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Role of Secretory Excretory Products of *Schistosoma Mansoni* Eggs in Modulating Hepatic Morbidity

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Abstract

In the present study the possible anti-morbidity effect of secretory excretory products (SEP) of Schistoma mansoni eggs (given to mice before infection) was investigated. Multiple small doses of SEP were injected intra-peritoneally into albino mice (100 µg of purified SEP followed 2 weeks later by two booster doses of 50 µg each at weekly intervals). Data revealed reduction in CD4+ cells and increase in CD8+ cells of hepatic granuloma in SEP-immunized infected group, resulting in significant decrease in CD4+/CD8+ ratio, in comparison to infected control group. The serum cytokine level of both TNF--alpha and IFN-gamma were also significantly decreased. Histopathological examination of liver revealed remarkable increase in degenerated ova within hepatic granuloma which decreased in diameter (12%). Significant reduction in worm burden (46%) and tissue egg loads (42.8% and 50% for hepatic and intestinal ova respectively) were observed. Mean while decreased percent of immature stages with increase in percent of dead ova in Oogram pattern was recorded. This work may help in decrease the severity of hepatic morbidity.

Introduction

Schistosomiasis *mansoni* is a tropical helminthic disease characterized by parasite egg-induced granulomatous inflammation and cumulative fibrosis. In a previous study, Boros (1989) told that the small granuloma size could lessen the possibility of tissue damage. At the same time, regulation of the host reaction to *Schistosome* egg antigen (SEA) by induction of specific T-cell unrespon-siveness could be potent prophylactic measure to prevent excessive destruction of host tissues by the granulomatous inflammation characteristic of acute schistosomiasis Stadecker (1992).

Recently, a variety of secretory-excretory products, from different stages of *S.mansoni*, have been identified to induce a level of host-protective immune responses with amelioration of morbidity (Maher *et al*., 2003; El-Ahwany *et al.,* 2006).

Parasitic helminthes secrete or excrete a variety of molecules (SEP) into their mammalian host' in some host- parasite systems, SEP may induce host-protective immune responses and their source of protective antigens has been utilized in successful vaccination model against helminthic infection (Lightowlers and Rickards, 1988). In infection with *S. mansoni*, hepatic granuloma formation is mediated by CD4+ T lymphocytes sensitized to egg antigens (Singh *et al.*, 2004). The systematic identification of immunogenic egg components is important to understand the specific basis of egg-induced immuno-pathology in schistoso-miasis. To gain further insight into the specific immune response against parasite eggs, Asahi *et al.* (2003), characterized several egg antigens with a molecular weight of 25 kDa (Sm-p25). They added that a recombinant Sm-p25 protein elicited significant proliferative and cytokine responses in addition to induced antibody responses. higher level of antibodies were detected in infected sera obtained after parasite oviposition. Doenhoff *et al.(*2003), reported that a 27kDa enzyme secreted by *S. mansoni* eggs is presumed to be responsible for the *Schistosome* egg fibrinolytic activity.

Several promising trials in experim-ental models of protective immunity in schistosomiasis have identified. (Pearce *et*

al., 1988 , Wolwezuk *et al*., 1989 , Botros *et al*., 1995, Hassanein *et al*., 1997 and Hassanein *et al* 1999). At the sametime ,an approach aims to identify proteins from *Schistosoma mansoni* that are capable of stimulating protective Th1 cell–mediated immune responses was considered (Mountford and Harrop 1998).

The present study was designed to investigate the response to injection of purified secretory excretory products (SEP) of *S. mansoni* eggs prior to infection with *S. mansoni* cercariae as an experimental trial to decreasing or modulating severe hepatic morbidity.

Materials and Methods

A- Animals

1- The animals were supplied and housed throughout the study in the Schistosome Biological Supply Center (SBSP), at Theodor Bilharz Research Institute (TBRI), an institution responsible for animal ethics.

