Immunohistochemical Analysis of the Immune Cells in Bilharzial Granuloma of the Urinary Bladder

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ABSTRACT

Background: Urinary bilharziasis represent a major health problem in Egypt. It is charactrarized by the formation of localized collection of immune cells i.e. granulomas.

In this investigation, we hypothesized that the evolution of the bilharzial granuloma is associated with recruitment of immune cells of diverse cell lineage. To explore this hypothesis and to fill this existing gap in the literature, we carried out this investigaiton. **Materials and Methods:**Granuloma cell population was immunohistologically examined in thirty cases of cellular bilharzial granulomas using immunoperoxidase staining methods and antibodies targeting antigens for B cells ($CD20^+$), T cells ($CD3^+$), histiocytes ($CD68^+$) and cytotoxic T cells (Granzyme B). **Results:** The mean values of positive cells in the cellular bilharzial granulomas were: 45.5±5.6 for $CD68^+$ cells; 14.8±1.1 for $CD3^+$ T cells; 9.1±1.1; for $CD20^+$ B cells and 1.5±0.8; for Granzyme B^+ T cells with cytotoxic activity.**Conclusions:** The numerical dominance of $CD68^+$cells suggests their critical role in the evolution of these lesions. Our study was the first to report immunophenotypic profile of the bilharzial grnaulomas.

Key words: Granulomas; Bilharziasis; cell population
Running title: Immunophenotyping of bilharzial granuloma

INTRODUCTION

In Egypt, Schistosomiasis (Bilharziasis) is the most common cause of morbidity and mortality with more than 100,000 new cases of urothelial carcinoma occurring per year. The ancient Egyptians knew the disease as "a-a-a" disease and the parasite causing it as "herrwt". *Schistosoma haematobium* is infection exclusively found in Egypt, results in the formation of Bilharzial granulomas, urothelial transformation, and lesions of proliferative cystitis. The latter includes Von Brunn's nests, cystitis cystica and cystitis glandularis. These lesions may undergo dysplastic changes and progress to urothelial carcinomas [1]. Squamous cell carcinoma of the urinary bladder, though uncommon in Europe and the United States, is the most common variety of bladder tumor in countries where urinary bilharziasis prevails .The Bilharzial granulomas is the hallmark of bilharziasis. The evolution of bilharzial granulomas passes by three stages: cellular, fibrocellualr and fibrotic stages

(granulomas). The Schistosomal granuloma consists of different cell types including lymphocytes, macrophages, eosinophils and neutrophils that interact with each other in a highly complex fashion. These interactions as well as the relative proportion of these cells seem to have determining roles in the outcome of *S. Haematobium* [2]. The deposition of the bilharzial ova in the submucosa of the urinary bladder induces cell injury. The outcome of this injury (granulomas and urothelial changes) is determined by the availability of particular immunocytes. The latter include lymphocytes, and histiocytes. To date, the immunophenotypic characterization of the bilharzial granuloma is still unknown. In this investigation,

we hypothesized that the evolution of the bilharzial granuloma is associated with recruitment of immune cells of diverse cell lineage. To explore this hypothesis and to fill this existing gap in the literature, we carried out this investigation. To achieve our goals, we examined thirty urinary bladder specimens with bilharial granulomas. We used immunoperoxiase staining methods and mouse monoclonal antibodies for B, T lymphocytes and macrophages [3]. evolution of the bilharzial granuloma is associated with recruitment of immune cells of diverse cell lineage. To explore this hypothesis and to fill this existing gap in the literature, we carried out this investigation. To achieve our goals, we examined thirty urinary bladder specimens with bilharzial granulomas. We used immunoperoxidase staining methods and mouse monoclonal antibodies for B,T lymphocytes and macrophages[3].

MATERIALS AND METHODS

Tissue specimens:

The specimens were obtained from patients with urinary symptoms suggestive of cystitis. The patients were admitted to the Department of Urology, Sohag Faculty of Medicine, South Valley University). In each case, diagnostic urethrocystoscopy was performed and a representative biopsy was taken from suspicious areas of the urinary bladder. All the specimens were formalin fixed, paraffin embedded and processed at the Departments of Pathology, Histology, and Parasitology, School of Medicine, South Valley University. The routine hematoxylin and eosin stained sections were initially examined and a total of 30 specimens with predominance of cellular bilharzial granulomas were selected for the study.

