Hepatic Histopathological and Histochemical Changes in Mice Infected with *Schistosoma Mansoni* After Vaccination with Gamma Radiation - Attenuated Schistosomules

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ABSTRACT

Current strategies for the control of schistosomiasis are based primarily on chemotherapy but successful vaccination against infection has been also demonstrated in several host parasitic models. The present study was designed to assess the effect of vaccination with schistosomulae attenuated by different doses of $\gamma$-radiation in mice challenged with *S. mansoni* cercariae regarding hepatic histopathological changes using Haematoxylin and Eosin (H & E) stain, and hepatic histochemical changes using periodic acid Schiff's (PAS), and bromophenol blue (BPB) to examine the polysaccharides and proteins contents. Sixty mice were used in four groups, the first group served as control normal group I (10 mice), the second group was infected by subcutaneous (S.C.) injection with 100 *S. mansoni* cercariae/mouse as infected control group II (10 mice). Groups III and IV (20 mice each) were subdivided equally into two subgroups A and B where subgroups III$_A$ and IV$_A$ were vaccinated by (S.C.) injection with 500 schistosomulae irradiated with 15 Krad and 20 Krad gamma-radiation. Meanwhile, subgroups III$_B$ and IV$_B$ were vaccinated by (S.C.) injection with the same doses of irradiated schistosomulae and then challenged after 4 weeks by S.C. injection with 100 *S. mansoni* cercariae. Hepatocytes, in the acute infected mice (group II) showed marked histopathological and histochemical changes with marked decrease in PAS $+ve$ materials and total proteins, whereas moderate changes were observed in the liver of vaccinated unchallenged subgroups III$_A$ and IV$_A$ (15 and 20 Krad respectively) and slight changes were detected in the livers of the mice vaccinated and challenged subgroups III$_B$ and IV$_B$. It has been shown that schistosomules irradiated at 20 Krad induced marked amelioration in both histopathological and histochemical changes that did schistosomules irradiated at 15 Krad. It could be concluded that gamma radiation is a good tool for vaccination against schistosomiasis mansoni and 20 Krad is most effective dose.

Key Words: *Schistosoma mansoni*/ Radiation/ Schistosomulae/ Histopathology/ Histochemistry/ Liver.

INTRODUCTION

Despite the strenuous control efforts, schistosomiasis remains to be a tropical disease that ranks with malaria and tuberculosis as a major source of morbidity affecting approximately 210 million people in 76 countries of the world (1). The repeated chemotherapy of schistosomiasis in endemic areas has resulted in the emergence of drug-resistant *schistosome* strains (2). Development of such resistance has drawn the attention of many authors to protect populations at risk against infection. Vaccination strategy in schistosomiasis is either anti-pathology or anti-infection vaccination that can be induced by exposure to radiation attenuated cercariae or larvae where they die at some point during their

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migratory pathway before maturity is reached \(^{(3)}\). This vaccine would contribute to the reduction of schistosomiasis morbidity through induced immune responses leading to decrease in parasite load and reduced egg production. It could be administered to children at ages of 3 to 12 years to prevent severe infection in a particularly high risk population \(^{(4)}\).

Radiation attenuated (RA) vaccine has enabled dissection of different immune response as putative effective mechanisms \(^{(5)}\). The action of the irradiation may not kill the parasite outright, but to incapacitate them to allow host immune response to kill them where the tegumental damage caused would expose epitopes on the worm surface that are normally not exposed and causes functional impairment of the parasite \(^{(6)}\). All information available indicated that the early-irradiated stages are required for induction of resistance. There is some evidence that cercariae lose some of its potency in induction of resistance immediately after artificial conversion to skin-stage schistosomula \(^{(7)}\). Therefore, schistosomula were considered the first important source of antigens. It was reported that in the early infection stage, newly transformed schistosomula tegument is able to activate dendritic cells and up regulate the expression of co-stimulatory molecules \(^{(8)}\). Irradiation modifies the expression of schistosomular antigens quantitatively temporally. However, both biochemical and morphological observations have failed to demonstrate difference between normal and irradiated parasites \(^{(9)}\). It was reported that cryopreserved schistosomules irradiated at 10 or 20 Krad induced greater levels of protection than did schistosomula irradiated at 2, 5, 30, or 50 Krad. Protective immunity developed as early as 3 weeks post-immunization \(^{(10)}\). However, Hafez et al. reported that vaccination of mice with 20 Krad irradiiated schistosomula and challenged with infective cercariae provides reduction in worm burden with mild histopathological changes in liver tissue \(^{(11)}\). A previous study reported that monkeys injected once with 104 irradiated schistosomula (50 krad at 4 krad/min) had 52% fewer challenge worms than the control group at necropsy. At 50 days post-challenge, the immunized monkeys excreted 80% fewer eggs than did the control animals \(^{(12)}\).

