Article

The Protective Role of Toll-like Receptors Agonist Monophosphoryl Lipid A Against Vaccinated Murine Schistosomiasis

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Abstract: Schistosomiasis, a crippling ailment that afflicts over 220 million people worldwide. Yet or up till now, there is no vaccine for schistosomiasis, and chemotherapy relies heavily on a single drug, the praziquantel. The present study was undertaken to investigate the therapeutic effect of Monophosphoryl Lipid A (MPLA) as an adjuvant in soluble egg antigen (SEA) vaccinated mice against the deleterious pathological impacts induced in hepatic tissues of mice by *Schistosoma mansoni* infection. In addition, to study the associated parasitological, immunological and biochemical parameters. Parasitological parameters showed that intraperitoneal injection of MPLA into SEA-vaccinated and *S. mansoni*-infected mice was effective to a significant degree in reducing the worm and egg burden, granuloma count and diameter as well as the total area of infection in their livers versus SEA-untreated but infected ones. In addition, MPLA showed ameliorative action on the elevated liver oxidative stress marker, including malondialdehyde (MDA) and decrease in the level of the antioxidant enzymes, reduced glutathione (GSH) and catalase (CAT) which may have a role in the liver damage and fibrosis due to *S. mansoni* infection. In conclusion, treatment with MPLA has multi-functions in attenuating the deleterious impacts of *S. mansoni* infection in mice livers. Its effects are mediated through a reduction of ova count, worm burden, granuloma diameter and amelioration of antioxidant defense systems, and liver function biomarkers.

Keywords: Schistosomiasis; Monophosphoryl Lipid A (MPLA); Chemotherapy; Oxidative stress; Antioxidant enzymes; SEA.

1. Introduction

Schistosomiasis, a neglected tropical disease, is predominantly in tropical and sub-tropical areas and affects ~300 million people worldwide, and at least 206.4 million people needed preventive treatment in 2016 (WHO, 2018). Chronic Schistosoma infection results mainly from the immune reactions against trapped Schistosoma eggs in the tissues, leading to the formation of large granulomas and fibrosis [2]. Toll-like receptors (TLRs) are a family of PRRs consists of nine different functional TLRs, named TLR1 through TLR9. TLRs 1-6 are expressed on the plasma membrane where they recognize various PAMPs in the extracellular environment, however, TLRs 4 recognize LPS, one of the most potent microbial stimuli for innate immune responses [3,4]. TLR signaling pathways involve the use of the adaptor protein MyD88 and activate the transcription factors nuclear factor κB (NF-κB), and activation protein 1 (AP-1), that stimulate inflammatory responses, including the synthesis of proinflammatory cytokines (e.g. tumor necrosis factor [TNF] and IL-1), responsible for activating the innate immune system [5]. During *S. mansoni* infection, acute signs of illness happen prior the existence of eggs in the faeces, the immune response is initially T helper 1 (Th1) response that is reflected by cytokine production (TNF, IL-1 and IL-6) [6–8]. Following the progression of the disease and onset of egg production, an immune deviation represented in Th2 response leading to downregulation in the secretion of these pro-inflammatory cytokines and the production of IL-10, IL-4 and IL-13 [9–12].

Monophosphoryl lipid A (MPLA), the LPS that is chemically modified, has the immunostimulatory activity of LPS but with less toxicity. MPLA serves as a TLR4 agonist. It has been approved in Europe as a vaccine adjuvant, and is a component of Hepatitis B and Human Papillomavirus Virus vaccines [13].

The present study aimed to evaluate the therapeutic effect of MPLA in SEA-vaccinated and *S. mansoni* infected mice during the beginning of egg laying (35-day post-infection). This can be achieved by estimating worm recovery, counting eggs, finding the number and the size of each granuloma, measuring the area of infection and liver histopathology. Also, the present study was extended to investigate the potential role of MPLA as antioxidant drug. This can be achieved by measuring MDA, non-enzymatic antioxidant; GSH and enzymatic antioxidants; CAT. Serum liver function biomarkers were also measured to document the capability of the used drug in changing the schistosomal induced pathology.

