

Study of Immunological Response to Early and Late Stages of Hydatid Cyst Formation

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Abstract

Background: Hydatid disease is a parasitic helminthes which triggers an immune response that was regulated by cytokines.

Aim of study: to investigate Th1 and Th2 cytokines production , eosinophil cationic protein gene expression and IgG subclasses in different stages of hydatid disease to evaluate their implication and interplay in the evolution of pathology, as a tool for clinical decision and future vaccine preparation.

Material and Methods: A total number of 42 patients with Hydatid cyst which was confirmed by radiological finding from those patients who consulted Baghdad Medical City Teaching Hospital at April – December, 2006 was enrolled in this study. Ten ml venous blood was taken for assessment of Th1 (IL2 and IFN- γ) and Th2 (IL4, IL5 and IL10) by ELISA method , eosinophil cationic protein gene expression by PCR technique and IgG subclasses(IgG1 and IgG4) by Radial Immunodiffusion Method in relation to different stages of Hydatid cyst.

Results: The commonest radiological finding was the late active hydatid cyst (71.42%), followed by (16.67%) and (11.91 %) for late inactive and early hydatid cyst respectively. The mean ECP gene expression was (33.45 $\pm$ 4.89) in late active hydatid cyst while it was (12.87 $\pm$ 1.43; 6.08 $\pm$ 1.13) for early and late inactive hydatid cyst formation respectively. The Th1 cytokines which include mean IL2 was (32.53 $\pm$ 3.03; 21.12 $\pm$ 4.41;12.9 $\pm$ 2.11) and mean IFN- γ was (7.9 $\pm$ 1.2; 4.2 $\pm$ 1.98; 9.62 $\pm$ 1.10) , while the Th2 cytokine which include meanIL4 (39.3 $\pm$ 2.90; 148.62 $\pm$ 12.81; 42.4 $\pm$ 3.44),;IL5 was(49.63 $\pm$ 5.38; 128.83 $\pm$ 13.6; 47.51 $\pm$ 4.23), IL10 was (30.34 $\pm$ 3.77; 178.11 $\pm$ 27.05; 21.36 $\pm$ 5.91) and the IgG subclass IgG1 was (930.14 $\pm$ 44.26 ;1885 $\pm$ 87.7;902.17 $\pm$ 70.8) while the IgG4 (61.6 $\pm$ 4.97;144.38 $\pm$ 20.25;59.52 $\pm$ 3.41) for early, late active and late inactive hydatid cyst respectively with statistical significant value (P value< 0.05).

Conclusions: There was Th1 cell activity in early hydatid cyst , Th2 cell activity in late active hydatid cyst and both Th1 and Th2 cell activity in late inactive hydatid cyst with marked ECP gene expression in early and late active hydatid cyst formation in comparison to those of late inactive hydatid cyst formation. IgG1 and IgG4 were hydatid cyst stage dependent and had interleukin 4,5 and 10 correlation and might be used as good laboratory markers to indicate the status of infection.

key words: Hydatid disease , echinococcosis, Th1&Th2

الخلاصة

الخلفية: يعتبر داء الحويصلات المائية من الامراض الطفيلية والتي تحفز الجهاز المناعي والتي تتم السيطرة عليه بالحركات المناعية.
الهدف من الدراسة: بغية التحري عن انتاج الحركات المناعية من الخلايا التائية المساعدة 1 و 2 والتعبير الجيني للبروتين الموجب للخلايا الحمضية والفئات الفرعية للغلوبين المناعي نوع جي في مراحل مختلفة من داء الحويصلات المائية لتقييم انعكاساتها والتفاعل في تطور علم الأمراض، كأداة لاتخاذ القرار السريري وتحضير لقاح في المستقبل.

المواد وطرق العمل: تمت دراسة 42 مريضا مصابا بداء الحويصلات المائية التي تم تأكيدها بالفحص الإشعاعي من المرضى المراجعين لمستشفى مدينة الطب في بغداد نيسان- تشرين الثاني 2006. تم سحب 10 مل من الدم الوريدي لتقييم افرازات الخلايا التائية المساعدة 1 (الانترلوكين 2 والانترفيرون جاما) والخلايا التائية المساعدة 2 (الانترلوكين 4 و 5 و 10) بطريقة الاليزا والتعبير الجيني للبروتين الموجب للخلايا الحمضية بطريقة تفاعل البلمرة المتسلسل والفئات الفرعية للغلوبين المناعي نوع جي بطريقة الانتشار الاشعاعي فيما يتعلق بمراحل مختلفة من داء الحويصلات المائية.

النتائج: أظهرت النتائج ان اكثر النتائج الشعاعية شيوعا هو داء الحويصلات المائية المزمن النشط بنسبة (71.42%) متبوعا ب (16.67% و 11.91%) لداء الحويصلات المائية المزمن غير النشط والمبكر على التوالي. وظهر التعبير الجيني للبروتين الموجب لخلايا الحمضة زيادة معنوية (4.89±33.45) لداء الحويصلات المائية المزمن النشط بالمقارنة مع (1.43±12.87 و 1.13±6.08) لداء الحويصلات المائية المزمنة المبكرة وغير النشطة على التوالي وكانت نتائج الحركيات المناعية للخلايا التائية المساعدة والتي تتضمن معدل الانترلوكين 2 (3.03±32.53 و 4.41±21.12 و 2.11±12.9) ومعدل الانترفيرون جاما (1.2±7.9 و 1.98±4.2 و 1.10±9.62) بينما كانت نتائج الحركيات المناعية للخلايا التائية المساعدة: معدل الانترلوكين 4 (2.90±39.3 و 12.81±148.62 و 3.44±42.4) والانترلوكين 5 (5.38±49.63 و 13.6±128.83 و 4.23±47.51) والانترلوكين 10 (3.77±30.34 و 27.05±178.11 و 5.91±21.36) وكانت الفئة الفرعية 1 للغلوبين المناعي نوع جي (44.26±930.14 و 87.7±1885 و 70.8±902.17) بينما كان معدل الفئة الفرعية 4 للغلوبين المناعي نوع جي (4.97±61.6 و 20.25±144.38 و 3.41±59.52) لداء الحويصلات المائية المبكر والمزمن النشط والمزمن غير النشط على التوالي مع فارق احصائي معنوي.