Histopathological lesions associated with infection with *S. mansoni* were characterized by large granuloma and significant reduction in glycogen content in liver tissue of mice \(^{(13)}\). There are strong evidences that in acute schistosomiasis the deteriorated antioxidant defense system plays an important role in liver pathology in schistosomiasis \(^{(14)}\). Activated kupffer cells may act both as effector cells in the destruction of hepatocytes by producing harmful soluble mediators and as antigen presenting cells during liver infection \(^{(15)}\). In liver injury and hepatocellular necrosis, activated kupffer cells are a major source of inflammatory mediators including cytokines, superoxide, nitric oxide, eicosanoids, chemokines, lysosomal and proteolytic enzymes and demonstrate increased cytotoxicity and chemotaxis \(^{(16)}\).

This work aimed to detect hepatic histopathological and histochemical changes regarding polysaccharides and total proteins in *schistosoma mansoni* infected mice and determined the effect of vaccination with 15 and 20 Krad gamma-irradiated schistosomula in controlling these changes.

**MATERIALS AND METHODS**

**Parasite and Experimental Animals**

An Egyptian strain *S. mansoni* cercariae were supplied by the Schistosome Biological Supply Program (SBSP) at Theodor Bilharz Research Institute, Imbaba, Giza, Egypt. This strain has been passed through outbreed golden hamsters and *B. alexandrina*, cared for maintainance at SBSP/TBRI.

Schistosomulae were obtained 14 days post-infection by perfusion technique using citrated saline of Hamster, s lung previously infected with 4000 cercariae according to BL. Mangold et al\(^{(17)}\). Schistosomulae were irradiated by 15 and 20 Krad gamma rays at dose rat 2.5 KGy/h at the time of the experiment. This was done in the National Center for Radiation Research and Technology (NCRRT), Cairo, Egypt.
Male albino mice of Balb/C strain, weighing 20–25 g for each, were obtained from the Ministry of Agriculture Breed House. They were completely healthy and were given access to water and standard diet and were monitored daily for health status at the animal house of the (NCRRRT), Cairo, Egypt. They were maintained according to the ethics committee of the National Research Center and in accordance to the “Guide for the care and use of laboratory animals” published by the US National Institutes of Laboratory Animal Resources. The local IAUCC or ethical committee has reviewed and approved the actions and protocols detailed in the report.

Experimental Design
In the present study, sixty mice were used and divided into four groups. Group I (10 mice) was kept without infection and served as normal control. Group II (10 mice) was infected with 100 S. mansoni cercariae and served as infected control. Groups III and IV (20 mice each) were equally subdivided into two subgroups A and B where subgroups IIIA, IV\textsubscript{A} were vaccinated with 500 schistosomulae irradiated with 15 Krad and 20 Krad gamma-radiation without challenge (as vaccine control). Subgroups IIIB and IV\textsubscript{B} were vaccinated with the same vaccine and then challenged 4 weeks by S.C. injection with 100 S. mansoni cercariae.

Histopathological Investigations
Portions of liver of each mice in all groups were preserved in 10% buffered formalin, cut into 1 cm. thick slices, dehydrated by alcohol, cleared with xylol and finally embedded in paraffin then processed into paraffin blocks. Using Reichert Rotary microtome serial paraffin sections of 5µm. thickness were made and the sections were then stained with Harries Hematoxylin and Eosin \textsuperscript{(18)}.

Histochemical Investigations
Liver tissues were rapidly excised from dissected mice and were taken and immediately fixed in Carnoy's fixative for 4 hrs. Dehydration, clearing and paraffin infiltration were done as usual. Paraffin sections were cut at 5 µm thickness. The slides were divided for staining by periodic acid Schiff's reagent (PAS) and mercuric bromophenol blue (BPB) reaction for PAS +ve materials and total proteins respectively \textsuperscript{(19)}. Stained slides were dehydrated, cleared and mounted in DPX. The histochemical alterations were examined and photographed using a research light microscope Axioscop 2 plus (Carl Zeiss, Germany).