Immunohistochemical evaluation of the immune cells in the bilharzial granulomas:

Immunostaining was carried out as previously described [4, 5]. Briefly, sections mounted on glass slides were deparaffinized and rehydrated through graded alcohols to water. Endogenous peroxidase activity was blocked with 0.6% H_20_2. Sections were then immersed in the retrieval solution (10-mM sodium citrate buffer, pH 6.0 for CD68, Granzyme B, and CD20) and subjected to heat-induced antigen retr-ieval for 15 minutes. The slides, in plastic Coplin jars containing retrieval solution were microwaved in a microwave set at high (~ 750 watts) for four cycles of five minutes duration each. Antigen retrieval of CD3 was perfor-med using trypsin (enzymatic retrieval; 0.062 gram of trypsin then dissolved in 50 ml distilled water for 3 minutes at 37). Non-spe-cific protein binding was blocked with 10 minutes exposure to 10% normal goat serum. Sections were then incub-ated with mouse monoclonal antibodies for 30 min. at room temperature (Clones PG-M1, L26, and UCHL1 for CD68, CD20 and CD3, respectively, DAKO Corporation, CA 93031 USA). Immunoech, Marseillie, France, provided aniTIA-1 antibody. Nonspecific protein binding was blocked with 10 minutes exposure to 10% normal goat serum. Sections were then incubated overnight with mouse monoclonal antibodies at 4 C. A secondarystaining system (LSAB2, DAKO, USA) was used according to the manufacturer instructions. Sections were next treated with peroxidaselabeled streptavidin for 30 min at room temperature and incubated with 14-diaminobenzidine and 0.06% H_20_2 for 5 min. They were counterstained with hematoxylin, dehydrated in alcohol, cleared in xylene and cover slipped. The slides were independently evaluated by; Drs. Mahmoud R.Hussein, Eman MS Muhammad, Hanaa A. Elhady and Eman E. AbuDief. A summary of the characteristics of the antibodies used was presented in Table1.

Positive controls:
The positive control specimens consisted of lymph nodes with reactive lymphoid hyperplasia (CD68, CD3, CD20, and Granzyme B) [6,7].

Negative controls:
Additional secti-ons, running in parallel but with omis-sion of the primary antibody served as the negative controls.

Evaluation of immunostaining results:
For evaluation of percentage of positive cells of CD3, CD20, CD68, and Granzyme B immunostaining, several rules were followed: 1) corresponding sections stained by hematoxylin and eosin were examined side by side with the immunostained sections; 2) in each case, the entire section was histologically examined by bright field microscope at low power magnification to detect the sites of the antibody positivity and then higher power magnification was used to evaluate the immunostaining; 3) the sections were examined independently by the authors; 4) the positive staining was evaluated by counting 100 cells in the areas of the highest expression; and 5) CD3 and CD20 signals were identified as membranous brown rim around the basophilic nucleus. Signals for CD68, and Granzyme B gave diffuse and granular cytoplasmic [6,7]. A summary of staining features of these antibodies was presented in Table 2.

Statistical Analysis:
Analysis of vari-ance (ANOVA) and differences were considered statistically significant at $p<0.05$.

RESULTS

The wall of the urinary bladder was studded with numerous bilharzial ova; most of them appeared calcified. They were demonstrated within the whole thickness of the wall especially in the submucosa (Fig.1 A & B) Bilharzial granuloma cell population was generally formed of lymphocytes, histiocytes, eosinophils and polymorphs. Further immunohistological analysis revealed that the cellular bilharzial granuloma was formed of mixed inflammatory cell infiltrate. Histiocytes/dendritic cells ($CD68^+$ cells) were predominant in all sites examined and the positive cells were demonstrated as large cells having cytoplasmic positive granules (Figure 2). Lymphocytes ($CD3^+$ cells) appeared as numerous small cells with positive membranous reaction (Figure 3). $CD20^+$ B lymphocytes were demonstrated also with positive membranous reaction and had two different distributions. The first entails the formation of lymphoid follicles (Figure 4). Conversely, the second pattern entailed the presence of few, dispersed cells through the granulomas (Figure 5). Active cytotoxic T cells (Granzyme B^+ cells) with cytoplasmic positive granules appeared as few scattered cells (Figure 6). In the order of decreasing frequency, the percentage of positive cells in the cellular bilharzial granulomas were 45.5±5.6 for CD68+ cells; 14.8±1.1 for CD3+ T cells; 9.1±1.1 for CD20+ B cells and 1.5±0.8 for Granzyme B + T cells with cytotoxic activity. A summary of these findings is shown in Table 3.