الاستنتاجات: يستنتج من هذه الدراسة وجود نشاط الخلايا التائية المساعدة 1 في داء الحويصلات المائية المبكر ونشاط الخلايا التائية المساعدة 2 لداء الحويصلات المائية المزمن النشط ونشاط لكليهما في داء الحويصلات المائية المزمن غير النشط مع وجود تعبير جيني للبروتين الموجب لخلايا الحمضية في المراحل المزمنة النشطة بالمقارنة لتلك المبكرة والمزمنة غير النشطة. ويستنتج ان الفئات الفرعية 1 و 4 للغلوبين المناعي نوع جي تعتمد على مرحلة داء الحويصلات المائية ولها ارتباط مع الحركيات المناعية (4 و 5 و 10) ومن الممكن ان تستخدم كعلامات مخبرية جيدة للإشارة الى حالة العدوى.

الكلمات المفتاحية: داء الحويصلات المائية، طفيلي الاكياس المائية، الخلايا التائية 1 و 2.

Introduction

Echinococcus infestations were among the most dangerous helminthic diseases in human (Al-Saimary, *et al.*, 2010). *Echinococcus* organisms were parasitic helminthes that had life cycles encompassing a carnivorous host (usually fox or dog) as a definitive host and intermediate host (human, ungulate or rodents) (Zhang *et al.*, 2008). Hydatid cyst (HC) in man was a serious condition and the surgical removal of cysts remained the mainstay of treatment because the initial phase of primary infestation was always asymptomatic, where they did not induce major pathology and might remain asymptomatic for many years but it is assumed that some may become symptomatic with the time (Gharbi, *et al.*, 1981). The larval stage of the parasite in human might progress and led to formation of cysts in virtually any organ, particularly in liver and lungs (Brunetti, & Vuitton, 2010) and triggered a cellular and humeral immune response commonly characterized by elevated of some serum immunoglobulines (Rigano, *et al.*, 1995). Cytokines were important in the regulation of the immune system and were secreted by a variety of cells in response to self and non-self-stimuli. Communication within cells occurred via cytokines which determine the quality and intensity of inflammatory and adaptive immune responses (Baz, *et al.*, 2006). It was well known that hydatid disease induced production of antibodies in human with total and specific Immunoglobulin's increase, T cells (T helper cells) produce distinct patterns of cytokines, and both Th1, Th2 cross regulate one another because their respective cytokines act antagonistically (Kakkos, *et al.*, 2001). Increasing evidence showed that parasites-derived substances play important role in initiating or maintaining the parasites growth (Margutti, *et al.*, 2001) because it has very complex multicellular (Zhang, *et al.*, 2008). In the immune response to infestations, cytokines produced by Th lymphocytes have a role in regulating antibody isotype production (Finkelman, *et al.*, 1990 ; Rigano, *et al.*, 1995). To date, Ultrasonography (US) and serum investigations were used to detect and monitor cystic echinococcosis, but a marker of cyst activity is still lacking. For this purpose, decision making clinically might be challenging, especially for relapsing cases

after an primary successful management (Junghanss,*et al.*, 2008; Brunetti, & Vuitton, 2010). Therefore the aim was to explore Th1 and Th2 cytokines profile and eosinophil cationic protein gene expression in different stages of hydatid disease in order to evaluate their implication in the evolution of pathology, as a tool for clinical decision and future vaccine preparation. An another aim was to assess IgG subclasses in relation to stage of disease and their interplay with cytokine profile.

Patients and Methods

A total no. of 42 (25 female and 17 male) patients with HC was enrolled in this study. The inclusion criteria for patients selection were: (i) presence of a minimum one HC cyst in liver, (ii) no prior surgery for HC, and (iii) no albendazole pretreatment or withdrawal minimally two years before serum collection time. The control group (16 individuals) who were aged and sex matched was people with CE free by both of abdominal US, Computed Tomography (CT) and serum investigations. Serum samples from patients and control were collected throughout (April–December ,2006) at Baghdad Medical City Teaching Hospital and stored at deep freezer until assayed. Patients clinically diagnosed and surgically confirmed in some of the cases. Diagnosis was accepted only when there were US and Computed Tomography (CT) Scan reports that confirm the presence of HC. Half of them were inpatients at Baghdad Medical City Teaching Hospital and the other were outpatients who consult hospital for other reasons and diagnosed by chance looking for other causes. HC was categorized according to the World Health Organization Informal Working Group on Echinococcosis (WHO-IWGE) in relation to standard US grouping for cystic echinococcosis (CE) as CE1 ,CE2 , CE3 , CE4 and CE5. CE1 indicate early HC formation while CE2 and CE3 indicated late active HC formation and finally CE4 and CE5 reflected late inactive HC formation (WHO-IWGE, 2003; Junghanss,*et al.*, 2008). Patients that had many cysts were categorized according to cystic activity, in agreement with Hosch et al. results (Hosch, *et al.*, 2008). Patients were classified in relation to different cystic stages at US because the later was correlated with the cyst biological activity(Hosch, *et al.*, 2008; Brunetti, & Vuitton, 2010).