Laboratory bred male albino mice of CDI strain, weighing 18-20 grams were used. Experimental animals were kept in air conditioned rooms at 25 °C, receiving food containing 24 % protein.

2- *Schistosoma mansoni* cercariae were obtained from (SBSP) at (TBRI) and infection was performed by subcutaneous injection of 100 *S. mansoni* cercariae to each mouse (Liang *et al.,* 1987).

3- Schistosome eggs were isolated according to Von Lichtenberg, (1962) from the liver of 8 weeks previously infected mice received 120 cercariae of *S. mansoni*. Eggs were suspended in normal saline (0.9/L) and put in culture medium containing RPMI-1640 (Sigma Chemical Co, St. Louis, USA) supplemented with antibiotics (300 IU/ml penicillin, 300 ug/ml streptomycin and 160 ug /ml gentamycin).All the steps of cultivation were done under sterile conditions .About 6000 eggs/5ml of culture medium were incubated for 1-3 days at 37 °C in 5% CO2 incubator, pH was adjusted to 7.5 by adding 0.1 N NaOH.

The total culture medium containing secretory / excretory products of eggs was concentrated and purified using ammonium sulfate precipitation method according to (Jaton *et al*., 1979). Protein content was measured using Bio-Rad method, sterilized, fractionated and stored at –70 °C until used. The antigenicity of SEP was tested using ELISA test (Bradford, 1976).

B- Experimental design:

Experimental animals were divided into 3 groups, each groups of 15 individual.

Group (1): SEP immunized group:

Each mouse was injected intraperitoneally with 100 µg/ml of secretory-excretory products (SEP) emulsified with complete Freunds' adjuvant. Animals were boosted two weeks later with 50ug/ml of SEP emulsified with incomplete Freund's adjuvant and boosted again one week apart. the mice were sacrificed 8 weeks following last dose of SEP.

Group II: - Each mouse was injected intraperitoneally with 100 µg/ml of SEP of egg antigen emulsified with complete Freunds' adjuvant. Then, the animals were boosted after two weeks with 50µg of SEP emulsified with incomplete Freund's adjuvant. Again, after one week ,the animals were boosted with 50µg of SEP emulsified with incomplete Freund's adjuvant. Mice were infected with 100 *S. mansoni* cecariae, one week after last immunization by sub-cutaneous injection and the mice were sacrificed 8 weeks post –infection.

Group III: *Shistosoma mansoni* infected control group, animals were infected sub-cutaneously with 100 cercariae and sacrificed 8 weeks later.

C- Parasitological parameters:

1- Worm burden: Perfusion of adult worms from the liver and porto-mesenteric system was performed 8 weeks after infection according to Duvall and Dewitt (1967).

2- Tissue egg load: The number of eggs per gram tissue (liver and intestine) was studied according to the procedure by Cheever (1968).

3- Oogram pattern: The percentages of immature, mature and dead ova in the small intestines were computed from a

total of 100 eggs per intestinal segment and classified according to the categories previously defined by Pellegrino *et al*. (1963).

D- Histopathological Study:

Liver specimens were fixed in 10% buffered formalin and processed to prepare paraffin blocks. Paraffin sections 4 µm thick were stained with haematoxylin–eosin and trichrome stains. The size of *Schistosoma* granulomas at x 10 was measured per section using ocular micrometer. Only lobular granulomas containing egg in the center and confluent were measured. The mean diameter of granuloma per group was calculated according to Von Lichtenberg (1962).

