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Cytokine and Antibody Response to Immunization of BALB/C Mice with *E.Granulosus* Using Various Routes

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Abstract

Antibody and cytokine response were both studied in BALB/c mice immunized with *Echinococcus granulosus* crude sheep hydatid fluid (CSHF), antigen B (AgB) and protoscoleces homogenate (PSH) via subcutaneous (sc), intraperitoneal (ip) and intramuscular (im) routes. The present study aims at determining the most suitable antigen source and route of immunization, which stimulates the production of T helper type 1 response known to induce a protective immunity against secondary hydatidosis. Hydatid cyst fluid (HCF, a mixture of parasite and host proteins) has lead to the expansion of both IgG1 and IgG2a antibodies regardless of the immunization route. This was accompanied with the expression of moderate IFN-γ gene in spleen cells being the highest user of im route of immunization. Animals immunized with PSH using the ip route induced the highest IFN-γ and IL-4 gene expression, which is an indicator of a mixture of Th1 and Th2 responses. In this group of mice, the titer of antigen specific IgG3 was significantly higher than that for the other groups. Moreover, immunization of mice with AgB (a lipoprotein immunoregulatory component of HF) lead to high levels of IgG1 and IgG2a regardless of the route of immunization. However, using the ip route of immunization, AgB induced higher levels of IL-4, an indication of polarization towards the Th2 response. Immunoblotting profile showed the inability of IgG3 subclass in all groups to recognize the 16 kDa band of antigen B, whereas the reactivity to the 24 kDa band of antigen B was absent only in mice immunized ip with AgB. In conclusion, the im route of immunization of mice with CSHF lead to high Th1 response. While the ip immunization with PSH induced both Th1 and Th2 responses. AgB immunization with the same route lead to dominant Th2 response. Moreover, the IgG3 response in this model requires further investigation using cytokine gene knockout mice to elucidate the primary cytokine responsible for high IgG3 levels.

الملخص

لقد تم دراسة الاستجابة المناعية ممثلة بالأجسام المضادة والسيتوكاينات المتكونة في عينات المصل المستخلصة من الفئران (سلالة BALB/c) نتيجة تحصينها بسائل الأكياس المائية الخام ، أوأنتيجين ب، أورؤوس اليرقات لطفيل إكينوكوكس جرانيولوسس *Echinococcus granulous* وذلك عن طريق حقن التجويف البطني، أو الحقن تحت الجلد، أوفي العضل. هدفت هذه الدراسة لتحديد أفضل الطرق وأفضل المحفزات المناعية اللازمة لتحفيز إنتاج استجابة مناعية للخلايا التائية T cells من النوع الأول بغرض تحقيق مناعة وقائية ضد تكون الأكياس المائية الثانوية. أدى حقن الفئران بسائل الأكياس المائية الخام (خليط من بروتينات الطفيل والعائل) لزيادة في الأجسام المضادة من نوع IgG1 و IgG2a بغض النظر عن طريقة الحقن. ترافق هذا مع تعبير (expression) معتدل لجين IFN-γ في خلايا الطحال والذي وصل أعلى مستوياته بالحقن العضلي.وقد أظهرت الفئران التي حقنت برؤوس اليرقات عن طريق التجويف البطني تعبيرا قويا لجينات IFN-γ و IL-4، كمؤشر لخليط من الاستجابات المناعية التائية من النوع الأول(Th 1) والثاني (Th 2). وقد كان تركيز الأجسام المضادة IgG3 المتخصصة في هذه المجموعة من الفئران أكبر مما كان عليه في المجموعات الأخرى. علاوة على ذلك، أدى التحصين المناعي للفئران باستخدام أنتيجين ب (بروتين دهني من المنظمات المناعية في محتوى الأكياس المائية) الى مستويات عالية من الأجسام المضادة IgG1 و IgG2a بغض النظر عن طريقة الحقن. ومع ذلك حفز أنتيجين ب باستخدام طريقة حقن التجويف البطني، تعبيراً جينياً لجين IL-4 وهذا مؤشرللانحراف باتجاه الاستجابة المناعية التائية من النوع الثاني. وأظهرت نتائج الرسم المناعي (Immunoblot) عدم قدرة الأجسم المضادة من ضرب IgG3 في أي من المجموعات على التعرف على الجزئ 16 كيلو دالتون من أنتيجين ب، بالمقابل لم يظهر أي تفاعل مع الجزئ 24 كيلو دالتون في الفئران المحصنة عن طريق حقن التجويف البطني بأنتيجين ب.

