL-10 and they are associated with susceptibility to the disease, whereas Th1 cells produce IL-2, IFN- $\gamma$  and they are related to protective immunity [8,22]. The evaluation of IL-10 could increase hydatid cyst survival and growth since IL-10 inhibits effector cellular immune response via its potent anti-inflammatory action [27]. Recent data indicated a bias toward Th2 response which also documented by [24] whom showed an increasing in IL-4 and IL-10.

Increasing evidence showed that some parasitic components play an important role in modulation of host immune system, by suppressing the function of certain immune cells as well as stimulating other cells [28,29].

The notion that a parasite antigen can exploit the host response to the parasite's advantage makes it worthwhile to continue to search for other antigenic components of sheep hydatid fluid that favor protective immunity by inducing a preferential Th1 polarization [6] . Cystic and alveolar echinococcosis induces two very distinct Th1 and Th2 cytokine secretion patterns.This result indicated that the parasite able to mimic immune response by the active escaping mechanism and AgB has important role in *E. granulosus* infection[30].

The results of [31] study provide a new evidence that *E. granulosus* AgB interferes with host dendritic cells (DC) functions through two strategies. First, it impairs monocyte precursor differentiation into DCs, rendering them unable to mature when they are stimulated with lipopolysaccharide LPS. Even though suppressing monocyte differentiation alone might be sufficient to prevent or impair the host immune response, AgB also modulates sentinel DC maturation, priming DCs to polarize lymphocytes into Th2 cells that benefit the parasite. AgB elicits a nonprotective Th2 cell response, thereby helping the parasite to evade the human response. This escaping from host immunosurveillance is achieved by AgB and other *E. granulosus* antigens interfering with monocyte differentiation and by modulating DC maturation via toll like receptor (TLR) [6, 31].

Consistently, a shift in the cytokine response toward a type 1 expression pattern in humans or mice reduces parasite growth [32]. Hence, blocking those antigens responsible for inducing host Th2 cell responses and vaccination antigens inducing a Th1 cell response may be an important pointer for future vaccine design, should be necessary [33]. Ref.[34] stated that when a cyst dies naturally, is killed by chemotherapy treatment or is removed by surgery, Th2 responses drop rapidly and Th1 responses become dominant. This can be interpreted as Th1 lymphocytes contribute significantly to the inactive stage of hydatid disease, with Th2 lymphocytes being more important in the active and transitional stages. This interpretation indicated that *E. granulosus* products have the ability to convert immune response to benefit the parasite [35].

The aim of the present study directed to high light on the immunogenicity of AgB because the information of structural characteristics the AgB immunodominant antigenic sites not only has potential value for further development of peptidomimetics for diagnosis but it also provides an interesting case of protein immunogenicity [36]. As a conclusion from recorded data, AgB may be represented as an immunogen related with immunomodulation and has the ability to shifting the cytokine profiles toward Th2 bias which can be interpreted as there was an evidence referred to Th2 type responses are predominant in patients with chronic extracellular parasite infections [6,37].

## V. REFERENCES

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