E- Immunological Parameters:

1- Enumeration of T-cell subsets:

Fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies for L3T4+ and Lyt2+ T-lymphocytes were used to determine the number of intralesional T-cells in formalin fixed tissues, embedded in paraffin using a modified method of Swoveland and Ghonson (1979). Sections were treated according to histological procedures to remove paraffin and taken through several washes in graded alchohol to rehydrate the tissues. Slides were washed in 0.05 M Tris buffer (pH 7.4), and incubated for 10 min in a humidified chamber after immersion in a solution of freshly prepared 1% trypsin. Slides were washed in 0.05 M Tris buffer and distilled water. FITC-labeled L3T4+ (CD4+) and Lyt2+ (CD8+) antibodies diluted 1:1 in Tris buffer, pH 7.6, were used to stain two slides per mouse. Slides were incubated overnight with the monoclonal antibodies in a humidified chamber at 4°C, washed in Tris buffer and mounted with entellan (Sigma) to enhance fluorescence prior to quantification. T-cells of each type were counted in two 50 mm wide bands perpendicular to each other in a single granuloma containing a single centrally positioned egg. The mean count per 50 mm band was obtained by dividing the sum of the two bands by two. A disaster Reichertjung fluorescent research microscope (Cambridge Instruments) objective 20X was used.

2- Detection of serum TNF-alpha and IFN-gamma by sandwich ELISA:

Serum murine TNF- and IFN-γ levels were measured with an ELISA kit (Quantikine M, R&D systems, Minneapolis, MN, USA). The detection limit of the assay was consistently 20 pg/ml. The concentration was calculated from the standard curve that was performed in the same assay.

Statistical analysis:

Comparison was performed between the treated groups and untreated control. The percentage change between each two groups to be compared was assessed using the formula: Differences between the mean scores of any of the two groups to be compared were tested for significance, using an unpaired 2-tailed Student's t-test. The data were considered significant if *p* values were less than 0.05.

Results

The results in table (1) are showing significant reduction (46%) in the mean number of *S. mansoni* adult worms in the group of infected mice immunized with purified eggs antigen compared to the infected controls ($P< 0.01$). Moreover, significant reduction in the mean number of ova / gram tissue (liver and intestine) was detected in the group immunized with purified egg antigen compared to infected controls ($P< 0.01$). The percent of immature ova was less in the immunized group than the infected one while the percent of dead ova was higher (78.54) in the immunized group than the infected control ($P<0.01$).

- Immunological Parameters: -

a- Enumeration of T cell phenotypes in hepatic granuloma:

In the SEP-Immunized infected group, the L3T4+ (CD4+) T cells significantly decreased ($p<0.001$) compared to the infected control group. However, Lyt2+ (CD8+) T cells were significantly increased ($p<0.001$) in the SEP-immunized group compared to infected control group. Also, there was a significant decrease in the

ratio of (CD4+/CD8+) T-cells in the immunized infected group.

b- Detection of serum TNF-alpha and IFN-gamma by sandwich ELISA:

In the SEP-immunized infected group, the serum cytokine levels of both TNF-α and IFN-γ were significantly decreased ($p<0.05$) compared to the infected control groups.

Histopathological Parameters:

The mean granuloma diameter in infected control group was 390.34±0.49 while in SEP-immunized infected group, it was 340.22±0.22, and the reduction in granuloma diameter was 12.84%.

Photomicro graphs 1,2,3 showing control infected, immunized and uninfected immunized groups.

Table-1: Different parasitological parameters detected 8 weeks post-infection in animal groups.

Animal groups	Worm Load	Hepatic ova	Intestinal Ova	Oogram Immature	pattern mature	Dead stage
Control infected group	32.0±0.31	8570.0±90.0	18090±88.55	45.2±0.22	44.36±0.60	10.44±0.1
Immunized group (SEP)	17.25±0.60*	4900.0±28.2*	8900.0±60.0*	37.1±0.33*	44.7±0.53	18.2±1.0*
%Reduction	46%	42.8%	50.8%	Reduction 17.92%	----------	Increased 78.54%

* Significant difference from infected control ($P < 0.01$).

Table 2: Number of granuloma T cell phenotypes per 50 µm band (Mean ± SEM) in the different studied groups.

Animal groups (n=10)	CD4+ Mean±SEM	CD8+ Mean±SEM	CD4+/CD8+ Mean±SEM
Infected Control Group	25.3±2.1	9.2±1.4	2.75±2.1
SEP-Immunized infected Group	14.2±+1.9*	18.9±1.9*	0.75±1.8*

* $p<0.001$ significant vs infected control group.