2- Materials and methods

2.1. Preparation of Monophosphoryl Lipid A working solution

MPLA derived from *Salmonella enterica* serotype Minnesota Re 595 (Sigma-Aldrich) was prepared according to **Romero et al. (2011)** [14] by dissolving 100 mg MPLA in 100 ml triethylamine (0.2%) to get final concentration of 1 mg/mL. The solution was heated to 60 °C and followed by sonication for 30 min. Prior to administration, MPLA solution was further diluted to 100 µg/mL using phosphate-buffered saline (PBS, pH 7.4). MPLA was injected intraperitonealy (20 µg in 0.2 ml) once daily for two successive days (total of 40 µg/mouse). Control mice received injections of vehicle in the same volume and by the route as that used in the respective treatment protocols.

3.2. Experimental design

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Closed random bred male mice (Swiss albino CD-1 strain), weighing 18-20 g, were obtained from the Schistosome Biological Supply Center (SBSC) at Theodor Bilharz Research Institute (TBRI), Giza, Egypt. Animals were housed in controlled temperature (22±3 °C) with a relative humidity of 50±15% and a 12-h light/dark photoperiod. Food and water were provided ad libitum. This study was approved (approval no. IP985019/2018) by the Medical Ethical Committee, TBRI, Giza, Egypt.

Sixty-four mice were assigned into two main groups, the normal, non-infected control (24 mice) and infected group (40 mice each infected with 80 *S. mansoni* cercariae). The animals of the normal control group were subdivided into 3 subgroups (8 mice/subgroup); subgroup 1 (untreated control), subgroup 2 administered with a single dose of Monophosphoryl Lipid A and subgroup 3 (vaccinated subgroup) administered with a single dose of 10 µg soluble egg antigen (SEA). The animals of the infected group were subdivided into 5 subgroups (8 mice/subgroup); subgroup 4 (untreated), subgroup 5 (SEA-vaccinated), subgroup 6 (MPLA-treated), subgroup 7 (MPLA-treated and SEA-vaccinated) and subgroup 8 administered a dual dose of PZQ (500 mg/kg body weight) orally at 6 weeks post-infection.

3.3. Parasitological Parameters:

For evaluation of the anti-schistosomal activity of MPLA, the following criteria were considered:

3.3.1. Worm burden

Worms in the Hepatic and portomesenteric vessels were recovered and counted following the method described by Duvall & DeWitt (1967) [15].

3.3.2. Tissue egg load

Ova found in the intestine or liver of every individual mouse were counted after digestion using KOH following the method described by Cheever (1968)[16] and Duvall and DeWitt (1967) [15].

3.3.3. Oogram pattern

Eggs at each stage/animal were determined and percentage was calculated at various developmental stages from each mouse following the method described by Pellegrino et al. (1962) [17].

3.4. Histopathology

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Liver samples were collected from all mice groups and fixed in 10% formalin buffer and embedded in paraffin wax to be sectioned (4 µm thickness). Some sections were stained with hematoxylin and eosin (H&E) for histological examination following the protocol of Hirsch et al. (1997) [18] and another were stained with Masson's trichrome for the determination of fibrosis [19].

3.5. Granuloma diameters and count

Measurement of the granuloma diameter was done using an ocular micrometer. This measurement was conducted on non-adjoining granulomas, which contained only one egg (regardless the miracidia were intact or degenerated). The mean diameter of each single granuloma was calculated according to Von Lichtenberg (1962)[20] by measuring two diameters (at right angles) of this single granuloma. Thirty granulomas were measured from each mouse. The cellular component of each granuloma was studied and viable/dead eggs percentage was calculated.

3.6. Oxidative Stress Markers assessment

All Oxidative stress markers used in this study were detected in the liver homogenate supernatant. The appropriate kits (Abcam Company, Cambridge science park, Cambridge, UK) were used for the determination of malondialdehyde (MDA) [21], glutathione reduced (GSH) [22], Catalase (CAT) [23] and Glutathione-S-Transferase (GST) [24].

