Human hydatidosis with different therapeutic modalities: cellular and immunological analysis

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Introduction In early phase of hydatidosis, the Th1 mediates protective immunity against the parasite and helps to eliminate the larval stage, whereas later, the Th2 subset promotes humoral immune response and reflects susceptibility to disease.

Aim of the study To investigate the local immunohistochemical pattern of T cell populations in Egyptian patients exposed to variable therapeutic modalities and to identify the dominating type of T cells in patients infected with hydatidosis using two immunocellular markers (STAT-4 and GATA-3), representing Th1 and Th2, respectively, and to evaluate systemic immune response using commercially available indirect hemagglutination (IHA) test.

Patients, materials, and methods Forty-five cases with human hydatidosis were enrolled and divided according to treatment they received into four groups. Fresh samples of hydatid cyst wall were fixed in 10% formalin for immunohistochemical examination. Antibody titer was measured using commercial available IHA test.

Results Significantly higher expression levels of GATA-3 in comparison with STAT-4 were recorded in all studied groups. Unexpectedly, IHA test recorded positive findings in only 22 of the 45 cases, exhibiting difficulty in the diagnosis and prognosis of such parasitic infection.

Conclusion These local and systemic immunological profiles may reflect the efficiency of the parasite to sequestrate its antigen away from the host immune system applying variable therapeutic modalities with pronounced overshadowed Th2 response. Consequently, these data may help to develop new immunotherapeutic strategies to successfully eliminate this serious helminthic infection.

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Introduction

Hydatidosis (cystic echinococcosis) represents an important public health problem owing to its wide geographical distribution as well as its medical and economic effects [1]. Diagnosis of hydatid infection is complex and necessitates a variety of parasitological, clinical, and laboratory investigations in addition to imaging techniques, which usually ascertain the clinical suspicion [2].

Serological diagnosis provides a cheap and easy method that can be applied on a large scale among population living in endemic areas or having history of residence in endemic areas [3]. In general, there are three therapeutic modalities for the treatment of hepatic hydatid cysts: chemotherapy, percutaneous conservative treatments, and laparoscopic surgery [4]. Hydatid cyst consists of three layers with hydatid fluid inside; the outermost layer is called the pericyst, ectocyst, or adventitial layer. It is formed by the host tissue's reaction to parasites. The next layer is the laminated membrane/layer. The innermost layer is the germinative layer or endocyst [5].

Upon oral ingestion of parasite-derived infective eggs by intermediate hosts including human, the oncosphere larva is released and penetrates the intestinal barrier, usually evoking a Th1-dominated immune response with interferon gamma (IFN-γ)-associated immune effector functions [6]. The early Th1 response is gradually replaced by a Th2 response, dominated by interleukin (IL-5) and IL-10 [7].

The production of various cytokines by Th1 and Th2 lymphocyte subsets plays a major role in the clinical outcome of disease [8]. As previously implied, in early phase of echinococcosis, a Th1 response is predominantly parasitocidal, whereas later, a Th2 response associates with parasite growth and disease progression [7,9,10].

Thus, and theoretically speaking, if there is a therapeutic approach succeeded to modulate the immune response toward parasite downturn, this will certainly help human cases to eliminate such serious infection or at least minimize its life-threatening hazards.

Signal transducer and activator of transcription (STAT-4) is an important transcription factor or what is called a sequence-specific DNA-binding factor, which is crucial for the differentiation of Th1 cells in promoting cellular-mediate immune response [11]. 'GATA-3' is another member of transcription factors characterized by its ability to bind to the DNA sequence 'GATA,' which is necessary for characterization of Th0 cells toward Th2 cells subtype while suppressing their differentiation toward Th1 [12].

Up to our knowledge, no data are reported concerning the effect of different therapeutic modalities on these vital immunological cells. Therefore, the aim of the current study, on one hand, was to investigate the local immunohistochemical pattern of T cells population in patients exposed to variable therapeutic modalities commonly performed in National Hepatology and Tropical Medicine Research Institute, and then identify the dominating type of T cells using two immunocellular markers (STAT-4, GATA-3) representing Th1 and Th2, respectively. On the other hand, the aim was to evaluate the systemic immune response using commercially available indirect hemagglutination (IHA) test.

Patients, materials, and methods

Study design

This was a cross-sectional study conducted in the period from March 2017 to May 2018.

