Immunomodulation of Hepatic Morbidity in Murine *Schistosomiasis mansoni* Using Fatty Acid Binding Protein

Ibrahim Rabia[1]; Eman El-Ahwany[2]; Wafaa El-Komy [1] and Faten Nagy[2]

[1]Parasitology [2]Immunology Department,Theodor Bilharz Research Institute,Giza, Egypt.
dr_mona_zaki@yahoo.co.uk

Abstract: Hepatic fibrosis and portal hypertension are responsible for morbidity in schistosomiasis *mansoni*.The objective of this study was to evaluate the possible anti-morbidity effect of fatty acid binding protein (FABP) of *Schistoma mansoni* when given to mice before infection. Multiple small doses of FABP were injected intra-peritoneally into experimental animals (100 µg of purified FABP followed 2 weeks later by two booster doses of 50 µg each at weekly intervals) and the experimental design included 3 groups of 15 mice each; the first group received FABP (immunized group), the second group was injected with the 3 doses of FABP one week prior to infection with 100 *S.mansoni* cercariae (immunized-infected group) and the third group served as infected control. Data revealed reduction in CD4+ cells and increase in CD8+ cells of hepatic granuloma in FABP-immunized infected group, resulting in significant decrease in CD4+/CD8+ ratio, in comparison to infected control group; the serum cytokine levels of both TNF-alpha and IFN-gamma were also significantly decreased. Histopathological examination of liver revealed remarkable increase in percent of degenerated ova within hepatic granuloma which decreased in diameter (12%). In this study, significant reductions in worm burden (46%) and tissue egg loads (42.8% and 50% for hepatic and intestinal ova respectively) were observed in addition to decreased percent of immature stages with increase in percent of dead ova in Oogram pattern .This work could present a trial contributing to shaping the severity of hepatic morbidity. [Journal of American Science 2010;6(7):170-176]. (ISSN: 1545-1003).

Key words: Schistosomiasis. – fatty acid binding protein – immunization. – histopathology.

1. Introduction

In schistosomiasis, the manifestations of immunopathology are mainly acute granulomatous inflammation and late fibrosis. The mechanisms driving fibrogenesis are distinct from those regulating inflammation (Wynn, 2008) .

Fibrosis is the end result of chronic inflammatory reactions induced by a variety of stimuli including infections, allergic responses, tissue injury and others. The key to fibrosis lies in the control of extracellular matrix (ECM) deposition in response to cytokines (Wang et al., 2009). In a previous study, Boros (1989) presumed that the diminished granulomatous response could be advantageous to host in a way that, if granuloma is retained in smaller size and less activated cell populations it could lessen the possibility of tissue damage. Stadecker (1992) added that, regulation of the host reaction to *Schistosome* egg antigen (SEA) by induction of specific T-cell unresponsiveness could be potent prophylactic measure to prevent excessive destruction of host tissues by the granulomatous inflammation characteristic of acute schistosomiasis.

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).

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 schistosomiasis. 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, and the highest levels of antibody were detected in infection 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.

The intracellular fatty acid-binding protein (FABP) Sm14 is essential for schistosomes in the

uptake, transport and compartmentalization of host derived fatty acids (*Esteves et al., 1997*). Lipids are necessary for schistosomes to synthesize and maintain their complex membrane systems; fatty acids act as precursor for lipid and phospholipids synthesis and have significant contribution in the parasite life cycle including membrane formation, functioning as lipid anchors for proteins, sexual maturation, and regulation of egg production (*Furlong, 1991*). These parasites lack the oxygen dependent pathways required for the synthesis of sterols and fatty acids, thus they must acquire host lipids. Sm14 was localized in *S. mansoni* by anti-Sm14 specific antibodies in the basal lamella of the gut and underlying the tegument (*Brito et al., 2002*). Experimental models of protective immunity in schistosomiasis have identified several promising trials (Pearce *et al.,* 1988 and Wolwezuk *et al.,* 1989) by using intraperitoneal injection of different doses of SEA (Botros et al., 1995, Hassanein *et al.,* 1997 and Hassanein *et al* 1999). Mountford and Harrop (1998) focused attention on an approach that aims to identify proteins from *Schistosoma mansoni* that are capable of stimulating protective Th1 cell–mediated immune responses.

The present study was designed to investigate different parasitological and immunological parameters in response to injection of purified fatty acid binding protein (FABP) of *S. mansoni* into mice prior to infection with *S. mansoni* cercariae as an experimental trial to decreasing or modulating severe hepatic morbidity.

2. Material and Methods

1- Animals

Laboratory bred male albino mice of *Mus musculus* strain, weighing 18-20 grams were used. Experimental animals were kept for 8 w responsible for animal ethics.