Blood sample:

Blood was drawn from the patients with hydatid cyst using a 10 ml disposable syringe from all cases then 8 mls of these samples were putted in a disposable centrifuged tube and lifted for half hour to clot. After that the serum was separated by centrifugation and putted in a small screw cupped tubes and a each was labeled with serial number. Sera were stored in a deep freeze (-20 C°) to be used for evaluation of the serum cytokines level of Th1 which include IL2 (Cedex , France) and IFN- γ (Enzyme Immunoassay Kits, Biosource, Finland) and Th2(IL-4,IL5 andIL10) (BioSource,Belgium) by enzyme linked immunosorbent assay (ELISA) in accordance to manufacturer instructions. Serum immunoglobulin G subclasses (IgG1 & 4) were assayed using Radial Immunodiffusion Method (RID)(RDI Fitzgerald industries Int, USA). RID (Mancini) method was the diagnostic method that used to determine IgG subclasses in agar plates (ready-to-use) that encompassing specific anti-IgG subclass antibodies . All the test samples, control sera and standard sera were prepared to be added to the agar plates. After 48-72 hours of serum incubation at room temperature the immunoprecipitation rings diameters were measured. The IgG subclass levels in the test samples was quantified by calibration curve procedure (

ring diameters and concentrations of the standards were plotted and the values of tested samples were determined by interpolation). The cycle of repeated thawing and freezing was avoided and no preservatives were added to the sera in all procedures used. The last two mls of blood samples were stored in deep freezer for detection of eosinophil cationic protein by PCR technique as the following:

Total RNA extraction

RNA was extracted through the use of (Accuzol kit/ Bioneer , Korea) by using autoanalyzer (Exiprep/ Korea). The RNA that had been extracted was evaluated and measurement by Nanodroper spectrophotometry.

DNase I Treatment: Extracted RNA were treated with DNase I enzyme to eliminate the trace amounts of genomic DNA from the eluted total RNA by using samples DNase I enzyme kit according to Promega company, USA instructions as following : cDNA synthesis (RT step) at 50 °C for 1 hour while the Heat inactivation at 95 °C for 5 minutes. Mixing total RNA 1µg, 10X buffer, DNase I enzyme, DEPC water with the following volume (10ul, 4ul, 2ul, 4ul) with a total volume of 20ul.

cDNA synthesis: This is by using AccuPower RocktScript RT PreMix kit (Korea) as following: total RNA 100ng/ul, DEPC water and Random Hexamer primer (10pmol) with the following volume 10 ul, 9 ul and 1ul respectively with a final volume of 20 ul. This RT PreMix was placed in AccuPower RocketScript RT PreMix tubes that contains lyophilized Reverse transcription enzyme at form. Then liquefied completely .

Quantitative Real-Time PCR (qPCR)

qPCR was attained for quantification of ECP mRNA transcript levels where gene expression study was carried out by using ($2^{-\Delta\Delta CT}$ Livak method). The qPCR reaction using Real-Time PCR system (BioRad, USA) by SYBER Green dye. qPCR master mix was used in recognition and amplification of ECP target gene and GAPDH housekeeping gene for normalization of gene expression. Primers were designed online using the primer3 plus (Primers sequences) as in the following:

Primer	Sequence		Amplicon size	Gene Bank
ECP	F	TTTGCCATCCAGCACATCAG	89bp	BC096062.3
	R	TGGTTTTTGCAACGCCATCG		
GAPDH	F	CCCATGTTCGTCATGGGTGT	145bp	NM_002046.3
	R	TGGTCATGAGTCCTTCCACGATA		

qPCR master mix was prepared for ECP target gene and GAPDH housekeeping gene according to (AccuPower™ 2XGreen Star qPCR master mix kit, Bioneer Company/ Korea) instructions as following table:

qPCR master mix	volume
cDNA	5 μ L
Forward primer	2 μ L
Reverse primer	2 μ L
DEPC	16 μ L
2X green star master mix	25 μ L
Total	50 μ L

After that, qPCR master mix reaction constituents that mentioned above placed in qPCR white tube strips and mixed by (Exispin vortex centrifuge, Bioneer Company/Korea) for 3 minutes, then the strips placed in MiniOpticon Real-Time PCR system BioRad. USA as following thermocycler conditions :

qPCR step	Temperature	Time	Repeat cycle
Early denaturation	50 °C	1 hour	1
denaturation	95 °C	20 second	45
Annealing \ Extention Detection (scan)	60 °C	30 second	
Melting	60-95°C	0.5 second	1

Statistical methods

All data were presented as means and the deviations were presented as standard deviation, and to test the significance in means of different quantitative data, independent sample t- test of significance was applied. Correlation analysis was done in SPSS version 15.0. P value below 0.05 was accepted as statistically significant value.

Results

Table (1): Frequency of HC according to radiological finding

Classification of HC according to radiological signs	No. of cases	Percentages
Early HC	5	11.91
Late active HC	30	71.42
Late inactive HC	7	16.67
Total	42	100

Table (2):ECP gene expression to early and late HC with control

Stage of HC formation	Fold change ($2^{-\Delta\Delta CT}$)* Mean $\pm$ St. error	P value
Early HC	12.87 $\pm$ 1.43	< 0.05
Late active HC	33.45 $\pm$ 4.89	
Late inactive HC	6.08 $\pm$ 1.13	
Control	1.00	

*($2^{-\Delta\Delta CT}$ Livak method) include: First, the CT of the target gene ECP was return to normal in relation to the reference (ref) GAPDH gene, for both the test patients group and control group as following two formula: $\Delta CT(\text{test}) = CT(\text{target, test}) - CT(\text{ref, test})$; $\Delta CT(\text{control}) = CT(\text{target, control}) - CT(\text{ref, control})$. Second, the ΔCT of the test patients group were normalized to the ΔCT of the control group as following formula: $\Delta\Delta CT = \Delta CT(\text{test}) - \Delta CT(\text{calibrator})$. Finally, Fold change of relative gene expression was calculated by following equation = ($2^{-\Delta\Delta CT}$) : Normalized expression ratio.