Statistical method:

All data were expressed as means ± SEM. In general, the data were analyzed by two-way ANOVA followed by the Bonferroni test and Duncan's multiple range test. Student's t test was used when only two data groups were compared with each other. The *p*-value of < 0.05 was considered as statistically significant. All calculations were performed using GraphPad Prism software 7 (La Jolla, CA, USA).

3- Results

3.1. Effect of MPLA on parasitological parameters

Treatment of *S. mansoni*-infected mice with MPLA as an adjuvant without or with vaccination with SEA induced a high significant reduction in the total worm burden with a percent reduction of 52.3% and 68% respectively compared to infected untreated and infected vaccinated mice (Table 1). A highly significant

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reduction in the mean total number of hepatic and intestinal egg load compared to infected untreated mice is shown in Table 1.

Table 1: Total worm burden and ova count in mice treated with MPLA.

Animal group	Mean no. of worms ± SEM	% reduction	Mean no. of ova count ± SEM/g tissue			
			Intestine	% reduction	Liver	% reduction
Infected untreated	25.80 ± 1.52	-	12,220 ± 1244	-	6,171 ± 316	-
Infected + SEA	20.5 ± 0.17$^{\#}$	20.5%	9,750 ± 1094 **	20.2%	4,420 ± 304 **	28.2%
Infected +MPA	$^{\#\#\#}$12.3 ± 1.30	52.3%	$^{\#\#\#}$2,352 ± 176	80.8%	$^{\#\#\#}$2,598 ± 173	57.9%
Infected+ SEA +MPLA	8.1 ± 0.29$^{\#\#\#}$	68.6%	$^{\#}$1,757 ± 204	85.6%	$^{\#\#}$1,484 ± 248	76.0%
Infected +PZQ	$^{\#\#\#}$1.3 ± 0.30	95.0%	$^{\#\#\#}$892 ± 108	92.7%	$^{\#\#\#}$454 ± 75	92.6%

#, ## and ### are a significance of 0.05, 0.01 and 0.001 respectively in mean number of worms compared to Infected untreated mice. * ** and *** are a significance of 0.05, 0.01 and 0.001 respectively in mean number of eggs compared to Infected untreated mice.

A significant decreases of a total immature egg number of infected but treated with the MPLA, MPLA+SEA and PZQ treated subgroups was obtained (Table 2), but no significant changes were shown in the number of mature eggs (all treated subgroups, Table 2).

Table 2: Oogram pattern in mice treated with the MPLA.

Animal Group	Oogram pattern (% ova)		
	Immature	Mature	Dead
Infected untreated	59.00 ± 4.84	35.4 ± 4.2	5.6 ± 0.16
Infected + SEA	60.00 ± 4.0	31.3 ± 2.1	8.7± 0.20
Infected + MPA	47.9 ± 3.9	29.9 ± 3.1	22.2 ±1.1* **
Infected+ SEA +MPLA	32.1 ± 2.8*	40.8 ± 2.3	27.1 ± 1.2* **
Infected +PZQ	29.5 ± 1.2* *	28.8 ± 1.1	41.7 ± 4.1* **

*, **, *** a significance of 0.05, 0.01 and 0.001 respectively

3.2. Effect of MPLA on Oxidative Stress Markers.

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The present data (Table 3) show the changes of different oxidative stress markers in the liver following different treatments. Treatment of normal mice with either SEA or MPLA significantly ($p<0.0001$) decreased the level of CAT. But, in infected mice, treatment with SEA did not change the level of CAT while the treatment with MPLA or MPLA+SEA significantly ($p<0.0001$) increased it. Treatment of normal mice with either SEA or MPLA significantly ($p<0.0001$) decreased the level of GSH while treatment with SEA, MPLA or SEA+MPLA significantly ($p<0.0001$) increased the levels of GSH in infected mice.

Treatment of normal mice with either SEA or MPLA significantly ($p<0.0001$) increased the level of MDA. Treatment of infected mice with SEA significantly ($p<0.0001$) increased the levels of MDA while the treatment with MPLA or MPLA+SEA significantly ($p<0.0001$) decreased it.

Table 3: - Oxidative Stress Marker in mice treated with MPLA.