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).

2- Preparation of fatty acid binding protein.

Adult *S. mansoni* worms were homogenized in 2 vol. of 20 mM Tris-HCL buffer (BDH Chemicals, England) containing 5 mM Phenylmethylsulfonyl fluoride (PMSF) as a protease inhibitor (Sigma–Aldrich, Louis, USA) at 20.000 rpm using IKA T20 homogenizer (IKA, Staufen, Germany). The homogenate was centrifuged at 30.000 rpm for 30 min. The entire process of homogenization and centrifugation was performed at 4˚C. The supernatant fractions were decanted and assayed for protein content and stored at -20˚C until used as crude extracts.

Thirty milligrams of crude extracts were loaded on a G-200 Sephadex column, and then eluted with 0.1 M PBS, pH 7.4. Four milliliters of fractions were collected and their absorbances were measured at 280 nm. Fractions from peak II and III were pooled and dialyzed against TBS (Tris-Buffered Saline) at 4°C and their protein contents were measured and stored at –20°C until used (Timanova *et al.,* 1999).

3- Experimental design:

The mice were divided into 3 groups of 15 animals each; all groups were sacrificed 8 weeks following the beginning of experiment:

Group (1): FABP immunized group:

Each mouse was injected intra-peritoneally with 100 µg/ml of FABP antigen emulsified with complete Freunds' adjuvant. Then, the animals were boosted two weeks later with 50ug/ml of FABP emulsified with incomplete Freund's adjuvant and boosted again one week apart. the mice were sacrificed 8 weeks following last dose of FABP.

Group II: - Each mouse was injected intraperitoneally with 100 µg/ml of FABP antigen emulsified with complete Freunds' adjuvant. Then, the animals were boosted two times at three weeks intervals with 50µg of FABP emulsified with incomplete Freund's adjuvant. Mice were infected with 100 *S. mansoni* cecariae, one week after last immunization by sub-cutaneous injection 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.

4- Parasitological parameters:

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

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

c- 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).

5- Histopathological Study:

Granuloma measurement:

Livers of mice were fixed in 10% buffered formalin, processed into paraffin blocks, serially cut at 4µm thickness, and stained with hematoxylin and eosin. Hepatic granuloma measurements were done according to Von Lichtenberg (1962) using an ocular

micrometer for those containing a central ovum only. The percent reduction in granuloma diameter relative to the infected controls was calculated as follows:

$$\% \text{ reduction of granuloma diameter} = \frac{\text{Mean diameter of control s - mean diameter of test s}}{\text{Mean diameter of control group}} \times 100$$

Counting was carried out in five successive microscopic fields (10x10) in serial tissue sections of more than 250 µm apart.

6- Immunological Parameters:

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

Serum murine TNF-α 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.

b- 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.

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.

3. Results

The results in (Table 1) are showing significant reduction (49.3%) in the mean number of *S. mansoni* adult worms in the group of infected mice immunized with purified FABP antigen compared to the infected controls (P< 0.001). Moreover, significant reduction in the mean number of ova / gram tissue (liver and intestine) was detected in the group immunized with purified FABP 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 (16.2) in the immunized group than the infected control (P<0.05).

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

Animal groups	**Worm Load**	**Hepatic Ova**	**Intestinal Ova**	**Oogram pattern**		
				Immature stage	**Mature stage**	**Dead ova**
Control infected group	30.0±0.28	8600.0±87.0	17909±81.0	63.2±0.31	34.2±11	2.6±0.1
FABP-Immunized group	15.21±0.33*	5100.0±29.2*	8610.0±57.0*	32.1±0.24*	51.7±0.22	16.2±1.0*
% Reduction	49.3 %	41.7%	52.9%	Reduced 49.2%	Increased 23.7 %	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
Uninfected Control	10.4 ±2.3	12.3 ±2.0	1.3 ±1.3
Immunized Group	12.6±1.1	14.4 ±2.2	1.4 ±2.0
Infected Control Group	25.3±2.1	9.2±1.4	2.75±2.1
FABP-Immunized infected Group	14.2±1.9*	18.9±1.9*	0.75±1.8*

* p<0.001 significant vs infected control group.

Immunological Parameters: -

a- Enumeration of T cell phenotypes in hepatic granuloma:

In the FABP-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 FABP -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 FABP -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.

Table 3: Serum TNF-α and IFN-γ levels against FABP antigen in different studied groups.