Statistical Analysis
Results were subjected to Student’s \textit{t}-test using SPSS program version 8 to determine the significance of the data. Data are expressed as mean ± standard error. Values with \( P < 0.05, P < 0.01 \) and \( P < 0.001 \) are significant, highly significant and very highly significant respectively.

RESULTS
Histopathological Investigations
Group I revealed normal lobular pattern of hepatocytes (Fig. 1). Group II (Control infected) revealed characteristic granulomata around the S. mansoni ova surrounded by inflammatory cells and sinusoidal dilatation (Fig.2). Vaccinated unchallenged subgroup III\textsubscript{A} (15Krad) showed striking sinusoidal dilatation and small granuloma mainly cellular with almost dead schistosomal ovum (Fig.3) while vaccinated challenged subgroup III\textsubscript{B} vaccinated showed small inflammatory cellular infiltrates in the portal area (Fig. 4). Subgroup IV\textsubscript{A} vaccinated with 20 Krad gamma-irradiated schistosomulae without challenge showed few cellular infiltration with signs of degeneration of hepatocytes (Fig.5), while vaccinated challenged subgroup IV\textsubscript{B} revealed smaller improved histopathology of liver parenchyma (Fig.6).
Fig. (1) Liver section of normal control group I showing normal hepatic architecture (H&E X200).

Fig. (2) Liver section of infected control group II showing fibrocellular granuloma of large size formed of schistosomal ovum surrounded by severe inflammatory cellular infiltrate (H&E X200).

Fig. (3) Liver section of subgroup IIIA (vaccinated unchallenged 15Krad) showing sinusoidal dilatation and small cellular granuloma formed of dead schistosomal ovum surrounded by inflammatory cellular infiltrate (H&E X200).

Fig. (4) Liver section of subgroup IIIB (vaccinated challenged 15Krad) showing small inflammatory cellular infiltrate (H&E X200).

Fig. (5) Liver section of subgroup IVIA (vaccinated unchallenged 20Krad) showing inflammatory cellular infiltration of liver parenchyma with interstitial congestion (H&E X200).

Fig. (6) Liver section of subgroup IVIB (vaccinated challenged 20Krad) showing almost normal hepatic cells with mild sinusoidal dilatation (H&E X200).

Polysaccharides (PAS + ve Materials)
Liver tissues of mice in normal control group (I) showed +ve PAS reaction. Normal polysaccharides granules were found in the cytoplasm of most hepatocytes as intense coarse granules (Fig. 7). Liver tissues of mice in infected control group (II) showed hepatic lobules with most hepatocytes having reduced content of PAS+ve materials (Fig. 8). In mice of subgroup III_A, vaccinated with 15 Krad gamma-irradiated schistosomulae without challenge, the hepatic tissues exhibited a decreased content of PAS+ve materials. The hepatocytes reacted in a weak pattern and showed few pink granules (Fig. 9). In subgroup IV_A, vaccinated with 20 Krad gamma-irradiated schistosomulae without challenge, most of the hepatocytes showed few to moderate PAS reaction (Fig. 10). In mice of subgroups III_B, and IV_B (vaccinated with 15 and 20 Krad gamma-irradiated schistosomulae and challenged), the hepatic tissues showed moderate increase in the PAS reaction (Figs. 11 and 12).

Liver sections stained with PAS stain (X100) for polysacharides granules showing reddish deeply stained in normal control group I (Fig. 7), negative PAS reaction in infected control group II (Fig. 8), decreased stain affinity in III_A (Fig. 9), few to moderate PAS content in IV_A (Fig. 10) and moderate content in both III_B and IV_B (Figs. 11 and 12).

Total Proteins
In the livers of mice of control normal group I, the total proteins appear as intense deep blue inclusions in the cytoplasm of the hepatic cells. The plasma membranes, nuclear envelopes and chromatin material were also stained (Fig. 13). In mice of control infected group II, the hepatocytes lost most of their protein content. The cytoplasm of degenerated, necrotic and neighboring non-infected cells was slightly stained with blue color due to a severe reduction of the protein content (Fig. 14). The liver of mice of subgroups IIIA and IVA (vaccinated with 15 and 20 Krad gamma-irradiated schistosomulae respectively) contained a large number of small vacuoles and subsequently appeared faintly stained (Figs. 15 and 16). In the same time, the liver tissues of mice in subgroups IIIB and IVB (vaccinated with 15 and 20 Krad gamma-irradiated schistosomulae and challenged) showed moderate protein content (Figs. 17 and 18).