DISCUSSION

In this investigation, we hypothesized that the evolution of the bilharzial granuloma is associated with recruitment of immune cells of diverse cell lineage. To explore this hypothesis and to fill this existing gap in the literature, we carried out this investigation. Our data demonstrated three observations: 1) the bilharzial granuloma is formed of admixture of $CD68^+$ histiocytes/dendritic cells, $CD3^+$ T cells and CD20+ B cells and 2) $CD68^+$ cells and $CD3^+$ T cells are the predominant

cell populations in the bilharzial granuloma and 2) a relatively small number of T lymphocytes ($CD3^+$ cells) had cytotoxic activity (Granzyme B^+).

The bilharzial granuloma is formed of admixture of $CD68^+$ cells, $CD3^+$ T cells and CD20+ B cells

In our series, the presence of admixture of immune cells in the bilharzial granulomas suggests that the evolution of these lesions entails the participation of several immunological pathways. The concomitant increase in $CD3^+$ T cell and $CD68^+$ histiocytes in bilharzial granuloma suggests that the activation of T lymphocytes occurs in close collaboration with antigen presenting cells i.e. the macrophages and the dendritic cells. The marked increase in the density of $CD3^+$, and $CD68^+$cells may be due to: 1) increased recruitment of these cells due to the release of soluble factors or adhesion molecules, 2) selective apoptosis and/ or reduced recruitment of other cells (CD20 B cells, eosinophils, and plasma cells), 3) an increase in the load of antigens in these damaged tissues, 4) local proliferation of these cells, 5) variable accessibility of the immune cells to the lesional tissues due to different tissue architecture rather than active recruitment.

$CD68^+$histioicytes and CD3 $^+$T cells were the predominant cell populations in the bilharzial granuloma

The high density of CD68+ cells and CD3 + and in the bilharzial granuloma strongly suggests their critical role in the development of these lesions. The direct association (position) between CD3+T-cells and CD68+cells reflects the close interaction between them in the execution of immune response. In this respect, the presence of CD68+macrophages/dendritic cells is essential for uptake, processing and presentation of the antigenic epitopes associated with the MHC-II to the CD3+ T-cells. The increased density of CD3+ cells may be due to: 1) increased antigenicity of the lesional cells [8a,b]; 2) production of soluble factors (chemokines); 3) increased expression of the adhesion molecules; and 4) increased tissue accessibility for immune cells. Several antigens were reported in the bilharzial granuloma such as egg soluble antigens [8a,b, 9].

Chemokines are small cytokines that induce leukocyte migration into inflammation sites or regulate leukocyte trafficking through lymphoid tissues (Yoshie O et al., 2001). They are secreted by virtually all somatic cells. Based on the spacing of the two cysteines in their N-terminal region, chemokines are grouped into four subfamilies: CXC, CC, C and CX3C chemokines. To date, more than 45 chemokines and 18 functional chemokine receptors (CXCR1-6, CCR1-10, XCR1, and CX3CR1) have been identified in humans. Chemokines mediate their biological effects through binding to cell-surface receptors. After priming, T cells differentiate into two functional subsets. Type 1 T helper cells (Th1) responsible for cell-mediated effector mechanisms. They produce tumor necrosis factor-ß/interferon (IFN)-γ. Type 2 T helper cells (Th2) that produce interleukin (IL)-4, IL-5, IL-6, IL-10, and IL-13 and play a greater role in the regulation of antibody production[10, 11, 12, 13, 14, 15].

Although all the previously mentioned factors are critical for the recruitment process of lymphocytes to the inflammation site of the granuloma, they are not sufficient for the lymphocytes to mediate their action. In this sense, other important participant for T cell action such as the adhesion molecules should operate. These molecules regulate the immune function as adhesion to the endothelial cells, lymphocyte migration into the extravasal tissues at