Animal Group	**Oxidative Stress Marker**		
	CAT	**GSH**	**MDA**
Normal control	1.43 ± 0.05	2.13 ± 0. 11	33.60 ± 2.32
Normal + SEA	1.17 ± 0.04[a]	1.85 ± 0. 12[a]	37.24 ± 3.42[a]
Normal + MPLA	1.22 ± 0.06[a]	1.92 ± 0.14[a]	38.68 ± 4.19[a]
Infected untreated	0.87 ± 0.01	1.03 ± 0.01	50.90 ± 0.32
Infected + SEA	0.88 ± 0.01[ns]	1.12 ± 0.06[b]	56.66 ± 1.90[b]
Infected + MPLA	1.11 ± 0.01[b]	1.52 ± 0.02[b]	35.06 ± 0.85[b]
Infected + SEA + MPLA	1.28 ± 0.01[b]	1.97 ± 0.15[b]	36.63 ± 1.70[b]
Infected +PZQ	0.98 ± 0.01	1.66 ± 0.13	38.00 ± 0.63

a= $p<0.0001$ significance compared to normal untreated mice.

b= $p<0.0001$ significance compared to untreated infected mice.

ns= nonsignificant

3.3. Histopathological examination

The liver of normal untreated control subgroup showed the normal architecture of hepatocytes which appear to radiate from the central vein. The hepatocytes have open face nucleus and acidophilic cytoplasm. No inflammatory cells are seen in the liver. Histopathological examination of the liver sections of the infected untreated mice (Fig. 1A) showed marked inflammatory cell infiltrate around a foreign body with chronic granulomatous reaction around the foreign body. A microphotograph of the liver of the infected and

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SEA-treated mice subgroup (Fig. 1B) showed a small chronic granulomatous reaction around a foreign body. After treatment with MPLA (Fig. 1C), it showed marked inflammatory cell infiltrated around the foreign body and some esinophilic cells were observed. Treatment of infected mice with MPLA+SEA showed slight decrease in the inflammatory cell infiltrate around the foreign body (Fig 1D). Figure 1E shows the architecture of the liver of the infected mice after treatment with PZQ.