Table 3: Serum TNF-α and IFN-γ levels against SEP of eggs in different studied groups.

Animal Groups (n=10)	TNF-α Mean Pg/ml±SEM	IFN-γ Mean Pg/ml±SEM
Uninfected Control	170±4.09	277.7±4.48
Immunized Group	275±4.2	510±5.4
Infected Control Group	565±25.3	1105±35.2
SEP-Immunized Group	315±32.1*	950±49.2*

*$p<0.05$ significant vs infected control groups.

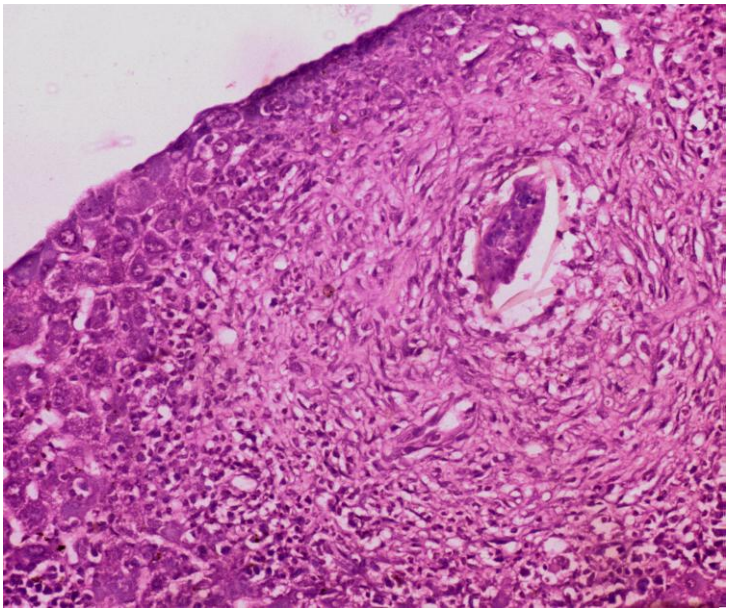

Fig (1) Photomicrograph showing Control infected group ,fibrocellular Granuloma intact mircidia. (H& E X200) .

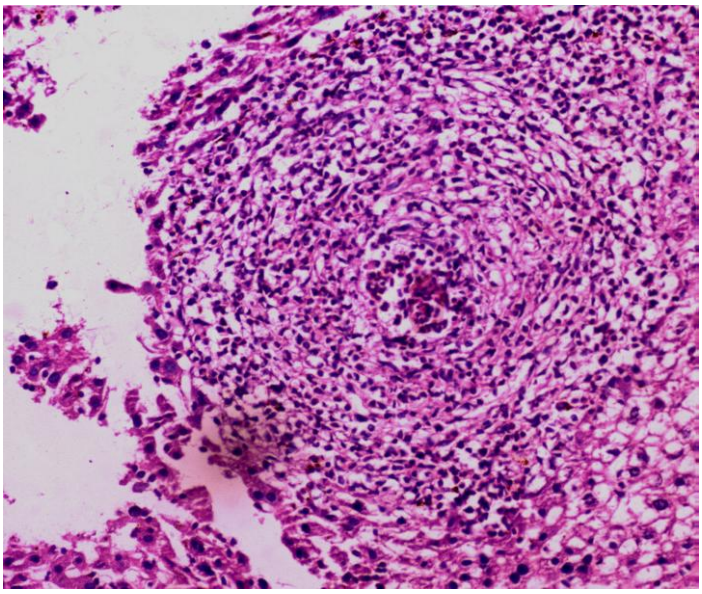

Fig (2) Photomicrograph showing Infected immunized group, fibrocellular granuloma surrounding degenerated ova with cellular inflteration lymphocytes and macrophages towards miracidia . (H& E X200)