Keywords: Crude Sheep Hydatid Fluid (CSHF), Antigen B (AgB), Protoscolices homogenate (PSH), T helper Type I (Th1), Interferon gamma (IFN-γ), Real Time PCR (RT-PCR).

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1. Introduction

Cystic echinococcosis (CE) is a cosmopolitan zoonotic parasitic disease caused by the larval stage (metacestode stage) of the tapeworm *Echinococcus granulosus*, which cycles between canines, mainly dogs, as definitive hosts and various herbivores as intermediate hosts. The infection rates in dogs and herbivores, as well as the prevalence including seroprevalence, and incidence of the disease in humans have been extensively studied in Jordan. This is one of the endemic areas among Middle East countries (Abdel-Hafez *et al.,* 1997; Al-Qaoud *et al.,* 2003; Qaqish *et al.*, 2003).

Like many helminthic parasites, *E. granulosus* develops sophisticated mechanisms for avoiding the cytotoxic effects of the immune response. Larvae can develop into hydatid cysts in various host organs, particularly liver and lungs. The cyst wall consists of an inner nucleated germinal layer, where protoscoleces bud are, and an outer a cellular laminated layer surrounded by a host fibrous capsule (Schantz *et al.,* 1995). The coexistence of the chronic infection with detectable humoral and cellular responses against the parasite represents one strategy of host-parasite relationship. CE is associated with induction of Th2 response, which appears to be important for parasite survival (Torcal *et al*., 1996; Rigano *et al*., 2001). Furthermore, humoral immune response studies on hydatid disease patients showed high levels of IgE and IgG4, which are induced by Th2 lymphocytes (K6ing and Nutman, 1993; Shambesh *et al.,* 1997). Further analysis of antibodies reactivity showed that IgG4 was predominantly bound to the highly specific AgB subunits of hydatid fluid antigens (Wen and Craig, 1994).

Vaccine-based control of disease transmission is the main objective of several research projects. Generation of a protective immune response against parasitic infections is associated with induction of Th1 type immunity. Evaluation of different parasite antigen preparations and different immunization routes may form a solid base for vaccination trials. Therefore, this study aims at evaluating three *E. granulosus* antigenic preparations (CSHF, AgB and PSH) versus three immunization routes (im, ip and sc) in mice to find the best immunization strategy that activates immune response of the mouse, where secondary hydatidosis develops.

2. Materials and Methods

2.1. Preparation of Antigens:

Crude sheep hydatid fluid (CSHF) and human hydatid fluid were prepared by the aseptic withdrawal of the cyst fluid from liver or lung cysts according to Moosa and Abdel-Hafez (1994). The fluid was centrifuged at 3500×*g* rpm, and the supernatant was freeze-dried using an Edwards lyophilizer (UK). The proper concentration was prepared by dissolving lyophilized powder in PBS, then dialyzing against PBS. Finally, protein content was determined according to Bradford (1976). *E. granulosus* protoscoleces homogenate (PSH) was prepared from washed PSc by homogenization in sterile PBS using a Braun homogenizer (B. Braun, Germany). The homogenate was centrifuged at 15,000×*g* for 30 min at 4°C.

Thermostable lipoprotein antigen B (AgB)-enriched cyst fluid was prepared according to McVie et al. *(*1997*),* based on Oriol et al. *(*1971*).* Hydatid fluid obtained from fertile sheep liver or lung cysts was dialyzed against 5 mM acetate buffer (pH 5); and was centrifuged at 15,000×*g* for 30 min. The precipitate was dissolved in 0.2 M phosphate buffer (pH 8); and boiled for 15 min. After centrifugation, the protein concentration of the supernatant was determined. And purity of AgB was assessed, using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

All antigens were stored at -20°C until analyzed. These antigens were diluted to the desired concentration for animal immunization, or used for cell culture stimulation in RPMI containing 5% fetal calf serum (FCS) after filtration through a 0.2 µm Millipore filter (Schleicher & Schuell, Germany).