**Total Proteins**

Liver sections stained with BPB stain (X100) for total proteins show deep blue granules in normal control mice group I (Fig. 13). Hepatocytes appeared losing most of its protein in infected control
group II (Fig. 14), few protein content in sub group III A (Fig. 15) few to moderate stain in subgroup IV A (Fig. 16) and moderate stained granules in subgroups III B and IV B (Figs. 17 and 18).

The effect of vaccination with 15 and 20 Krad gamma-attenuated schistosomules on hepatic PAS and total proteins was shown in Table 1. The PAS content was significantly high (P < 0.001) suppressed in S. mansoni-infected mice while this content was significantly suppressed (P < 0.01) in vaccinated unchallenged subgroups III A and IV A. On the other hand, in vaccinated challenged subgroups III B and IV B, PAS content showed non-significant decrease as compared to normal control group I. Regarding the total proteins content, infected mice exhibited a highly significant decrease (P < 0.001) compared with normal control mice. Meanwhile, vaccinated-challenged subgroups showed non-significant decrease as compared to control normal group and significant increase (P<0.01) as compared to infected control group II.

Table (1) Effect of vaccination with 15 and 20 Krad gamma - attenuated schistosomules on hepatic PAS and total proteins contents in mice of experimental groups

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I Normal control</th>
<th>Group II Infected control</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group I Normal control</td>
<td>Group II Infected control</td>
<td>Group III</td>
<td>Group IV</td>
</tr>
<tr>
<td></td>
<td>Normal control</td>
<td>Infected control</td>
<td>III A vaccinated – unchallenged 15 Krad</td>
<td>III B Vaccinated – unchallenged 15 Krad</td>
</tr>
<tr>
<td>PAS mg/g tissue</td>
<td>123.6±1.3</td>
<td>68.4±5 a</td>
<td>81.8±2.8 b</td>
<td>106±3.6 n.s. b₁</td>
</tr>
<tr>
<td>Total proteins mg/g tissue</td>
<td>124±0.8</td>
<td>56.6±13.3 a</td>
<td>81±4.1 aₐ₁</td>
<td>108±4.7 aₐ₁</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SE. Number of mice in each group is ten.
P values a < 0.001; b < 0.01; c < 0.5 and n.s non significant compared to control normal group.
P values a₁ < 0.001; b₁ < 0.01; c₁ < 0.5 and n.s. non significant compared to control infected group.

DISCUSSION

Schistosomiasis continues to be one of the most prevalent parasitic diseases in the world. Despite the existence of a highly effective antischistosome drug, the disease is spreading into new areas, and national control programs do not arrive to complete their tasks particularly in low endemic areas. The availability of a vaccine could represent an additional component to chemotherapy (30). In this context, the advent of a schistosomiasis vaccine would be a significant addition to current methods of control of this disease, one vaccination strategy that remain the “gold standard” against which second and third generation molecule specific vaccines are judged is the attenuation of invasive cercariae with ionizing radiation (gamma, X ray and UV ray) (31). Attenuation of infective cercariae has traditionally been achieved using a gamma source of radiation and the dose usually between 20 to 50 Krad (32).

Key questions arising in this context are: how and why radiation attenuated (RA) schistosomulae generate a high level of protection against subsequent challenge, whereas an equivalent number of normal parasites do not. One possible explanation for the increase immunogenicity of RA larvae in their delayed and truncated pattern of migration. In contrast to normal parasite, RA larvae move slowly (33). Radiation- induced defects in the neuromuscular coordination of the developing larvae are likely to be responsible for this impaired ability to migrate. Another explanation is that radiation alters the surface antigens present on the parasites. In this hypothesis, it converts a poorly immunogenic surface molecule to one that is highly immunogenic (34). The third explanation for immunogenicity of RA larvae is that irradiation ablates certain proteins present in the parasite that function to down-regulate immune response (35).
Harrison et al reported that baboons immunized 2 or 3 times with schistosomula irradiated with 20 krad were significantly more protected (85-90%) against challenge infection than baboons similarly immunized with larvae receiving 60 krad (56-50% protection). Baboons immunized with schistosomula irradiated with 20 krad were better protected against challenge infection at 8 weeks after immunization than at 28 weeks after immunization. Protection was manifest by a reduction in worm numbers, excreta egg counts and gross pathology and, to a lesser extent, by stability of body weight and haematological indices following challenge (26).