Inclusion criteria

The study in accordance with the WHO included large liver cysts (>5 cm) with multiple daughter cysts, single liver cysts (>5 cm) based on ultrasound examination, cysts that were located superficially that could have the risk of spontaneously rupture or as a result of trauma, viable cysts with signs of active infection, cysts communicating with the biliary tree, cysts that cause local pressure to adjacent organs, and complicated cysts [4].

Exclusion criteria

Pregnant women, patients with liver cysts less than 30 mm in diameter, patients with cysts that are difficult to access, and patients with an active malignant disease were excluded [4].

Study groups

Forty-five patients with cystic echinococcosis attending Surgery Department, National Hepatology and Tropical Medicine Research Institute, Cairo, Egypt, were enrolled in the present study and divided according to treatment they received into the following groups:

Group 1 included six cases that did not receive any form of treatment.
Group 2 included 15 cases previously treated with only medical therapy before the last surgical intervention in the form of albendazole (400 mg twice daily for 4 weeks repeated up to 12 cycles separated by 2 weeks rest).
Group 3 included 10 cases previously treated with a single course of medical treatment plus single PAIR technique (percutaneous, aspiration, injection, and reaspiration).
Group 4 included 14 cases previously treated with multiple courses of medical treatment plus multiple PAIR techniques.

Other classification was done according to the number of the lesions in the involved organs: 35 cases had a single lesion in the liver, two cases had multiple lesions in the liver, and eight cases had multiple lesions in multiple organs.

Processing of hydatid cyst wall

Fresh samples of hydatid cyst wall were fixed in 10% formalin for immunohistochemical examination.

Immunohistochemical assay

According to Prophet *et al.* [13], tissue sections of 4–5-μm thickness were put onto an adhesive-coated glass slides. Deparaffinization and rehydration steps were done for the sections. Then epitope retrieval was performed using the tris-EDTA buffer to unmask calcium ion-covered tissue, then incubated for 10 min in buffer, and then washed twice. Ultravision protein block was applied and incubated for 5 min to block nonspecific background staining. Primary antibodies were used for the individual markers (GATA-3 and STAT-4) and then secondary antibodies were applied. Incubation was done with biotinylated goat anti-polyvalent for 10 min. After each step, the sections were washed four times in phosphate buffered saline. One to two drops of 3,3′ Diaminobenzidine chromogen were added to 1 ml of Diaminobenzidine substrate, mixed by swirling and applied to the tissues, which were then incubated for 10 min (all chemicals were supplied from Biological, Swampscott, Massachusetts, USA).

Data analysis by real-time quantitative morphocytometry

The pathological and morphometric analysis were performed at the Pathology Department, National Research Center, Cairo, Egypt, using the Leica Qwin 500 Image Analyzer (Leica Imaging Systems Ltd, Cambridge, England). Each slide was observed with a light microscope at ×100× and ×200 magnification. Ten fields were randomly selected, and their color index was measured. Area percentage of each marker was measured in 10 fields as well on a real-time image from the microscope.

Reading of the results

Brownish color reflects positive expression. The area of the reaction to be measured was covered automatically by a blue mask which was called binary image. The area of this binary image was calculated automatically by the software.

Serological assay

Anti-hydatid antibody titer was detected by IHA test using the commercially available kit echinococcosis Fumouze (ELITech Microbio, France, Paris).

Statistical analysis

Results were collected, tabulated, and statistically analyzed by SPSS software, version 20 (SPSS Inc., Chicago, IL, USA). Data were reported as mean values for quantitative variables and percentages for qualitative variables. Analytic statistics, for example, χ^2 and analysis of variance test, were used. *P* value less than 0.05 was considered statistically significant.

Ethical considerations

The study was conducted according to the institutional ethical and professional guidelines for the management and follow-up of patients for postoperative care. Informed written consent was provided by each patient before the procedures were performed.

Results

Concerning the results of immunohistochemical assay, the mean±SD of area percentage values of GATA-3 and STAT-4 was used to localize the local cellular patterns of T cells and presented in Table 1 and Fig. 1. In general, there was a higher local expression of the GATA-3 marker (Th2) in relation to STAT-4 marker (Th1) in all studied groups. This high expression levels of GATA-3 was visually evidenced by the densely stained huge brown areas detected in tissue sections and confirmed by the application of digital real-time image analysis, which can be easily measured by software system following accurate identification of the local markers (appeared as bluish colouration during the analysis). Figure 1c clarifies the wide gap between the two markers, reflecting the significant domination of Th2 subset on Th1 subdivision of such immune cells (Table 2).