2.2. Immunization and Infection of Mice

Groups of 8 female BALB/c mice aged 6-8 weeks, bred at animal facilities (Yarmouk University) were immunized with 120 ug/mouse/dose of CSHF, PSH or AgB using the ip, im, or sc routes for each antigen. Antigens were mixed with equal volumes of complete Fruend`s adjuvant as first injection, and followed by 3 boosters of antigens in incomplete Fruend`s adjuvant. Similar groups of mice were immunized with the same volumes using PBS mixed with the relevant adjuvant. The interval between first injection and subsequent boosters was 7 days. The experiment was terminated after 12 days of the last booster. Mice were killed by ether overdosing, and blood was collected for serum preparation while spleens were collected for mRNA preparation**.**

2.3. Determination of Specific Antibodies

E. granulosus-specific IgG1, IgG2a, and IgG3 were quantified by ELISA. Microtiter plate wells were coated with 200 ul of 5 µg/ml crude human hydatid fluid diluted in carbonate buffer (pH 9.6). After incubation with 1:100 dilutions of sera, plates were washed four times and then incubated with a predetermined dilution of goat anti-mouse antibodies including IgG1, IgG2a, and IgG3 (Sigma, USA). HRP anti-goat IgG was added after 1 hr incubation. After addition of substrate solution, *o*-phenylene diamine (OPD, Sigma), and H_2O_2 (C.B.H.: UK), optical density (OD) was read on 450- and 630-nm reference filters. Reactivity index (RI) was calculated to overcome the interplate variation. A pool of positive samples with high titer was used as reference values in each plate. And OD of each sample was divided by reference value to obtain RI for all antibodies. For each plate, six wells with pooled negative control sera from normal mice were included. The same was done to other two wells containing sera from pooled positive control, immunized and infected mice from other studies carried out in our lab.

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2.4. SDS-PAGE and Immunoblot

SDS-PAGE was performed along the lines of Lammeli (1970). Low molecular weight protein standards (Bio-Rad, CA, USA) ranging from 14.4 to 97.4 kDa were used after being processed equally to antigens. After electrophoresis of CSHF samples and protein marker, gel was fixed, stained, and destained. To be used in immunoblot, the gel was neither fixed, nor stained. Immunoblotting technique was performed similarly to Towbin et al (1979). At end of electrophoresis, separated proteins were transferred to a nitrocellulose sheet by using the transblot cell (Bio-Rad, USA). After the end of transfer, cellulose sheet was removed and cut into 2 mm wide strips; and blocked with 2% bovine serum albumin in PBS (BSA-PBS) incubated with mouse sera (diluted 1:50) after being washed for three times. HRP conjugated goat anti mouse IgG1, IgG2a, and IgG3 were added after being diluted at 1:1000 for each antibody. The strips were incubated for 1 hour at R.T. After washing steps, three ml of substrate solution was added for 15 minutes at R.T. until color develops.