Table (3): showed the mean level of Th 1 cytokine level in early and late HC

Parameter Means $\pm$ SD(pg/ml)	Early HC formation	Late active HC formation	Late inactive HC formation	Control	P value
IL2	32.53 $\pm$ 3.03	21.12 $\pm$ 4.41	12.9 $\pm$ 2.11	4.77 $\pm$ 2.98	< 0.05
IFN- γ	7.9 $\pm$ 1.2	4.2 $\pm$ 1.98	9.62 $\pm$ 1.10	5.3 $\pm$ 0.46	<0.05

Table (4) : showed the mean levels of Th 2 cytokine

Parameter Mean $\pm$ SD (pg/ml)	Early HC formation	Late active HC formation	Late inactive HC formation	Control	P value
IL4	39.3 $\pm$ 2.90	148.62 $\pm$ 12.81	42.4 $\pm$ 3.44	33.7 $\pm$ 6.55	< 0.05
IL5	49.63 $\pm$ 5.38	128.83 $\pm$ 13.6	47.51 $\pm$ 4.23	44.48 $\pm$ 3.72	<0.05
IL10	30.34 $\pm$ 3.77	178.11 $\pm$ 27.05	21.36 $\pm$ 5.91	13.2 $\pm$ 4.5	<0.05

Table (5) : showed the mean levels of IgG subclasses(IgG1 &IgG4)

Parameter Mean±SD (mg/dl)	Early HC formation	Late active HC formation	Late inactive HC formation	Control	P value
IgG1	930.14±44.26	1885±87.7	902.17±70.8	812.2±59.9	< 0.05
IgG4	61.6±4.97	144.38±20.25	59.52±3.41	51.51±7.84	<0.05

Discussion:

Table (1): showed the percentages of HC radiological presentation that had been categorized in relation to WHO-IWGE standard US organization for CE (WHO-IWGE,2003). The commonest radiological finding was the late active HC (71.42%), followed by (16.66%)and (11.9 %) for late inactive HC and early HC respectively. This might be due to the ability of HC to be actively live for many years in mammalian hosts (Spruance, 1974; Roneus, *et al.*,1982.). Furthermore, the *Echinococcus* organisms had developed a variety of further strategies by which the immune evasion resulted would enable its survival in active live form which include antigenic dissimilarity, detachment of protein in its surface, production of protease, immunosuppression , sloping of the Th1/Th2 cytokine profile, T cell suppression and modulation, molecular masking and mimicry, discharge of some antigens that were proteins in nature, mitogenic components and carbohydrates of the oncosphere/metacestode that effect antigen presentation and inhibition of effector cell chemotaxis, (Alkarmi, and Behbehani, 1989; Shepherd, *et al.*,1991 Dematteis, *et al.*, 2001; Rigano, *et al.*,2001; Jenne, *et al.*, 2001; Lorenzo, *et al.*, 2002; Zhang, and McManus,2003 Gottstein, *et al.*, 2006; Kanan, and Chain, 2006; Graichen, *et al.*, 2007; Rigano, *et al.*,2007) that finally support the survival of the parasite in active form away from immune response.

Table (2): showed that there was marked ECP gene expression in early HC formation in comparison to those of late inactive HC formation and control. This might be due to a more significant infiltration of neutrophils and macrophages that occurred during the early phases of *E. granulosus* infestation followed by leukocytosis, resulting in an amplified number of myeloid cells such as macrophages, lymphocytes, and eosinophils (Fotiadis, *et al.*, 1999; Zhang, and McManus, 2003) which led to obvious pathologic changes (Finkelman, *et al.*, 1991; Allen, *et al.*,1996; Barnes, *et al.*, 2007). While in the late active phase of CE , there was also significant gene expression of ECP reflecting very high eosinophil activity as a part of shifting of immune response from Th1 into Th2 with concomitant increase in IL4 and IL5 level as it had been illustrated below in addition to increase in neutrophils and macrophages. Eosinophils degranulate the eosinophil cationic protein at the host *Echinococcus* interface, and extents points in HC that been destructive to the parasite (Ramos, *et al.*, 2006). So it can be concluded that ,during the echinococcal infestation, there was a striking activation of cell-mediated immunity with cellular inflammatory responses and pathological modifications but the cellular infiltration of eosinophils and other cells (neutrophils, macrophages, and fibrocytes) in human infestations (Peng, *et al.*, 2006; Magambo, *et al.*, 1995) usually did not produce a severe inflammatory reaction and matured cysts tend to be enclosed by a fibrous layer that split up

the laminated cystic layer from host tissue and protect it from host immune attack physically. It had been reported that the eosinophils had been participated in capsule creation (Slais, and Vanek, 1980) but the exact immunological reactions involved require further elucidation.