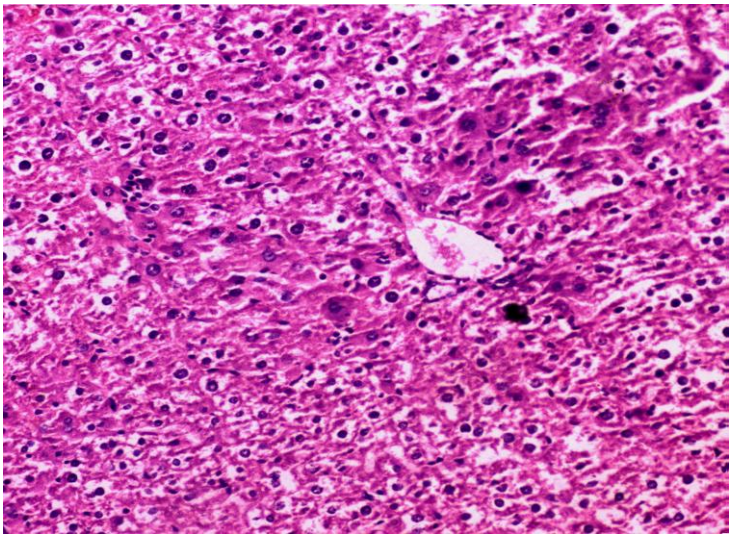

Fig (3) Photomicrograph showing Uninfected immunized group showing hydropic degeneration. (H & E x 200)

Discussion

The morbidity in schistosome infection is primarily due to fibrosis resulting in large part from healing of the inflammatory granulomatous focal damage around deposited eggs. This granulomatous reaction is most vigorous at the acute stage of infection (8-10 weeks post-infection), when T helper lymphocytes produce high levels of inflammatory lymphokines (Stadecker, 1992) and induces activation of granuloma macrophages (El-Ahwany *et al.*, 2000). Some investigators indicated that, early in infection, probably even prior to egg production, schistosomes induce an immunologic environment that is highly conductive to the establishment of strong immunoregulatory mechanisms.

A lot of trials have been conducted to find a possible way for amelioration of the disease severity or morbidity by inhibition of host reaction around *S.mansoni* eggs. Schistosomal granuloma is mediated by class II MHC CD4+ T helper (Th) lymphocytes and is specifically directed to egg antigens (Zouain *et al.,* 2002). The magnitude of schistosome granuloma depends upon the type of activated Th cell population in response to the quality and quantity of inducing antigen (Stadecker *et al.,* 2001; Hanallah *et al.,* 2003).

In the murine model, cells displaying different functions can be partially differentiated by cell surface phenotype markers such as CD4+ and CD8+ (Smith *et al.,* 2004). In this work, phenotypic T cell subsets showed decrease in CD4+/CD8+ T cell ratio, in the SEP-immunized infected group compared to the corresponding infected control group. This finding was mainly due to an increase in the percentage of CD8+ subset in the SEP-immunized infected group. A shift in CD4+/CD8+ T cell ratio in favor of CD8+ lymphocytes in the circulation of chronically *S. mansoni* infected patients was reported by other investigators (Lukacs and Boros, 1993). The differences in T cell subset profile within the hepatic granuloma might be reflected by the functional activity of T cells. Thus, the reduction in granuloma diameter was concurrently associated with reduction in CD4+ cells and increase in CD8+ cells in SEP-immunized infected group. Although the decrease in granuloma diameter was not high, yet a marked increase in percent of degenerated ova was observed in SEP-treated infected group. In a study by Hassanein *et al. (*1997), they attributed hypo-responsiveness and decreased granuloma diameter to T-cell anergy following intravenous injection of SEA.