Table 1 Mean±SD of area percentage values of GATA-3 and STAT-4 related to different studied groups

Groups	Marker (mean±SD)	
	GATA-3	STAT-4
Group 1	32.8±8.4	0.24±0.1
Group 2	38.1±5.2	3.03±1.3
Group 3	39.02±3.1	0.46±0.23
Group 4	41.7±4.1	0.83±0.65
ANOVA	4.586	28.730
P	<0.007*	<0.001**

ANOVA, analysis of variance. **P-Value < 0.001 was considered statistically highly significant.

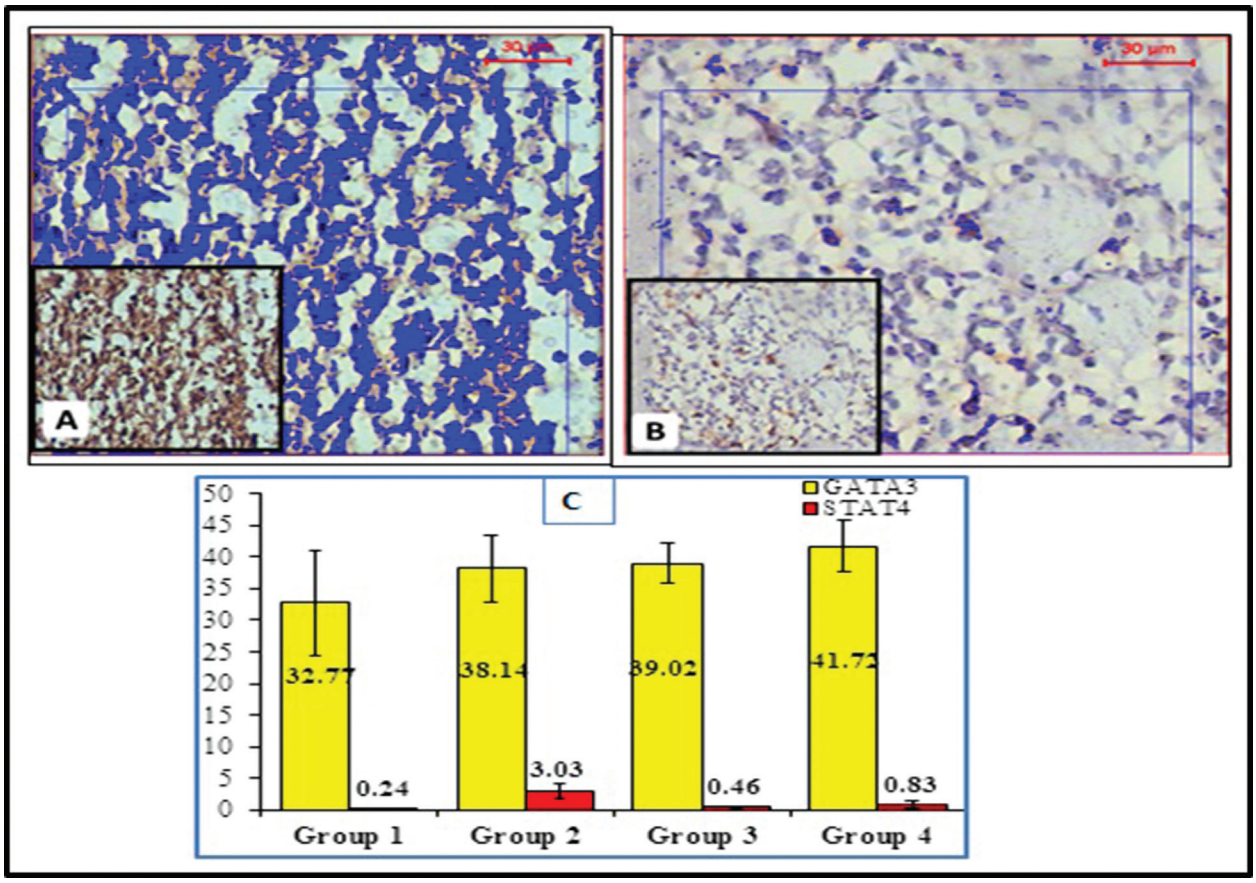
On the contrary, and unexpectedly, IHA test recorded positive findings in only 22 (48.9%) cases of the 45 proven surgically to be cystic hydatidosis, whereas 23 (51.1%) of the 45 cases showed negative results (Table 3).