Table (3):showed that there is significantly higher level of IL-2 and IFN- γ in early and late active HC formation period than in late inactive HC formation period and control group. As IL-2 and IFN- γ are product of Th1, so Th 1 response was more in early period than in the late period with inactive HC formation. This reflected a strong protective reaction against *E. granulosus* in early HC formation which remain increased when the HC formation become active and late. In early HC ,the cellular immunity and Th1 cytokines increase to in an attempt to control total parasite growth in some individuals and to limit the lesions size in the patients diseases (Rigan`o, *et al.*, 2004). Initial Th1-polarized cytokine production, which can destroy the metacestode at premature stages of establishment and development (Vuitton, 2003; Rigano, *et al.*, 2007) but after its establishment , the immune response would changes to a chiefly Th2 cytokine predominant reaction in the later late active stage of HC that was why the cyst can live for a long period (Bresson, *et al.*, 2008) and finally lead to gradual increase in the late active stage , a feature that can be considered as a characteristic immunological phenomena of *E. granulosus* infestation. In addition to that, when the HC calcified and become inactive the Th1 cytokines production will reduced reflecting decreased inflammatory changes in this period. Also the increase in both IL-4 and IL-10 that had been found in this study and illustrated later also impairs the increasing Th1 protective response to a significant levels making Th2 response more predominant ,so this allowed the parasite to survive to late and active cyst (Chandrasekhar, and Parija, 2009;Mezioug and Touil-Boukoffa, 2009; Vuitton, and Gottstein, 2010). The increase in IFN- γ would lead to NO synthase production by the host in response to an IFN- γ -activating signal (Ait, *et al.*, 2006) that prime host defense against *E. granulosus* (Amri, *et al.*, 2007) but as the IFN- γ - start to decrease when the immune system begin to shift from Th1 into Th2 (table 3) and the antigens in the laminated-layer of CE increased , so this would lead to decrease NO production (Andrade, *et al.*,2004) in addition to the Th2 cytokines inhibition of parasite killing due to the anti-inflammatory action of IL-10 (Bauder, *et al.*, 1999; Vuitton, 2003).

Table (4) showed that there was significant increase in Th2 cytokines (IL 4, IL 5 and IL10) in late active HC in comparison to early, late inactive HC and control group (P value < 0.05). This indicate that there was a shift in immune response from Th1 into Th2 which might be due to an immune evasion molecule exaggerated by antigen B that inhibited elastase activity and neutrophil chemotaxis and eliciting a non-protective Th2 immune reaction that enable the parasite to avoid the immune response (Fraize, *et al.*, 2005; Rigano, *et al.*,2007). The striking feature was the dramatic increase in mean level of IL 10 in late active HC which had a principal function in limitation and ultimately termination of inflammatory reaction. IL-10 also shows a crucial role in function and differentiation of T cells vivo (Moore, *et al.*, 2001) and results in more rapid metacestode growth. T regulatory cells secrete large amounts of IL-10 that inhibit the immune response and activate immune evasion (Amri, *et al.*, 2009; St`ager, *et al.*, 2010). As the IL 10 was typically associated with immunoregulation of effector responses (Moore, *et al.*, 2001), so its high levels could be considered as a hallmark of late *Echinococcus* infestation (Vuitton, 2003; Zhang, *et al.*, 2008). A new type of effector CD4⁺ T cells, which mainly secrete IL-9 and IL-10 named T helper 9 (Th9) cell subsets (Dardalhon, *et al.*, 2008). Th9 and IL-9 were involved in *E.*

granulosus immunopathogenesis and might play helpful roles possibly in *E. granulosus* growth at the late stage after infestation. Thus in addition to Th1/Th2 cells imbalance, there also might be imbalance in Th1/Th9 in patients with CE. It is stated that *E. granulosus* progressive growth during late stages of infestation resulted in the persistence of the Th2 shift (Touil-Boukoffa, *et al.*, 1998 ; Amri, *et al.*, 2007). In this study, the increased level of IL4 might enable the CD4⁺ T cells to differentiate into Th9 cells by the aid of TGF- β (Veldhoen, *et al.*, 2008). Consequently, as Th2 is principal at the *E. granulosus* infestation late stage , Th9/IL-9 may associate with the potentially beneficial roles in the growth of *E. granulosus* at the late stage after infestation. However, the exact underlying mechanism of IL-9 role in the *E. granulosus* inflammation response needs further study. Table (3&4) also showed that patients with late inactive HC generates both responses. Because each one regulate each other (Pearce, and MacDonald, 2002), this is possible due to echinococcal antigens involving different epitopes (Ortona, *et al.*, 2003; Fraize, *et al.*, 2005). It is unclear why hydatid infestation can prompt high values of both Th1 and Th1 cytokines (Rigano, *et al.*, 1995) since they usually downregulate each other (Pearce, and MacDonald, 2002). The antigen itself and the quantity of antigens released might show significant roles. However, a remarkable feature of late inactive CE infestation is the co-occurrence of IFN- γ , IL-4 and IL-10 at increased values in CE (Mezioug, and Touil-2Boukoffa, 2009). It can be decided that ,Th1 reaction was responsible for destruction of the parasite while Th2 reaction, such as IL-4, IL5 and IL10 were useful for parasite development and suppression of parasite death through IL-10 role in addition to intermediate mediator that can prevent the cellular immune response effector phase. Constantly, a change in the cytokine reaction to Th1 immune response reduces parasite growth as it had been shown in table (3&4) in late inactive HC. Hence, blocking the responsible antigens for host Th2 cell responses induction and vaccination with antigens that would encourage a Th1 cell reaction may be an vital point vaccine design in future.

Table (5) : showed that the level of IgG1 and IgG4 in relation to stage of HC formation. IgG1 and IgG4 started to increase in early HC formation but reached to a significant level in late active HC that return to significantly low level in the late inactive HC formation (P value < 0.05) when compared to control group. This was linked with whether the state of cyst was active and live or infiltrated and calcified (Daeki, *et al.*, 2000). The increased production of IL-4 , IL5 and IL-10 was concomitant with high levels of IgG1 and IgG4. This increase was linked with the predominant immune response (Al-Qaoud, and Abdel-Hafez ,2008) and might be used as good laboratory markers to indicate the status of infestation.