the site of immune response and T cell interaction with target cell antigens via binding to transitional cells and other resident cells in the lamina propria as well as to the extracellular matrix proteins (collagen, fibronectin and laminin). These adhesion molecules have been classified into various families. They include several categories such as the immunoglobulin subfamily (ICAM1-3, PECAM-1, and VCAM-1), selectins (ELAM-1, LECAM-1, and GMP-140), integrins (LEA-1). These adhesion molecules seem to play a crucial role in the pathogenesis of bilharzial granu-loma [2,16,17,18, 19]. The increased staining intensity for CD68+ cells may reflect their increased activity as well as their contents of lysosomes. The numerical dominance of CD68+ cells in our study may be due to the broad expression of CD68 antigen in other cell types. The later include dendritic cells and fibroblast.The marked increase of CD68+ histiocytes/ dendritic cells in the bilharzial granulomas is in line with previous findings in other granuloma-tous lesions. Therefore, the presence of CD68+ signals in the lamina propria (bilharzial reaction) may reflect the presence of dendritic cells (DCs) and histiocytes [20a,b]. The CD68+ cells act as sentinels for receiving the danger signals. In this respect, the presence of macrophages is essential for uptake, processing and presentation of the antigenic epitope associated with MHC-II to the CD3+ T cells. The DCs seem to undergo a self-recruitment process in bilharziasis. In support, there is marked increase in DCs both in the lamina propria. Interestingly, DCs also express both S100 and CD11c. These markers are typically found in interdigitating DCs of nodal T cell areas. Also mature DCs co-express CCR7, thus indicating the development of a nodal migratory phenotype upon activation. Of note, DCs were also found to contain Granzyme B and thus induce target cell apoptosis [20a,b]. Taken collectively, it is tempting to speculate that the CD68+ cells share to the development of these lesions. Thus it is conceivable that targeting these cells may help abort their development.

A relatively small number of CD3$^+$ T lymphocytes had cytotoxic activity Cytotoxic T-lymphocytes are able to recognize and destroy target cells through the release of cytoplasmic granules such as Granzyme B. The latter are exogenous serine proteinases (enzymes) that can enter the cytosol of the target cell, activate the intracellular cascade of caspases resulting in the killing of the target cells. Monoclonal antibodies, against Granzyme B, used in this study define a subpopulation of cytotoxic T-cells and natural killer (NK) cells that have cytotoxic activity. The presence of Granzyme B $^+$cells in bilharzial granulomas suggests possible pathogenetic roles for Granzyme B in these lesions [20a,b].Granzyme B plays a key role in the cytocidal activity during cytotoxic T lymphocyte mediated apoptotic cell death. In this respect, multiple caspases have been identified as direct substrates for Granzyme B suggesting that the activation of caspases constitutes an important event during cytotoxic T cell induced cell death [20a,b]. Also, Granzyme B can operate via caspase-independent apoptotic pathway. This is achieved by direct cleavage of the 45-kDa unit of DNA fragmentation factor (DFF45) by Granzyme B and thus mediating DNA fragmentation [20a,b]. Despite its ability to enter target cells, Granzyme B is unable to gain access to cellular substrates in the absence of perforin [20a,b].

CD20+ B lymphocytes were scarce in the bilharzial granuloma lesions In our series, the presence of CD20+ B-lymphocytes suggest

that these cells are recruited to the site of inflammation but once activated, they migrate to the bone marrow where they secrete antibodies that rapidly transit in the blood to the site of inflammation. It is still possible that these cells do not play as much of a role in the evolution of these lesions. To summarize, here we report for the first time the immunophenotypic characterization of the bilharzial granulomas. It is conceivable that the evolution of granulomas is associated with skewing of the immune response towards cell -mediated immunity. These results might explain different pathologic diseases associated with developing bladder-wall morbidity with S. haematobium infection. The possible pathogenetic and prognostic ramifications of our findings are open for further investigations

Table 1: The antibodies used in the immunohistochemical evaluation of the bilharzial granuloma lesions

Antibody	Specificity	Retrieval	Dilution	Incubation time
CD68	Histiocytes	Citrate, pH=6	Ready to use	30 min at 37 °C
CD3	Pan T cells	Trypsin	1:100	30 min at 37 °C
CD20	Pan B cells	Citrate	1:200	Overnight at 4°C
Granzyme B	Cytotoxic T-cells	Citrate, pH=6	1:25	30 min at 37 °C

Table 2: Immunohistochemical staining characteristics in the positive control specimens

Antibody	Control tissue (LN)	Distribution of staining
CD68	Sinusoidal	Cytoplasmic, granular, diffuse
CD3	Paracortical	Membranous
CD20	Follicular	Membranous
Granzyme B	Paracortical	Cytoplasmic granular, focal & diffuse