Regarding the relation between positivity and number of the lesions, 13 of 22 positive cases belonged to patients with single lesion in the liver, only one case with multiple lesions in the liver, and eight cases belonged to patients with multiple lesions in multiple organs. High positive results (i.e. titer 640 or above) were recorded in 10 cases, where two of them belonged to patients with single lesion in the liver and the remaining eight cases belonged to patients with multiple lesions in multiple organs. Regarding the relation between negativity and number of the lesions, 22 of the 23 negative cases belonged to patients with single lesion in the liver and only one case with multiple lesions in the liver, with highly significant statistical difference ($P<0.001$) (Table 4).

Discussion

Despite the established therapeutic approaches targeting cystic echinococcosis, this parasitic disease still continues to cause sizable morbidity and mortality in many parts of the world. Immunological imbalance toward the anti-inflammatory side of immune response with upregulated Th2 mechanism is reported in chronic phase of the disease. There is not enough data concerning the effect of variable medicinal protocols on such immunological disparity. So, the main goal of the present study was to investigate the local immunohistochemical pattern of T cells subsets,

Figure 1



Local expression of GATA-3 marker (a) and STAT-4 (b) immunohistochemical-stained tissue before analysis (small squares) and during real-time analysis (bluish discoloration in the larger squares). (c) Chart represents (mean±SD) both markers in different studied groups.

Table 2 Relation between GATA-3 and STAT-4 in each group

GATA-3	STAT-4	
	R	*P* value
Group 1	−0.490	<0.001**
Group 2	−0.460	<0.001**
Group 3	−0.247	0.040*
Group 4	−0.513	<0.001**

Th1 and Th2, in patients infected with CE and exposed to different therapeutic modalities using STAT-4 and GATA-3 markers representing Th1 and Th2, respectively, to identify the dominating type of these immune cells.

Data analysis in the current work was based on accurate calculation, applying a digital real-time quantitative morphocytometric analytic software program. Such type of automated analysis is reported to be more reliable and accurate than manual ocular calculation, which was reported to be a source of biased results. Ocular estimation is less accurate in distinguishing very fine dissimilarities within cellular staining at which IHC is mostly related to target antigen concentration using such type of colorimetric assessment [14].

In the current study and based on such immunohistochemical accurate evaluation, GATA-3 (Th2 marker) was reported to be significantly higher than STAT-4 (Th1 marker) in all the studied groups. The relatively higher expression of STAT-4 in group 2 was obviously far beyond the minimum level of GATA-3, reflecting the expected failure of medical treatment to up-regulate or even modulate the immune system toward the proinflammatory side. It was noticed that the highest level of GATA-3 was reported in group 4, in which the patients were exposed to combined repeated treatment (41.7±4.1), followed by group 3 (39.02±3.1), and then group 2 (38.1±5.2), whereas the lowest expression was in group 1 (32.8 ±8.4). These results indicated that the Th2 type secured the upper hand regardless of the type of treatment and is responsible for imperfect parasite killing, evidenced by recurrence in all our groups, except the group 2, which received medical treatment only.

These data to a certain extent were shocking, denoting the lack of immunomodulatory mechanisms even in repeated combined therapy, keeping in mind the

Table 3 Titer of indirect hemagglutination test in different groups of the current study

Groups IHA test	Group 1 (*N*=6) (*n*)	Group 2 (*N*=15) (*n*)	Group 3 (*N*=10) (*n*)	Group 4 (*N*=14) (*n*)	Total (*N*=45) [*n* (%)]
Negative	**4**	**6**	**6**	**7**	**23 (51.1)**
Total positive	**2**	**9**	**4**	**7**	**22 (48.9)**
1/320	**2**	**5**	**2**	**3**	12 (26.66)
1/640	0	**1**	0	**2**	3 (6.66)
1/1280	0	**1**	**2**	**2**	5 (11.1)
1/2560	0	**2**	0	0	2 (4.44)
χ^2 between negative and total positive	1.333	1.200	0.800	0.000	
P	0.248	0.273	0.371	1.000	

IHA, indirect hemagglutination.

Table 4 Titer of indirect hemagglutination test in relation to the number of hydatid cystic lesions

	IHA test						
		Positive (*n*)					χ^2
Number of lesions	Negative (*n*)	1/320	1/640	1/1280	1/2560	Total [*n* (%)]	*P* value
Single lesion in the liver	22	11	2	0	0	**35** (77.8)	<0.001**
Multiple lesions in the liver	1	1	0	0	0	**2** (4.4)	0.926
Multiple lesions in multiple organs	0	0	1	5	2	**8** (17.8)	<0.001**
Total	23	12	3	5	2	45 (100)	

IHA, indirect hemagglutination. **P-Value < 0.001 was considered statistically highly significant.

known local cellular immune profile in cystic echinococcosis with dominating Th2 type. This is extremely disappointing, especially after statistical validation of such negative correlation, confirming the leading role of anti-inflammatory cellular side over the proinflammatory side.