Conclusion

- 1-The late active HC was the commonest cyst detected radiological.
- 2- Eosinophil cationic protein gene expression was over expressed in active infestations than in the early and inactive HC reflecting a dramatic eosinophil participation in Th2 immune response.
- 3-Collectively, Th1 cytokines which had protective immunity might be associated with the early HC which was defective or modulated in case of active HC. On the other hand, Th2 cytokines were predominant in late active HC and might be responsible for the disease susceptibility to clinical complications and secondary locations. Any successful attempt to block antigens responsible for induction of host Th2 cell responses and vaccination with antigens that would induce a Th1 cell reaction may be an essential

point for vaccine design in future. In the established *Echinococcus* cystic stage, the typical immune response of the Th2 cell type involves the cytokines IL-4, IL-5, IL-10 and the antibody isotypes IgG1 and IgG4 together with expanded populations of eosinophils.

4-IgG1 and IgG4 were HC stage dependent and had interleukin 4,5 and 10 correlation and might be used as good laboratory markers to indicate the status of infestation.

Recommendations:

1-Our results suggest that we need national control programs including a multi-sectorial collaboration to eradicate hydatidosis and effective actions to control cystic echinococcosis

2-A better understanding of the immunology of echinococcosis in humans has led to new developmental therapy, such as immunomodulation.

3-Further studies of HC with allergic asthma and allergic rhinitis or other IgE mediated disease are mandatory specially those cases treated with allergen specific immunotherapy that lead to shift of immune response from Th2 into TH1.

References

- Ait Aissa, S., Amri, M.; Bouteldja, R.; Wietzerbin, J.& Touil-Boukoffa, C. (2006). Alterations in interferon and nitric oxide levels in human echinococcosis. *Cell. Mol. Biol.*; 52: 65–70.
- Alkarmi, T., and Behbehani, K. (1989). *Echinococcus multilocularis*: inhibition of murine neutrophil and macrophage chemotaxis. *Exp. Parasitol.*;69:16–22.
- Allen, J. E., Maizels, R. M. (1996). Immunology of human helminth infection. *Int. Arch. Allergy Immunol.*;109: 3–10.
- Al-Qaoud, K. M. & Abdel-Hafez, S. K. (2008). The induction of T helper type 1 response by cytokine gene transfection protects mice against secondary hydatidosis. *Parasitology Research*; 102(6):1151–1155.
- Al-Saimary, I. E.; Al-Shemari, M. N. & Al-Fayadh, M. M. A. (2010).Epidemiological and immunological findings on human hydatidosis. *Medical Practice and Review*;1(2):26-34.
- Amri, M. ; Mezioug, D. & Touil-Boukoffa, C. (2009). Involvement of IL-10 and IL-4 in evasion strategies of *Echinococcus granulosus* to host immune response. *European Cytokine Network*; 20(2): 63–68.
- Amri, M.; Aissa, S. A. ; Belguendouz, H. ; Mezioug, D. & Touil-Boukoffa, C. (2007). In vitro antihydatic action of IFN- γ is dependent on the nitric oxide pathway. *J.Interferon Cytokine Res.*; 27: 781–787.
- Andrade, M. A.; Siles-Lucas, M. : Espinoza, E. ; Perez Arellano, J. L. ; Gottstein, B. & Muro, M.(2004). *Echinococcus multilocularis* laminated-layer components and the E14t14–3-3 recombinant protein decrease NO production by activated rat macrophages in vitro. *Nitric Oxide*; 10: 150–155.
- Barnes, T. S., Hinds, L. A. ; Jenkins, D. J.& Coleman, G. T. (2007). Precocious development of hydatid cysts in a macropodid host. *Int. J. Parasitol.*;37:1379–1389.
- Bauder, B.: Auer, H. ; Schilcher, F.; Gabler, C.; Romig, T. ; Bilger, B. & Aspöck, H.(1999). Experimental investigations on the B and T cell immune response in primary alveolar echinococcosis. *Parasite Immunol.*; 21: 409–421.
- Baz, A.; Ettlin, G. M. & Dematteis, S. (2006). Complexity and function of cytokine responses in experimental infection by *Echinococcus granulosus*. *Immunobiol.*; 211: 3-9.