2.5. Measurement of Cytokine Gene Expression

To analyze cytokine gene expression, RNA was extracted from spleens of immunized mice and reverse-transcribed into cDNA. Quantitative analysis was done using a Real time PCR machine. Approximately, one third of fresh spleen samples were prepared by homogenizing the sample in TRI-REAGENT^{TM} (SIGMA, USA) according to manufacturer protocol. The RNA pellet was dried, for 5-10 minutes, and reconstituted in the appropriate volume of DEPC-treated dH_2O. Concentration and purity of isolated RNA were determined spectrophotometrically at 260 and 280 nm. The first strand cDNA synthesis was performed in 20µl reactions at which 2-5 µg of RNA were added to 1 µl of oligo(dT); and continued with DEPC-treated dH_2O to a volume of 11µl. The reaction mixture was incubated at 70^{o}C for 5 min. Then, the contents were centrifuged quickly after being chilled on ice for 1 minute. The reaction mixture was supplemented with 5µl of 4X M-MLV buffer, 1µl (40U/µl) RNAase inhibitor, 1µl (200U) M-MLV-RT enzyme and 2µl dNTPs mix (10mM each). The mixture was incubated at 42^{o}C for 60 minutes after being vortexed gently. The synthesized cDNA was stored at -70^{o}C until usage. Amplification and detection of synthesized cDNA for each sample were accomplished - using the real time PCR machine (Rotor-Gene 3000A, Corbett Research, Australia). SYBR-green dye was used for the purpose of DNA quantification. The PCR- reaction mixture constituted of 1µl cDNA, 2.5µl 10x reaction buffer, 2 mM $MgCl_2$, 400 µM dNTP mix, 200 nM sense and anti-sense primers, platinum DNA-polymerase enzyme (2.5 U), SYBR-Green (0.5x final conc.) and finally, sterile distlled H_2O was added to 25 µl total reaction volume. For primer design see Al-Qaoud and Abdel-Hafez (2008). Primers were purchased from Promega (Madison, Wisconsin, USA). The sequence of primers was as follows: for β-actin (forward primer CCC CGG GCT GTA TTC CCC TCC A, reverse primer TCC CAG TTG GTA ACA ATG CCA); for IL-4 (forward primer CAC TTG AGA GAG ATC ATC GGC, reverse primer TGC GAA GCA CCT GGA AGC CC); and for IFN-γ (forward primer CCT GCA GAG CCA GAT TAT CTC T, reverse primer TCG CCT TGC TGT TGC TGA AGA A). The optimization of real time PCR reaction was performed according to the manufacturer's instructions, and as described by Bustin (2000). Amplification steps consisted of initial denaturation at 95^{o}C for 5 min; and followed by 40 cycles of denaturation at 95^{o}C for 30 sec, and annealing and extension were at 60^{o}C. Relative quantification of cytokine gene expression was calculated using comparative C^T method in relation to β-actin gene as internal control. After determination of the threshold cycle (C^T), the relative expression level (Expr.) of each cytokine was calculated in relation to the expression level of β-actin gene as follows:

Expr. of cytokine gene = $2 - \Delta\Delta CT$

$\Delta\Delta CT = \Delta CT$ sample - ΔCT no template control (NTC)

ΔCT sample = CT sample – CT positive control.

$$\text{Relative Expr. level of each cytokine gene} = \frac{\text{Expr. level of cytokine gene}}{\text{Expr. level of } \beta\text{-actin gene}} \times 100\%$$

The positive control is a plasmid construct (kind gift from Prof. Fleischer, Bernhard Nocht Institute, Hamburg, Germany) containing similar sequence for that of the target cytokine gene; and is amplified by the same primers, which were used to amplify the target. Gel electrophoresis, using 1.5% agarose, was performed for all samples to test real time PCR product for both target gene and β-actin.

2.6. Statistical Analysis

Statistical analysis for differences among groups was done using the non-parametric (Mann-Whitney) *t* test. Results were considered significant at $P < 0.05$.

3. Results

AgB induced both high IgG1 and IgG2a whereas PSH induced the highest levels of IgG3 Evaluating IgG antibody subclasses revealed that IgG1 is the predominant subclass in all immunized groups with the im route being comparatively the lowest (Figs. 1 a, b & c). The highest level of IgG1 was recorded in AgB immunized mice with no significant differences among routes. However, a significant difference (P=0.007) was noticed in the titer of IgG1 antibody in CSHF immunized mice between ip and sc routes. Furthermore, immunization with PSH indicated significantly lower IgG1 in im route when compared to the ip (P=0.001) and to sc routes (P= 0.003) (Figure 1).

The levels of IgG2a were highest in AgB immunized mice when compared with the other two antigens for all immunization routes (Figure 1b) with a significant difference between ip and sc routes (P=0.03). Moreover, immunization with PSH resulted in significant differences in IgG2a titers between the im and ip routes (P=0.002) and the im and the sc (P=0.001).

With respect to IgG3, the highest level was noticed in PSH immunized mouse group regardless of immunization route (Figure 1c). Moreover, a significant difference in IgG3 levels was noticed between the ip and im (P=0.006), also between the sc and im immunization routes of mice (P=0.024).