Table 3: The mean count of the inflammatory cells in the bilharzial granuloma

Cell type	**Bilharzial granulomas**
T cells ($CD3^+$)	14.8±1.1
$CD68^+$ cells	45.5±5.6
Cytotoxic T cells (Granzyme B^+**)**	1.5±0.8
B cells ($CD20^+$)	9.1±1.1

Fig. 1,A

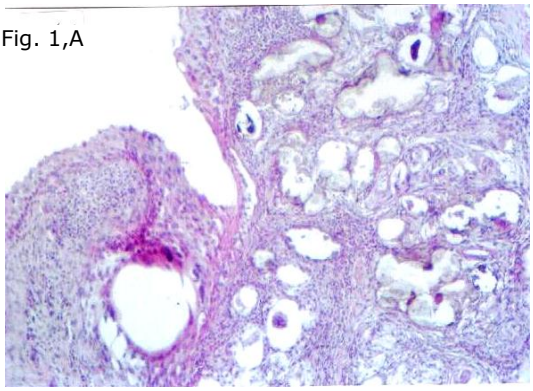

Fig. 1,B

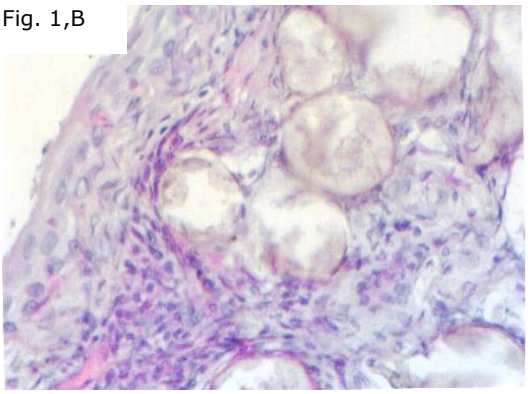

Figure 1: Bilharzial cystitis with numerous bilharzial granulomas in the submucosa (1-A: X200 and 1-B: X 400)

Fig. 2

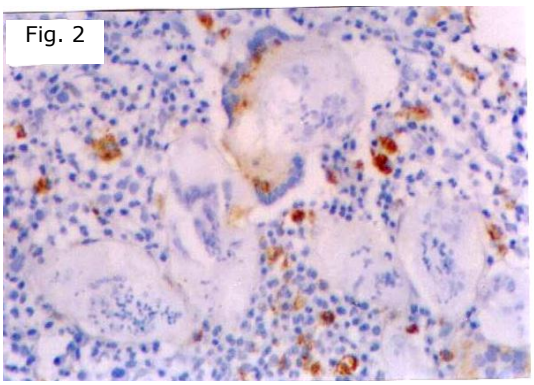

Figure 2: Bilharzial cystitis with numerous CD68 $^{+}$ histiocytes

Fig. 3

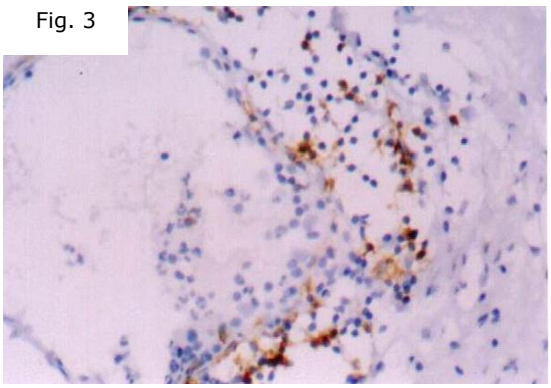

Figure 3: Bilharzial cystitis with CD3 $^{+}$ T cells (X400)

Fig. 4

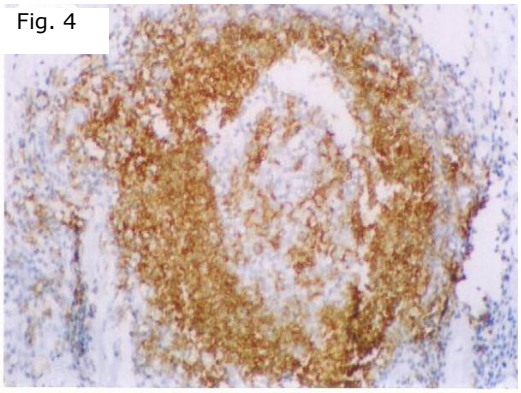

Figure 4
: Bilharzial cystitis with CD20 $^{+}$ B cells forming lymphoid follicles (X 400)