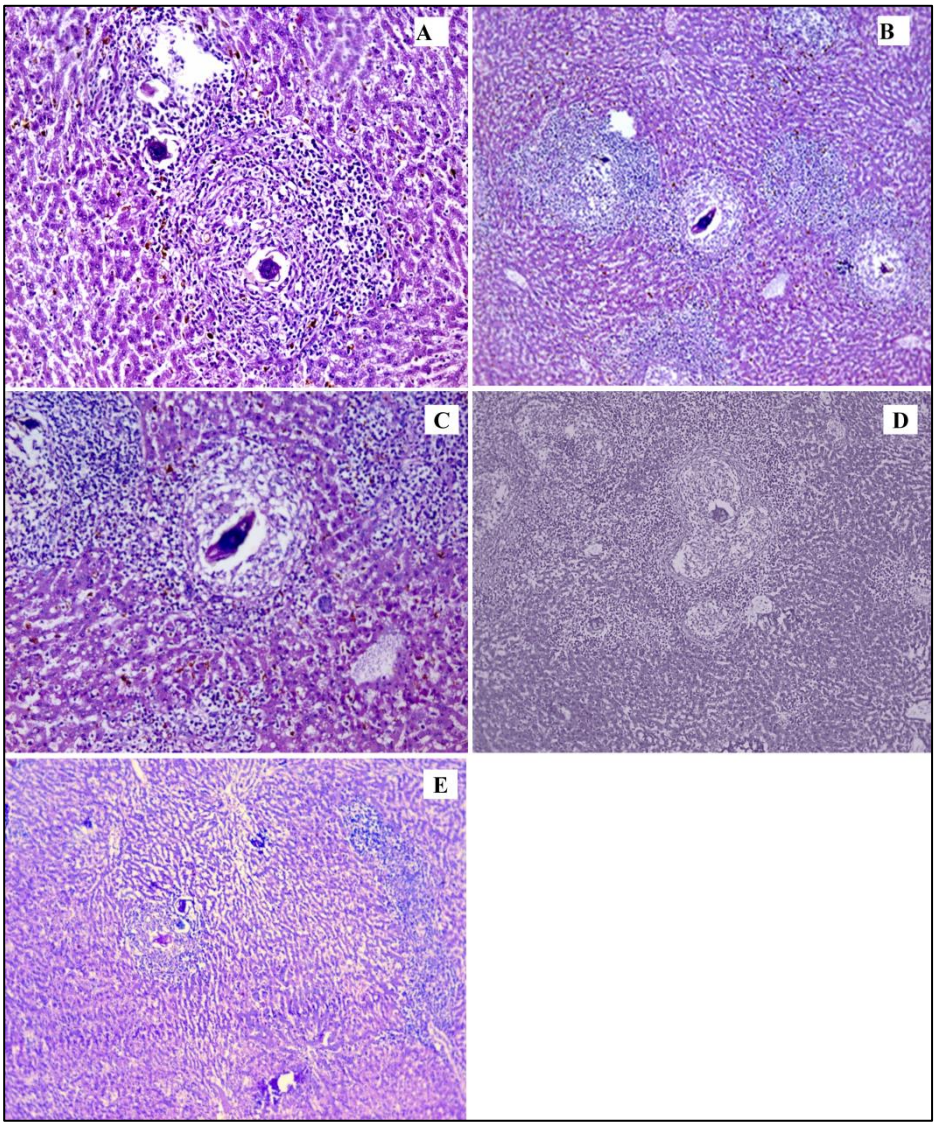

Fig. 1: Histopathological study of H&E-stained liver sections of different subgroups of mice infected with *Schistosoma mansoni* (×400). A: Infected untreated subgroup; **B:** Infected SEA-treated subgroup; **C**: Infected MPLA-treated subgroup; **D**: Infected MPLA+SEA-treated subgroup and **E**: Infected PZQ–treated.

Hepatic granuloma diameter showed a significant decrease (<0.0001) in all treated subgroups of *S. mansoni* infected mice. Also, the number of eggs in liver significantly ($p<0.0001$) decreased (Table 4).

Table 4: - Hepatic granuloma diameter in infected untreated and treated mice.

Animal Group	Hepatic granuloma diameter	% Reduction	Number of granuloma	% Reduction
Infected untreated	251.8±14.7		**23**	
Infected+SEA	216.0 ±11.3[#]	**14.2%**	**19**[*]	**17.4%**
Infected+MPA	184.4±13.2[#]	**26.8%**	**14**[*]	**39.1%**
Infected+SEA+MPLA	139.9± 20.1[#]	**44.4%**	**12**[*]	**47.8%**
Infected +PZQ	111.9±18.9[#]	**55.6%**	**10**[*]	**56.5%**

and *: a significance of 0.0001 in mean number of hepatic granuloma diameter and number respectively compared to Infected untreated mice.

4. Discussion

The combination of protection using SEA and adjuvant was recommended in several studies [25–29] as it provided many complementary goals, a reduction of egg-induced pathology, minimal parenchymal changes and the eradication of worms. Therefore, the assessment of the effect of MPLA adjuvant with protective antigen SEA against infected mice is important by studying several criteria related to the parasitic intensity, stages and distribution through the tissues of the host for the evaluation of the magnitude of infection and efficacy of the treatment [30]. Hepatosplenic schistosomiasis is a serious manifestation of *S. mansoni* infection that may lead to an irreversible sequelae [31,32]. In terms of the result of histopathological liver analysis with 6 weeks after infection, using a combination of MPLA and PZQ treatment improved the histopathology of the liver with respect to the ganuloma number and diameter (5.8 $\pm$1.03 and 191.1 $\pm$ 7.8 respectivelly) and the reported changes were in accordance with El-Beshbishi et al. (2013)[33], who found that hepatic tissues of untreated-infected rats (6 weeks after infection) showed moderate cloudy swelling of the liver parenchyma and cells irregularly outlined granulomata encircling recently deposited intact or partially degenerated ova. Also, El Ridi et al. (2012)[34] noted that the schistosomicidal effects of Arachidonic acid (ARA) were associated with an improvement with respect to liver histopathology.