3.1. Immunization Route Affected The Type and Reactivity of Igg Subclasses to Parasite Components

Immunoblotting of sera against CSHF fractions showed reactivity of sera with AgB fractions (8, 16, and 24 kDa bands) in CSHF, and in AgB immunized mice regardless of the immunization route (Figure 2 I, II and III). Further bands of 45-48 and 66 kDa, in addition to high molecular weight bands, were also prominent. However, in these mouse groups, a remarkable observation was that the IgG3 subclass using all routes did not recognize the 16 kDa polymer of AgB.

3.2. Intraperitoneal Immunization with PSH Induced Both Th1 and Th2 Cytokine Genes

The highest expression levels for both IL-4 and IFN-γ genes were induced in PSH immunized mice, by using ip r oute (Figure 3). Th1 cytokine (IFN-γ) gene expression dominated im and sc immunized mice, by using CSHF as antigen source. But lower levels were expressed in ip immunized mice. In contrast, AgB induced a Th2 cytokine (IL-4) gene expression using the ip route only.

4. Discussion:

This study aimed at exploring the most suitable hydatid cyst antigenic sources and the most appropriate injection route that lead to the development of Th1 response in mice. Such response is advantageous to the host but critical to the metacestode stage of the parasite.

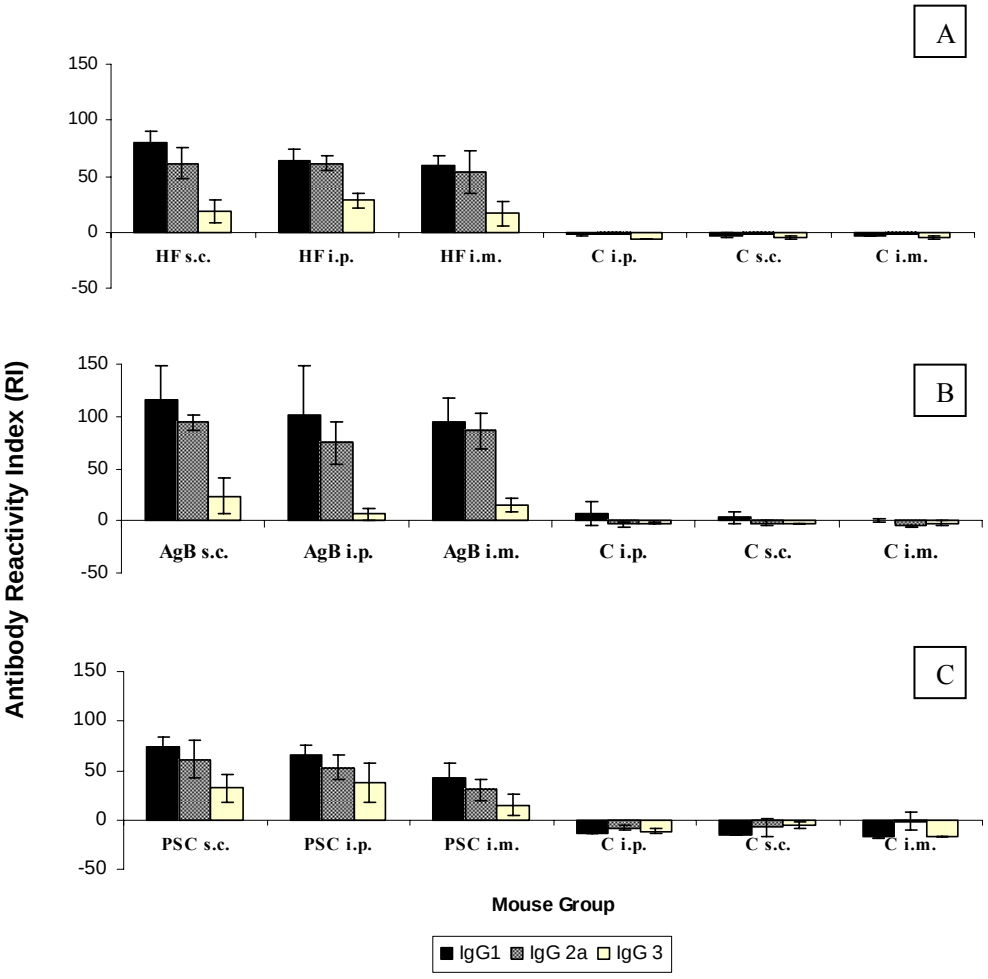

Figure 1 : Antibody isotype profile (IgG1, IgG2a and IgG3) of mice immunized with CSHF **(a)**, AgB **(b)**, and PSC antigens **(c)**, using sc, ip and im routes. Mice were killed on day 12 after final booster and anti-CSHF, anti-AgB and anti-PSC specific antibodies were measured using ELISA technique. Each block represents the mean value of reactivity index (R.I.) for 8 mice of each immunized group and 4 mice of control groups.

$$\text{R.I.(reactivity index)} = \frac{\text{Optical density of sample (O.D.) cut off point (COP)}}{\text{O.D. of positive control}} * 100\%$$

The COP was measured as mean O.D. +3 standard deviations of 6 wells containing pooled negative control sera.