Histopathological study revealed marked inflammatory reaction with large granuloma around the ovum. This was in accordance to the work of Chesney et al. (27), who described the infiltration of circulating fibroblasts into granulomas and speculated that these cells may be important for attracting lymphocytes as well as forming collagen.

Histochemical alterations were not only restricted to the site of infections, but also to various parts of organ tissues due to parasite replication, invasion and secretion of some toxins which may produce disruption, tissue injury and subsequently reduce the immune response and increase the histochemical alterations of the infected animals (28). In the current study, severe depletion of hepatic glycogen was observed in the liver of infected control group II. Similarly, other investigators recorded severe and significant decrease in the hepatic glycogen in schistosomiasis and fasciolosis (29). This was in agreement with Ahmed and Gad who recorded depletion of glycogen on month post infection (30). Another study attributed this to the effect that occurs on the membrane of endoplasmic reticulum or to the elevation of calcium that can trigger the conversion of the enzyme phosphorylase b (inactive form) to phosphorylase a (active form) which degrades glycogen into glucose (31). Also, a considerable decrease in liver glycogen during acute phase of Eimerian infections was recorded (32). It was found that the livers of mice infected with Babesia microti, showed slight decrease followed by extensive drop in the glycogen content and the livers showed very weak PAS-reaction at 14th and 28th day post infection, respectively (33). A study of Brossier et al. reported that the depletion in glycogen content in Toxoplasma gondii infected livers may be related to consumption of glycogen not only by the parasite itself but also by releasing some enzymes inside the invaded hepatocytes. Some toxins may cause disruption in glucokinase activities and subsequently defectiveness in the process of glycogenesis, in addition to acceleration of both hexokinase and phosphorylase activities to promote glycolysis and glycogenolysis (34).

Histochemical assessment of total proteins in the current work revealed that most hepatocytes lost their protein contents in infected control group II of mice, as a result of acute infection. This was in agreement with previous study that reported severe depletion in protein in S. mansoni-infected mice (35). Excess superoxide radicals interact with hydrogen peroxides and organic peroxides with generation of highly reactive entities that can attack DNA, membrane lipids and other essential cell components. This interprets depletion of total protein level as well as glycogen level explored in most hepatocytes, particularly those around blood vessels (36). Also, Kuzna-Grygiel and Kolodziejczyk (37) found an increase in the activity of oxidative enzymes in the acute and chronic phases of babesiosis infection and this type of activities can be considered as a source of highly reactive free radicals “hydroxyl radicals” being included spontaneously in peroxidation of lipids which lead to damages of cellular membranes, genetic material, and intimately—to death of the host cells. Similarly, a weak reaction for proteins (Millon’s test), in the form of small cytoplasmic granules, was noted in host cell cultures with a flagellate parasite, Trichomonas vaginalis. In addition, eimerian infection causes significant depletion of total protein in the liver and intestine of rabbit and pigeon, respectively (38).

Previous study proved that vaccination with UV irradiated T. gondii tachyzoites resulted in immunological responses against challenge that can maintain normal histochemical characteristics and ameliorates severe depletion of polysaccharides, proteins and DNA of the hepatocytes as a result of acute infection (28). The current work revealed that most hepatocytes in vaccinated non challenged subgroups IIIA and IV-A lost their protein contents due to the toxic and pathologic effect of radiation-attenuated schistosomules. While, In vaccinated –challenged subgroups III-B and IV-B, restoration of...
protein level in hepatocytes was detected and this was more marked in group IV vaccinated with 20 Krad irradiated schistosomulae than in group III vaccinated with 15 Krad irradiated schistosomules.

It could be concluded that irradiated schistosomulae-vaccines can respond positively to reduce the histochemical alterations and mice gain considerable or partial protection against challenge. Also, 20 Krad irradiated schistosomulae gave better immune response so alterations were reduced and the liver parenchyma start to restore its normal level of PAS +ve materials and total proteins.

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