Animal Groups (n=10)	TNF-α Mean Pg/ml±SEM	IFN-γ Mean Pg/ml±SEM
Uninfected Control	166±5.11	299.8±4.55
Immunized Group	292±5.3	613±10.1
Infected Control Group	612±19.6	1312±31.1
FABP-Immunized Group	300±24.3*	989±53.7*

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

Histopathological Parameters:

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

4. 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 or would be acting as assort of primary infection that somewhat hinders the challenge one. 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 schistosomiasis *mansoni.* Nevertheless, relatively little is known about the mechanisms that lead to excessive collagen deposition during infection with *Schistosoma mansoni*.

Our findings revealed that immunization with FABP of *Schistosoma mansoni* antigen 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 and are ongoing to helpfully show more promise.

5. References:

1. Asahi H, Oke T, Lopes da Rosa J, Williams DL, Stadecker MJ. (2003): sm-p25: A new egg component stimulating T-cell and antibody responses in murine schistosome infection. *Am J Trop Med Hyg, 52nd Annual meeting, suppl.* 69(3): 346.
2. Boros, D.L. (1989): Immunopathology of *Schistosoma mansoni* infection. *Clin. Microbiol. Rev*., 2:250-269.
3. Botros S., Hassanein H., Hassan S., Akl M., Sakr S., Shaker Z., Hafez G., Ghorab N. and Dean D. (1995). Immunoregulatory potential of exogenous *S. mansoni* soluble egg antigen in a model of experimental in Schistosomiasis 1- regulation of granuloma formation in *vitro*. *Int.J. Immunopharmac*, 12:707-718.
4. Brito CFA, Oliveira GC, Oliveira SC, Street M, Riengrojpitak S, Wilson RA, Simpson AJG, Oliveira RC (2002). Sm 14 gene expression in different stages of the *Schistosoma mansoni* life cycle and immunolocalization of the Sm 14 protein within the adult worm. *Braz J Med Biol Res 35*: 377-381.
5. Bradford M.M. (1976). A rapid sensitive method for the quantization of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem* .72: 248-254.
6. Cheever A.W. (1968). Condition affecting the accuracy of potassium hydroxide digestion technique for counting *S.mansoni* eggs in tissues. *Bull wld Hlth Org*, 39: 328-331.
7. Chensue S.W., Warmington K.S., Hershey S.D., Terebuh P.D., Othman M. and Kunkle L. (1993). Evolving T cell-response in