Immunization of mice with protoscoleces using the ip route (Figure 3) induced both Th1 and Th2 typ responses. Rogan (1998) phased the two cell subsets in mice injected with protoscolices. It appears that, at the early stage, exposure to protoscoleces is accompanied by Th1 response, which is intended for the clearance of injected protoscoleces. Moreover, the death of considerable quantities of protoscoleces, which occurs in the first 3 weeks in secondary experimental hydatidosis may account for the development of Th1 (Rogan and Craig 1997). Consistently, in mice inoculated with live protoscoleces, the Th2 markers (high IL-4, IL-5 and IL-10 as well as IgG1) were found dominant during early days of infection, whereas inoculation with dead protoscoleces induced reversed type of response (Dematteis et al 1999, 2001).

Prominent levels of IgG3, which were induced by PSH immunization, agree with a (Al-Qaoud and Abdel-Hafez 2005). Induction of IgG3 secretion is mainly through T-independent carbohydrate antigens that are dominant on the surface of protoscolices (Ferragut and Nieto 1996; Dematteis et al. 2001), supported by high titres of IgG3 found in the peritoneal lavage of PSH-inoculated mice (Dematteis et al. 1999). Studies correlating the type of cytokine response with the IgG3 titers do conflict. While IFN-γ induced IgG3 production in response to T cell independent type 2 antigens IL-4 inhibited it (Snapper and Mond 1996). Absence of IFN-γ blocked IgG3 production completely (Snapper and Paul 1987). Data revealed that lack of IFN-γ receptor reduced IgG2a production, but not IgG3 (Huang et al 1993). The parallel expression of both IFN-γ and IL-4 cytokines genes in PSH immunized mice may explain why IgG3 may be induced by any or both of the opposing responses.

Upon immunization of mice with HF, all routes induced the expression of IFN-γ gene, and same groups demonstrated low levels of IL-4 gene expression, but still higher than that of control groups (Figure 3).