In this study, administration of SEP prior to infection resulted in decreased worm load, hepatic and intestinal ova together with change in Oogram pattern. This could be due to enhancement of

immune response. Similarly, immunization with SEP of lung stage schistosomula prior to infection induced protective effect, manifested by reduction in parasitological parameters, increased levels of specific immunoglobulins as well as raised hepatic m-RNA expression of TNF-alpha and TGF-beta (Maher *et al.*, 2003). In the present work, at 8 weeks post infection the serum levels of IFN-γ and TNF-α were significantly reduced compared to the infected controls, showing the most pronounced reduction of granuloma diameter. The cytokines play an important role in regulation of the inflammatory granulomatous response in schistosomiasis (Garraud and Nutman, 1996). IFN-γ and TNF-α appears to play an important role in the generation and maintenance of egg-induced granuloma (Chensue *et al.*, 1993 and Hoffman *et al.*, 1998). The diminished focal and systemic production of IFN-γ and TNF-α may be implicated in the downmodulation of the granulomatous response (Joseph and Boros, 1993 and Hassanein *et al.*, 1999). In a studt by Singh *et al.*, (2004), they reported that the decrease of the gene expression of TNF alpha and TGF-beta few months following successful treatment *of S. mansoni* infected mice, was correlated with resorption of liver fibrous tissue.

The development of hepatic fibrosis and portal hypertension is the principal cause of morbidity and mortality in schisto-somiasis *mansoni*. Nevertheless, relatively little is known about the mechanisms that lead to excessive collagen deposition during infection with *Schistosoma mansoni*.

Our findings revealed that immuni-zation with SEP of *Schistosoma mansoni* eggs induced some sort of protective effect manifested by, reduction in worm burden, egg load and granuloma size 8 weeks post-infection; the miracidia inside granulomas were mostly degenerated. This was accompanied by decreased ratio of T cell subsets (CD4+/CD8+) and decreased serum levels of both IFN-γ and TNF-α.

Further achievement trials concerned with immunization protocols against schistosomiasis are recommended helpfully to reach more promising results.

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دور الناتج الخارج من بيض البلهارسيا فى تقليل مضاعفات الكبد

إبراهيم ربيع - زينب فهمي - إيمان الأهوانى - هدى صبري
قسم الطفيليات والمناعة بمعهد تيودر بلهارس (الجيزة – القاهرة)

تستهد ف هذه الدراسة الى دارسة تأثير الناتج الخارج من بيض البلهارسيا فى تقليل مضاعفات الكبدوذلك باستخدام كميات قليله من الناتج الخارج من بيض البلهارسيا عن طريق الحقن تحت الغشاء البرتونى فى حيوانات التجارب بجرعة 100 ميكرون وبعد أسبوعين أخذ جرعة أخرى بقدار 50 ميكرون و تكرر هذه الجرعة مرة أخرى بعد أسبوع. ويتم العدوى 100 سركاريا ببلهارسيا المستقيم و يتم التشريح بعد 45 يوم.

وقد استخدمت ثلاثة مجموعات من ذكور الفيرأن البيضاء عمر 8 أسابيع وزن 18- 20-جم ,كل مجموعة 15 فأر
المجموعة الأولى: تم عدوى الفيرأن100 سركاريا ببلهارسيا المستقيم.
المجموعة الثانية:مجموعة مصابة وتم إعطائها الناتج الخارج من بيض البلهارسيا.
المجموعة الثالثة: مجموعة غير مصابة وتم إعطائها الناتج الخارج من بيض البلهارسيا.

وقد أوضحت التجارب نقص فى CD4 & CD8 فى المجموعة التى رفع منعتها نقص فى نسبهCD4& CD8 . ونقص فى TNF-alpha and IFN gamma . بالمقارنة بالمجموعة المصابة فقط.

وأدى أيضا الى نقص فى عدد ديدان البلهارسيا المعويه بنسة 46 % وزيادة فى عدد البويضات الميتةو نقص فى عدد البويضات فى أنسجة الكبد والأمعاء . و نقص فى حجم الورم الحبيى بنسة 12 %.

وتوصى هذه النتائج على إمكانية استخدام إعطائها الناتج الخارج من بيض البلهارسيا وذلك لنقص مضاعفات الكبد بعد الإصابة بالبلهارسيا المعوية .