- Bresson-Hadni, S. ; Miguet, J.-P.; Manton, G. ; Giraudoux, P. & Vuitton, D-A. (2008). Alveolar echinococcosis: a disease comparable to a slow growing cancer, Bulletin de l'Academie Nationale de Medecine;192(6): 1131–1139.
- Brunetti, P. K. & Vuitton, D. A.(2010). Expert consensus for the diagnosis and treatment of cystic and alveolar echinococcosis in humans.Acta Tropica;114(1):1–16.
- Chandrasekhar, S. & Parija, S. C.(2009). Serum antibody & Th2 cytokine profiles in patients with cystic echinococcosis. Indian Journal of Medical Research; 130(6): 731–735.
- Daeki, A. O.; Craig, P. S. & Shambesh, M. K.(2000). IgG-subclass antibody responses and the natural history of hepatic cystic echinococcosis in asymptomatic patients. Ann. Trop. Med. Parasitol.; 94: 319–328.
- Dardalhon, V. ; Awasthi, A.; Kwon, H. et al.(2008). IL-4 inhibits TGF- β -induced Foxp3+ T cells and, together with TGF- β , generates IL-9+ IL-10+ Foxp3– effector T cells. Nature Immunology; 9(12): 1347–1355.
- Dematteis, S. ;Pirrotto, F. ; Marques, J.; Nieto, A.; Orn, A. &Baz, A. (2001). Modulation of the cellular immune response by a carbohydrate rich fraction from Echinococcus granulosus protoscoleces in infected or immunized BALB/c mice. Parasite Immunol.;23: 1–9.
- Dvoroznakova, E.; Hrcakova, G. ; Boroskova, Z. ;Velebny, S. &Dubinsky, P. (2004). Effect of treatment with free and liposomized albendazole on selected immunological parameters and cyst growth in mice infected with Echinococcus multilocularis. Parasitol. Int.; 53: 315–325.
- Finkelman, F. D.; Holmes, J.; Katona, I. M.; Urban, J. F.; Beckmann, M. P.; Park,L.S.; Schooley, K. A.; Coffman, R. L.; Mosmann, T. R. & Paul, W. E. (1990). Lymphokine control of in vivo immunoglobulin isotype selection. Annu. Rev. Immunol.; 8:303-333.
- Finkelman, F. D.; Pearce, E. J. ; Urban, J. F. & Sher, A. (1991). Regulation and biological function of helminth-induced cytokine responses. Immunol. Today; 12: 62–66.
- Fotiadis, C.; Sergiou, C.; Kirou, J.; Troupis, T. G.; Tselentis, J. ; Doussaitou, P.;Gorgoulis, V. G. & Sechas, M. N.(1999). Experimental Echinococcus infection inthe mouse model: pericystic cellular immunity reaction and effects on the lymphoid organs of immunocompetent and thymectomized mice. In Vivo;13:541–546.
- Fraize, M.; Sarciron, M. E.; Azzouz, S. ; Issaadi, N. ; Bosquet, G. &Petavy,A.F.(2005). Immunogenicity of two Echinococcus granulosus antigens EgA31 and EgTrp in mice. Parasitol Res; 96: 113–120.
- Gharbi, L.L.; Ilassin, W.; Brauner., M. & Dupuch, K. (1981).Ultrasound examination of the hydatid live. Radiology;139: 459-63
- Gottstein, B.; Haag, K. ; Walker, M. ; Matsumoto, J. ; Mejri, N. and Hemphill, A. (2006). Molecular survival strategies of Echinococcus multilocularis in the murine host. Parasitol. Int.; 55: 45–49.
- Graichen, D. A.; Gottstein, B.; Matsumoto, J.; Muller, N. ; Zanolto, P. M. ; Ayala, F. J. & Haag, K. L.(2007). Expression and diversity of Echinococcus multilocularis AgB genes in secondarily infected mice: evaluating the influence of T-cell immune selection on antigenic variation. Gene ;392:98–105.
- Hosch, W. T. ; Junghanss, M. Stojkovic et al.(2008). Metabolic viability assessment of cystic echinococcosis using high-field ^1H MRS of cyst contents,NMR in Biomedicine;21(7):734–754.

- Jenne, L.; Arrighi, J. F. ; Sauter, B.& Kern, P. (2001). Dendritic cells pulsed with unfractionated helminthic proteins to generate antiparasitic cytotoxic T lymphocyte. *Parasite Immunol.*;23:195–201.
- Junghanss, T. A. ; Horton, J. ; Chiodini, P. L. & Brunetti, E. (2008). Clinical management of cystic echinococcosis: state of the art, problems, and perspectives. *American Journal of Tropical Medicine and Hygiene*; 79(3): 301–311.
- Kakkos, S. A.; Mouzaki, A. & Vagianos, C. E. (2001). Modifications of the immune system caused by the cestode. *Echinococcus granulosus: A review Annals of Gastroenterol.*, 14:91-98.
- Kanan, J. H., and Chain, B. M. (2006). Modulation of dendritic cell differentiation and cytokine secretion by the hydatid cyst fluid of *Echinococcus granulosus*. *Immunology* ;118:271–278.
- Livak, K. J. and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real time quantitative PCR and the $2\Delta\Delta C(T)$ method. *Method*; 25(4):402–408.
- Lorenzo, C.; Salinas, G. ; Brugnini, A.; Wernstedt, C.; Hellman, U. & Gonzalez-Sapienza, G. (2002). *Echinococcus granulosus* antigen 5 is closely related to proteases of the trypsin family. *Biochem J.*; 369: 191–198.
- Magambo, J. K.; Zeyhle, T. M.; Wachira, J. & Raasen, T. (1995). Cellular immunity to *Echinococcus granulosus* cysts. *Afr. J. Health Sci.*;2: 250–253.
- Marco, M.& Nieto, A.(1991). Metalloproteinases in the larvae of *Echinococcus granulosus*. *Int. J. Parasitol.*; 21:743–746.
- Meeusen, E. N.& Balic, A. (2000). Do eosinophils have a role in the killing of helminth parasites? *Parasitol. Today*;16:95–101.
- Mezioug, D. & Touil-Boukoffa, C. (2009). Cytokine profile in human hydatidosis: possible role in the immunosurveillance of patients infected with *Echinococcus granulosus*. *Parasite*;16(1): 57–64.
- Moore, K. W.; Waal Malefyt, R. de; Coffman, R. L. & O'Garra, A.(2001). Interleukin-10 and the interleukin-10 receptor. *Annu. Rev. Immunol.* 19: 683–765.
- Ortona, E. ; Margutti, P. ; Delunardo, F.; Vaccari, S.; Rigano, R. ;Profumo, E.;Buttari, B. & Siracusano, A.(2003). Molecular and immunological characterization of the C-terminal region of a new *Echinococcus granulosus* heat shock protein 70. *Parasite Immunol.*; 25: 119–126.
- Pearce, E. J. & MacDonald, A. S.(2002). The immunobiology of schistosomiasis. *Nat. Rev. Immunol.*; 2: 499–511.
- Peng, X.; Li, J.; Wu, X.; Zhang, S.; Niu, J.; Chen, X.; Yao, J. & Sun, H.(2006). Detection of osteopontin in the pericyst of human hepatic *Echinococcus granulosus*. *Acta Trop.*;100:163–171.
- Ramos, A. L.; Discipio, R. G. & Ferreira, A. M.(2006). Eosinophil cationic protein damages protoscoleces in vitro and is present in the hydatid cyst. *Parasite Immunol.*; 28:347–355.
- Richards, K. S.; Arme, C. & Bridges, J. F.(1983). *Echinococcus granulosus equinus*: an ultrastructural study of murine tissue response to hydatid cysts. *Parasitology*; 86: 407–417.
- Rigano, R. & Buttari, B. ; De Falco, E. et al.(2004). *Echinococcus granulosus*- specific T-cell lines derived from patients at various clinical stages of cystic echinococcosis. *Parasite Immunology*; 26(1): 45–52.