Supporting our results, Rigano *et al*. [8,15] reported a positive correlation between resistance to pharmacological treatment and Th2 response in terms of IL-4 and IL-10 but not IL-5 and IL-6 production, whereas IFN-γ production was correlated with successful treatment. So, it could be conducted that, Th1 cell activation is possibly related to protective immunity, whereas Th2 cell activation is probably associated with susceptibility to hydatid disease.

Rigano *et al*. [9] reported that the progress of the parasite and the subsequent clinical outcomes depend mainly on the activated subsets of T cell, Th1 or Th2 cells, favoring parasite's survival. Sakamoto and Cebera [16] as well previously studied the immune response during normal growth of unilocular hydatid using immunohistochemical staining and observed that most cases with progressive hydatid cysts had relatively small number of Th1 cells in the pericystic adventitia.

Mourglia-Ettlin *et al*. [17] hypothesized that activated natural killer cells could play an important role in killing of the parasitic stage through the production of Th1 proinflammatory cytokines, which could be partially responsible for the activation of resident macrophages, inducing their known protoscolicidal activity. The immune mechanism effects seem to be relevant to the development of protective immune responses [18,19].

Our findings confirm previous observations and provide further information on the usefulness of induction of Th1-like response for effective elimination of cystic echinococosis. This can be achieved by cytokines such as IFN-γ in treatment of CE either systemic or with PAIR technique.

Consistent with our observations, induction of Th1-like response can mediate protective immunity against certain parasitic helminths. Mountford and Pearlman [20] used a radiation attenuated vaccine with IL-12 as an adjuvant to induce Th1-like response in vaccination of *Schistosoma* spp.

Several studies have shown that IFN-γ production, which is required for immunity against *Taenia crassiceps*, can be induced via both STAT-4 dependent and STAT-4 independent signaling pathways [21,22].

The other surprising results in the current study were related to the serological reports. These results which are more or less equivalent to a previous study indicated

that 58% of the patients were negative, whereas 42% were positive [23]. Moreover, Kaur *et al*. [24], Ortona *et al*. [25], and El-Shazly *et al*. [26] have previously reported lower sensitivity of IHA test than ELISA in diagnosis of CE. Therefore, the negative serological results do not exclude the infection with CE especially in patient with characteristic feature of ultrasound examination and positive history.

On the contrary, a significantly high number of positive results were recorded in patients with multiple lesions in multiple organs. So, if a physician finds high positive titer, he/she should search for infections in other organs as well. Moreover, finding viable scolices in some of the hydatid cyst fluid samples should alert the surgeons to suspect the activity of such serious parasitic disease, and thus, all the needed precautions to prevent secondary seeding of protoscolices before, during, and after surgical interventions should be taken. However, the relatively high negative results in the current study may reflect the efficiency of the parasite to sequestrate its antigen away from the host immune system.Barnes *et al*. [27] reported that there was a relation between the seropositivity and the number, besides the location of the hydatid cysts. They reported higher positivity in patients with multiorgan involvement of approximately 90–100% positivity, and also, in cases having ruptured cysts.

Moreover, false-negative results in immunodiagnostic tests for CE may be seen in patients with small cysts, intact cysts, cysts in extrahepatic locations, heavily calcified cysts, or cyst in privilege sites (brain or eye). Akbulut *et al*. [28] reported that 15 of 40 patients with pancreatic echinococcosis, found in the literature have had negative serological testing for CE. Among 65 patients with CE in Germany, false-negative serological results were reported in 18% by IHA test and in 15% by ELISA [29]. In a study by Akcam *et al*. [30], more than 20% of patients with extrahepatic cysts were reported to be negative by IHA test. Zapatero *et al*. [31] reported that complicated cyst usually had positive serology. They also reported 100% sensitivity of IHA test in patients with ruptured cysts.

Conclusion

For complete cure of these parasitic cystic lesions, the therapeutic strategy should target the parasitic cystic stage at both structural and immunological levels to prevent recurrence of this parasitic cystic lesion after treatment. These local and systemic immunological profiles in the current study may reflect the efficiency of the parasite to sequestrate its antigen away from the host immune system applying variable therapeutic modalities with pronounced overshadowed Th2 response. Consequently, these data may help to develop new immunotherapeutic strategies to successfully eliminate this serious helminthic infection.

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Conflicts of interest

There are no conflicts of interest.

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