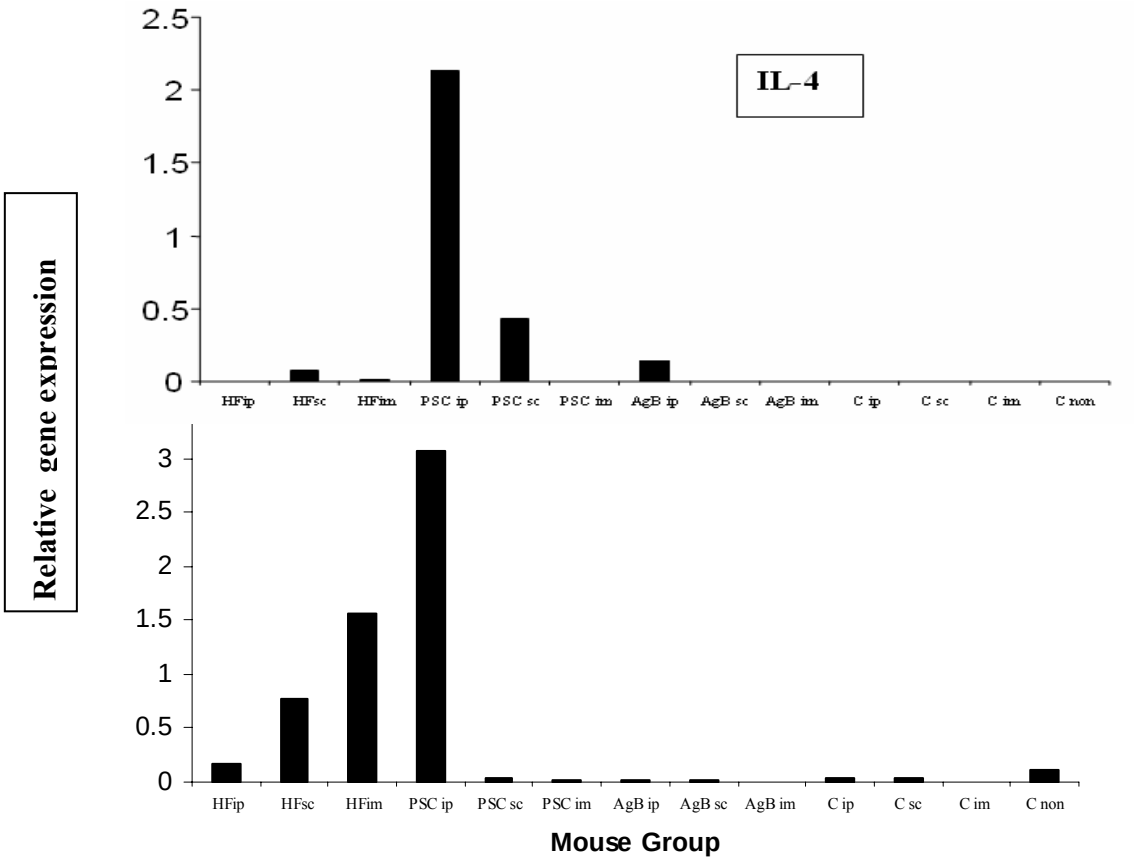

Figure 2 :Relative expression (Expr.) levels of IL-4 (a) and IFN-γ (b) genes determined in mice immunized with CSHF, PSH antigen and AgB, using sc, ip and im routes using the SYBR-Green I assay. Mice were killed on day 12 after final booster and the expression level of each cytokine gene was measured using RT-PCR. Each block represents the average of expression level for two mice from each group. Comparative C^T method ($\Delta\Delta C^T$) was used to calculate the expression level of each cytokine gene relative to β-actin gene as internal control as per text.