- Rigano, R. ; Profumo, E. ; Di Felice, G. ; Ortona, E.; Teggi, A. & Siracusano, A. (1995). In vitro production of cytokines by peripheral blood mononuclear cells from hydatid patients. *Clinical and Experimental Immunology*; 99(3): 433–439.
- Rigano, R. ; Profumo, E.; Ioppolo, S. ; Notargiacomo, S. ; Teggi, A. & Siracusano, A. (1999). Serum cytokine detection in the clinical follow up of patients with cystic echinococcosis. *Clinical and Experimental Immunology*;115(3): 503–507.
- Rigano, R.; Buttari, B. ; Profumo, E.; Ortona, E.; Delunardo, F.; Margutti, P.; Mattei, V.; Teggi, V.; Sorice, M. & Siracusano, A.(2007).Echinococcus granulosus antigen B impairs human dendritic cell differentiation and polarizes immature dendritic cell maturation towards a Th2 cell response. *Infect. Immun.*;75:1667–1678.
- Rigano, R.; Profumo, E. ; Buttari, B.; Teggi, A. & Siracusano, A.(1999). Cytokine gene expression in peripheral blood mononuclear cells (PBMC) from patients with pharmacologically treated cystic echinococcosis. *Clinical and Experimental Immunology*;118(1):95–101.
- Rigano, R.; Profumo, E.; Bruschi, F.; Carulli, G.; Azzara, A. ; Ioppolo, S.; Buttari, B.; Ortona, E.; Margutti, P.; Teggi, A. & Siracusano, A.(2001). Modulation of human immune response by Echinococcus granulosus antigen B and its possible role in evading host defense. *Infect. Immun.*;69:288–296.
- Rigano, R.; Profumo, E.; Ioppolo, S.; Notargiacomo, S.; Ortona, E.; Teggi, A. & Siracusano, A. (1995). Immunological markers indicating the effectiveness of pharmacological treatment in human hydatid disease. *Clin. Exp. Immunol.*; 102: 281–285.
- Roneus, O.; Christensson D.; and Nilsson, N. G.(1982). The longevity of hydatid cysts in horses. *Vet. Parasitol.*;11:149–154.
- Shepherd, J. C.; Aitken, A. and McManus, D. P. (1991). A protein secreted in vivo by Echinococcus granulosus inhibits elastase activity and neutrophil chemotaxis. *Mol. Biochem. Parasitol.*; 44: 81–90.
- Slais, J., and Vanek. M. (1980). Tissue reaction to spherical and lobular hydatid cysts of Echinococcus granulosus (Batsch, 1786). *Folia Parasitol.*; 27:135–143.
- Spruance, S. L.(1974). Latent period of 53 years in a case of hydatid cyst disease.*Arch. Intern Med.*;134:741–742.
- St`ager, S. ; Joshi, T. & Bankoti, R. (2010). Immune evasive mechanisms contributing to persistent Leishmania donovani infection. *Immunologic Research*; 47, (1–3): 14–24.
- Touil-Boukoffa, Bauvois, B.; Sanc`eau, J.; Hamrioui, B. & Wietzerbin, J. (1998). Production of nitric oxide (NO) in human hydatidosis: relationship between nitrite production and interferon- γ levels. *Biochimie*;80(8-9): 739–744.
- Veldhoen, M.; Uyttenhove, C. ; van Snick, J. et al.(2008). Transforming growth factor- β “reprograms” the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nature Immunology*; 9(12): 1341–1346.
- Vuitton, D. A. (2003). The ambiguous role of immunity in echinococcosis: protection of the host or of the parasite? *Acta Trop.*; 85: 119–132.
- Vuitton, D. A.& Gottstein, B. (2010). Echinococcusmultilocularis and its intermediate host: amodel of parasite - host interplay,” *J Biomed Biotechnol.* 2010 ;2010: 923193. doi: 10.1155/2010/923193. Epub 2010 Mar 21.
- WHO-IWGE(2003). International classification of ultrasound images in cystic echinococcosis for application in clinical and field epidemiological settings. *Acta Tropica*;85(2):253–261.

- Zhang, S.; Hue, S.; Sene, D.; Penfornis, A.; Bresson-Hadni, S.; Kantelip, B. Caillat-Zucman, S. & Vuitton, D. A. (2008). Expression of major histocompatibility complex class I chain-related molecule A, NKG2D, and transforming growth factor in the liver of humans with alveolar echinococcosis: new factors in the tolerance to parasites? *J. Infect. Dis.*;197:1341–1349.
- Zhang, W., Li J. & McManus, D. P. (2003). Concepts in immunology and diagnosis of hydatid disease. *Clin. Microbiol. Rev.*;16:18–36.
- Zhang, W.; Ross, A. G. & McManus, D. P. (2008). Mechanisms of immunity in hydatid disease: implications for vaccine development. *The J. of Immunol.*,181:6679-6685.