The present study was therefore undertaken to investigate the effect of MLPA on *S. mansoni* infectivity and its complications in mice. Previous studies revealed that the intensity of schistosomal infection, which represented by the worm burden and egg count, increases the degree of liver fibrosis and granulomatous

reaction [35]. This is in agreement with the present histopathological findings of *S. mamsoni* infected liver, which revealed increased number and diameter of granuloma, total area of infection as compared with infected mice. Treatment of infected mice with MLPA improved the histopathological picture of the liver. This was ensured by significant diminution in number and diameters of granulomas, reduction in their fibrotic content accompanied with a reduction in the total area of infection as compared with infected mice. The role of free radicals and oxidative stress in the progression of liver injury in various chronic liver diseases such as viral hepatitis, alcoholic hepatitis and hepatic cirrhosis were studied [36]**.** Schistosomiasis is no exception, oxidative stress occurs in the liver at the site of inflammation in the vicinity of eggs of *S. mansoni*. This state of oxidative stress is attributed to increased generation of ROS and exhaustion of endogenous antioxidant enzymes [37–39]. Oxidative processes occurred at the site of granulomatous inflammation and on the other hand the antioxidant capacity of the liver decreased, leading to the generation of lipid peroxides which may play a central role in the pathology associated with schistosomiasis [40]. In the present study elevation of MDA as a result of infection with *S. mansoni*; Poli (2000)[41] and Mahmoud et al. (2002)[40] has been suggested to be due to the release of significant amount of O^{-} from macrophages of hepatic granulomas. At the same time, liver GSH was drastically depleted in infected mice. Such depletion is critical, as shown by the increased cytotoxicity of H_2O_2 in endothelial cells, as a result of inhibition of glutathione reductase which keeps glutathione in its reduced state [38,39,42]. There are other examples of an infectious disease-associated decrease of hepatic catalase and GSH levels [43,44] leading to a greater sensitivity to inflammation-derived products [45]. The activity of the anti-oxidant enzyme, catalase, in the liver tissue of infected mice with *S. mansoni* also decreases where catalase detoxifies hydrogen peroxide produced by inflammatory cells to water [36,46]. Therefore, treatment with nucleotids may protect hepatocytes from damage, demise and dysfunction that caused by oxidative stress at the sites of inflammation [23].

5. Conclusions

In conclusion, treatment of SEA-vaccinated and *S. mansoni*-infected mice with MPLA has many good effects in attenuating the deleterious impacts in livers of these mice. Its effects were clear in reducing ova count, worm burden, granuloma diameter and amelioration of antioxidant defense systems, and liver function biomarkers.

Author Contributions

Conceptualization, Ibrahim Aly, Essam Ibrahim, Rabab Hamad, Hoda Sayed, Mohammed Alshehri and Mona Kilany; Data curation, Essam Ibrahim, Sama Attiyah, Wafaa Komy, Hamed Ghramh, Ali Alshehri, Khalid Alsyaad and Mohammed Alshehri; Formal analysis, Rabab Hamad, Hamed Ghramh, Ali Alshehri and Mona Kilany; Funding acquisition, Essam Ibrahim; Investigation, Sama Attiyah, Wafaa Komy, Ali Alshehri and Mona Kilany; Methodology, Ibrahim Aly, Rabab Hamad, Hoda Sayed, Sama Attiyah, Wafaa Komy and Mona Kilany; Supervision, Ibrahim Aly and Essam Ibrahim; Validation, Hoda Sayed; Writing – original draft, Ibrahim Aly, Essam Ibrahim, Rabab Hamad, Hoda Sayed, Sama Attiyah, Wafaa Komy, Hamed Ghramh, Ali Alshehri, Khalid Alsyaad and Mona Kilany; Writing – review & editing, Ibrahim Aly, Essam Ibrahim, Khalid Alsyaad, Mohammed Alshehri and Mona Kilany.

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Conflict of Interest:

All authors state that they haven't any financial/commercial conflict of interest regarding this work.

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