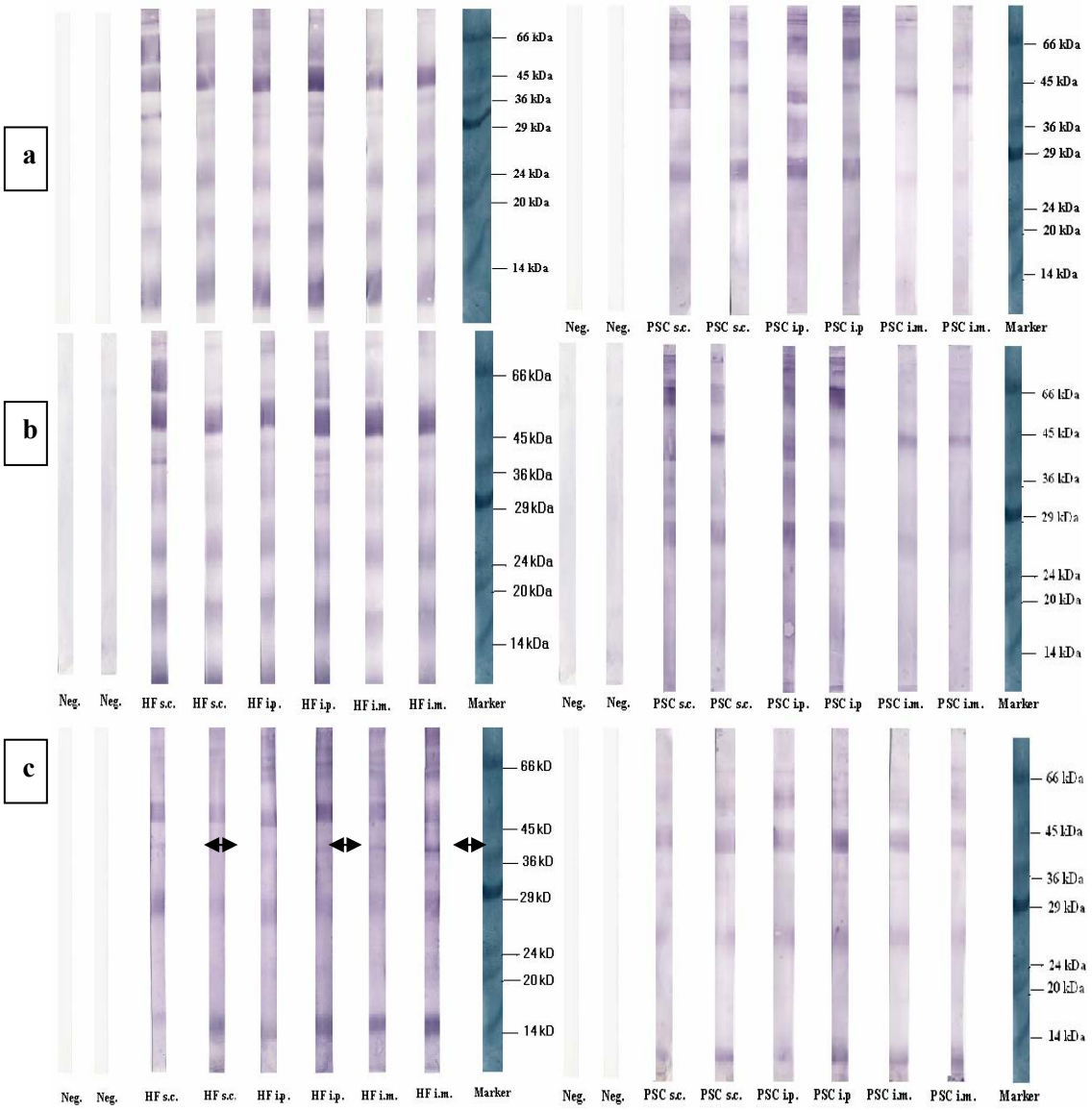

Figure 3 :Immunoblotting pattern of sera collected from mice immunized with CSHF (I), PSC homogenate antigens (II) and AgB (III) using sc, ip and im routes. The peroxidase conjugated secondary antibodies used were anti mouse IgG1(a), IgG2a (b) and IgG3 (c). The first 2 lines are blotting strips incubated with negative control sera and the final lane is a low molecular weight marker. CSHF, PSC homogenate and AgB were electrophoresed on 12.5% SDS-PAGE and blotted into nitrocellulose membrane.

Similar results were reported by Haralabidis *et al*. (1995), Rigano *et al*. (1999 and 2001), who attributed this dichotomy (Th1 and Th2) to the presence of a mixture of host and parasite antigen in the CSHF. Yet, using the same antigen in our laboratory resulted in variable responses and protection rates. This may be explained by using different antigen preparations from different antigen sources of variable purities. Promising data, in this study, stresses the importance of using either highly purified antigens or recombinant proteins in the immunization trials.

The absence of IgG3 reactivity to the 16 kDa subunit of AgB in all immunization routes was remarkable (Figure 2). It is well known that AgB is a polymeric lipoprotein that is made of an 8 kDa molecular weight building blocks. Until recently, it was thought that all AgB monomers are homologous. However, new studies indicated that AgB is encoded by a multigene family (EgAgB8/1, EgAgB8/2, EgAgB8/3, and EgAgB4) (Kamenetzky et al, 2005), each code for one subunit of 8 kDa. Recent data from our laboratory showed that a monoclonal antibody, which is raised against AgB8/2 subunit, did not react with all batches of parasite hydatid fluid (Al-Qaoud *et al.*). Moreover, polyclonal antibodies produced by mouse gene immunization recognized only one subunit of AgB. Since one batch of HF was used in this study, this phenomenon can be attributed to the heterogeneity of the AgB subunits.

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