murine schistosomiasis. *J Immunol*, 151: 1391-400.
8. Doenhoff M, Stanley RG, Curtis RHC and Pryce D (2003).A fibrinolytic enzyme in *Schistosoma mansoni* eggs. *Am J Trop Med Hyg, 52nd Annual meeting, suppl.* 69(3): 347.
9. Duvall R.H. and Dewitt W.B. (1967). An improved perfusion technique for recovering adult *Schistosoma* from laboratory animals. *Am. J. Trop. Med. Hyg.* 16: 483- 4.
10. El- Ahwany E.G, Hanallah SB, Zada S,El-Ghorab N M,Badir B,Badawy A,Sharmy R,and Hassanein HI (2000). Immunolocalization of macrophage adhesion molecule-1 and macrophage inflammatory protein-1 in schistosomal soluble egg-induced granulomatous hyporesponsiveness. *Int J Parasitol*, 30:837-84.
11. El- Ahwany E.G., Nosseir M.M. and Aly I.R. (2006). Immunompulmonary and hepatic granulomatous response in mice immunized with purified lung- stage schistosomulae antigen. *J. Egypt. Soc. Parasitol.* 36(1): 335-350.
12. Esteves A, Joseph L, Paulinos M, Ehrlich R (1997). Remarks on the phylogeny and structure of fatty acid binding proteins from parasitic platyhelminths. *Int J Parasitol 27*: 1013-1023.
13. Furlong ST. (1991). Unique roles for lipids in *Schistosoma mansoni. Parasitology today*, 2: 59 - 62
14. Garraud O., Nutman T.B. (1996). The role of cytokines in human B –cell differentiation into immunoglobulin –secreting cells. *Bull. Pasteur*. 94: 285-309.
15. Hanallah SB,El-Lakkany N,Mahmoud S,Mousa m and Botros S (2003).Altered immunoglobulin isotype profile and anti-immature worm surface immunoglobulins in mice harboring a praziquantel resistant *Schistosoma mansoni* isolate.*APMIS*,111:1125-1132.
16. Hassanein H., Akl. M., Shaker Z., El-Baz H., Abo- Elala I., Rabia I., Sharmy R. and Doughty B. (1997). Induction of hepatic egg granuloma hypo responsiveness in murine schistosomtasis *mansoni* by intravenous injection of small doses of soluble egg antigen. *APMIS.* 105:77-83.
17. Hassanein, H., Kamel, M., Badawy , A., El-Gorab, N., Abdeen , H., Zada, S., El-Ahwany , E. and Doughty, B. (1999). Anti-miracidial effect of recombinant glutathione S- transferase 26 and soluble egg antigen on immune response in murine schistosomtasis *mansoni. AMPIS*, 107: 723-736.
18. Hoffmann K.F., Caspar P, Cheever A.W. and Wynn T.A. (1998). IFN-gamma, Il-2 and TNF-alpha are required to maintain reduced liver pathology in mice vaccinated with *Shistosoma mansoni* eggs and IL-2. *J Immunol*, 15: 4201-4210.
19. Jaton J., Brandt D.C. and Vassalli P. (1979). The isolation and characterization of immunoglobulins, antibodies and their constituent polypeptide chain. *J. Immunol. Meth.* 2: 43- 67.
20. Joseph A.L., and Boros D.L. (1993). Tumor necrosis factor plays a role in *Schistosoma mansoni* egg induced granulomatous inflammation. *J. Immunol.*, 151: 5461-5471.
21. Maher, K. M., El- Shennawy, A. M.; Ibrahim, R. B.; Aly, E. and Mahmoud, S. (2003): Protective and antipatholy potential of immunization with *Schistosoma mansoni*-secretory/ excretory product. *New Egypt J Med* , 28: 173-182.
22. Mountfourd,A.P and Harrop, R. (1998). Vaccination against schistosomtasis: The case for lung –stage antigens. *Parasitol. Today*, 14: 109- 114.
23. Liang U.S., Bruce J.I. and Boyd D.A. (1987). Laboratory cultivation of *Schistosoma* vector snails and maintenance of schistosome life cycle. Proceedings of the 1st Sino-*Amercian Symposium*, 1: 34-48.
24. Lightowlers, M.W. and Rickard, M D. (1988). Excretory secretory products of helminthes parasites: Effects on the host immune responses. *Parasitol.* 96: 5123- 66.
25. Pearce E.J., James S.L., Hieny S., Lanar D.E. and Sher A. (1988). Induction of protective immunity against S. mansoni by vaccination with *S. mansoni* paramyosin (SM97), a non-surface parasite antigen. *Proceedings of the National Academy of Science, USA.* 74:5463-5467.
26. Pellegrino J., Oliveira C.A., Faria J. and Cumha A.S. (1962). New approach to the screening of drugs in experimental *Schistosoma mansoni* in mice. *Am. J. Trop. Med. Hyg.*, 11: 201-215.
27. Singh KP, Gerard HC, Hudson AP, Boros DL. (2004): Expression of matrix metalloproteinases and their inhibitors during the resorption of schistosome egg-induced fibrosis in praziquantel-treated mice. *Immunology*. 111(30:343-52.

28. Smith, P.; Walsh, C. M.; Mangan, N. E; Fallon, R.E.; Sayers, J.R.; Mckenzie, A.N. and Fallon, P.G. (2004): *Schistosoma mansoni* worms induce anergy of T-cells via selective up-regulation of programmed death ligand 1 on macrophages. *J.Immunol*., 173: 1240-1248.
29. Stadecker, M.J. (1992): The role of T-cell anergy in immunomodulation of schistosomiasis. *Parasitol. Today*, 8: 199-204.
30. Stadecker, M.J.; Hernandez, H.j. and Asahi, H. (2001): The identification and characterization of new immunogenic egg components: implications for evaluation and control of the immunopathologic T-cell response in schistosomiasis. *Mem. Inst. Oswaldo. Cruz.*, 96:29-33.
31. Swoveland, p. and Ghonson, K. (1979): Enhancement of florescent antibody staining of viral antigens in formalin fixed tissue by trypsin digestion, *J. Infect. Dis.*, 140:458-462.
32. Von Lichtenberg, E.V. (1962). Host response to eggs of *Schistosoma mansoni* .Granuloma formation in the unsensitized laboratory mouse. *Am. J. Pathol.,* 41: 711.
33. Wolwezuk C., Auriault H., Gras-Mass G., Vendeville J., Balloul, M., Tartar A. and Capron, A. (1989). Protective immunity in mice vaccinated with *Schistosoma mansoni. J Immunol*, 142: 1342-1350.
34. Wynn A. (1999). Immune deviation as a strategy for schistosomtasis vaccines designated to prevent infection and egg induced immunopathology. *Microbes and infection,* 1: 525-534.
35. Zouain C.S., Gustavson S., Silva-Teixeira D.N., Contigli C., Rodrigues V., Leite M.F. and Goes A. M. (2002). Human immune response in schistosomtasis: The role of P24 in the modulation of cellular reactivity to *S. mansoni* antigens. *Hum Immunol.,* 63: 647-656.

3/1/2010