Fig.5

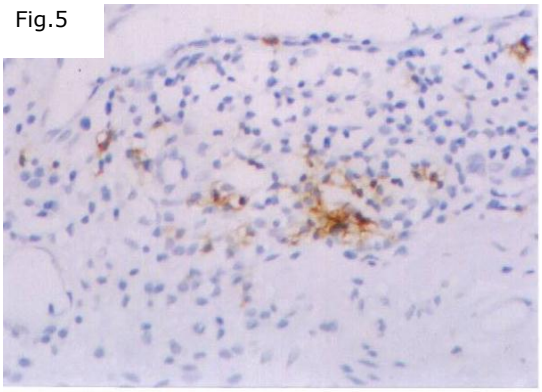

Figure 5: Bilharzial cystitis with CD20 $^{+}$ B cells scattered through the granuloma (X 400)

Fig. 6

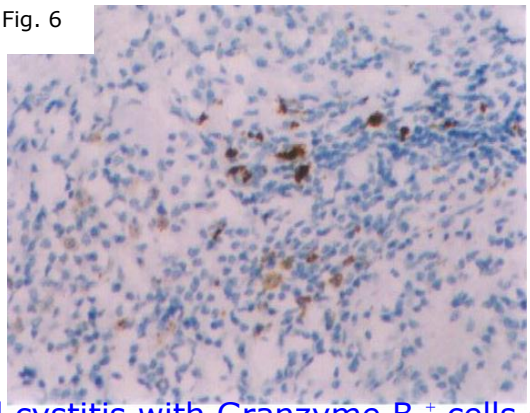

Figure 6: Bilharzial cystitis with Granzyme B $^{+}$ cells cytotoxic T cells (X400)

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الإيجاز العربى

التحليل كيميائي نسيجي مناعي للخلايا الالتهابية في بلهارسيا المثانة البولية

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تمثل الخلفية:
الإصابة بمرض البلهارسيا مشكلة صحية كبرى في مصر, وهو يتميز بتكوين تجمعات محلية من الخلايا المناعية ويعني بذلك الأورام الحبيبة. وقد افترضنا في هذا البحث أنه أثناء تكوين الأورام الحبيبة الناتجة عن البلهارسيا يكون مصحوباً باستقطاب الخلايا المناعية المختلفة. ولكي نتحقق من هذا الافتراض ونبحث في هذه الجزئية الغير مطروقة, حاولنا تصنيف مجموعات هذه الخلايا لهذه الأورام الحبيبة.

أدوات وطرق البحث:
- أخذت عينات من (30) مريض حضروا إلى قسم جراحة المسالك البولية بأعراض مختلفة مثل أعراض التهابات المثانة, دم مصاحب بالبول مع وجود بويضات الشيستوسوما هيماتوبيوم في البول. وقد تم عمل منظار على المثانة البولية مع أخذ عينة من أي إصابات.
- تم فحص العينات نسيجياً بعد عمل صبغات كيمياء مناعة للأنسجة المصابة باستخدام طريقة البيرأوكسيداز المناعية مع استخدام أجسام مضادة للخلايا البائية (CD_{20}^{+}) والخلايا التائية (CD_3^{+}). والخلايا الالتهابية (CD_{68}^{+}) والخلايا التائية السامة (GRB^{+}).

النتائج:
أوضحت النتائج أن نسبة الخلايا الإيجابية للأجسام المضادة المختلفة كالأتي :
45.5 ± 506 للخلايا الالتهابية (CD_{68}^{+}), 14.8 ± 1.1 للخلايا التائية (CD_3^{+}), 9.1 ± 1.1 للخلايا البائية (CD_{20}^{+}), 1.5 ± 0.8 للخلايا التائية السامة (GRB^{+})

الخلاصة:
وجود العدد الأكبر من نوع الخلايا الالتهابية (CD_{68}^{+}) يقترح أن هذه الخلايا لها دور كبير في تكوين الأورام الحبيبة الناتجة عن البلهارسيا. وتعتبر هذه الدراسة الأولى من نوعها التي توضح التميز النمطي للأورام الحبيبة الناتجة عن الإصابة بمرض الشيستوسوما هيماتوبيوم.