RESEARCH PAPER

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Granulomatous-like immune reaction and hepatic fibrosis induced by *Schistosoma haematobium* immature worms

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Golden hamsters were inoculated with *Schistosoma haematobium* cercariae to examine histological lesions at different time points over an 18 month period of infection. Hamsters were sacrificed 26 weeks and 82 weeks after inoculation. The parasite was found in the blood and in the liver of infected animals as was expected, but we found exclusively male worms, no female worms nor eggs. Interestingly we observed unexpected hepatic lesions induced by *S. haematobium* immature male worms alone in the golden hamster, characteristic of schistosome eggs. Samples from liver, kidneys, lungs, bladder and gastrointestinal tract were collected during necropsy to evaluate injuries induced by *S. haematobium*. Notably we observed hepatitis in the liver of infected hamsters, no lesions were found in other organs. We also found liver fibrosis in infected hamsters. This study provides further experimental evidence for the role that schistosome worms, and their derived antigens, may play in the pathology of the infection and modulation of liver chronic inflammation in the murine model of schistosomiasis.

Introduction

Human schistosomes currently infect more than 200 million people in 76 countries worldwide in the endemic areas of Africa, the Caribbean, Central America, South America, southeastern Asia and the Middle East. Prevalence is thought to be rising mainly due to increasing travelers from the US and Europe to these endemic regions for business or leisure. Wars are also known to increase the impact of schistosomiasis as demonstrated by a case recently published by our group.[1]

Of the three major human schistosome species, *S. haematobium*, causing urinary schistosomiasis, is the most prevalent species in sub-Saharan Africa where it is responsible for a substantial amount of schistosome-associated pathology.[2]

The histopathologic hallmark of an infection with schistosomes is periovular granuloma formation in the primary target organs, e.g., the liver. As the infection ages, important hepatic fibrosis develops, which in murine schistosomiasis is mainly egg granuloma-associated. Schistosomal granuloma formation is generally considered to be the result of a delayed-type hypersensitivity response generated by the host toward antigens secreted by tissue-deposited parasite eggs.[3-6] Schistosomal egg granuloma formation and ensuing hepatic fibrosis are intriguing pathophysiological processes demonstrating the complex interactions that exist between the host and the parasite. Parasite stage-specific factors as well as host organ-specific modalities and the genetic background have been identified or implicated in the pathogenesis of schistosomiasis.[6] However, experimental evidence previously obtained in the murine model of schistosomiasis mansoni also points toward the involvement of adult *S. mansoni* worms and their secreted antigens in the modulation of the egg antigen-induced liver granuloma and in the fibrotic response.[6-12]

In the present work, while studying schistosomiasis-associated inflammation, interestingly we observed unexpected hepatic lesions induced by *S. haematobium* immature male worms in the golden hamster. Here we characterized these lesions and the nature of the local immune response by examining the hepatic inflammatory infiltrate. We demonstrate the induction of hepatic fibrosis and hepatitis, characteristic of egg granulomas, induced by male adult worms of *S. haematobium* alone. This study provides further experimental evidence for the role that schistosome worms, and their derived antigens, may play in the pathology of the infection and modulation of chronic inflammation in the murine model of schistosomiasis.

Results

Animals' general aspect. During the experimental work no animal died and all animals exhibited normal cage activity.

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Table 1. Challenge worm recoveries and antibody production analyzed by IHA

Group	Hamster	Weeks between challenge and perfusion	Nº challenge cercariae	Nº worm recovered	% recovery	IHA titre
Group1 (Control)	1	26	-	-	-	-
	2	26	-	-	-	-
Group 2 (Infected)	3	26	90	23	25.56%	1:160
	4	26	100	26	26.00%	1:160
	5	26	150	41	27.33%	1:320
Group 3 (Control)	6	82	-	-	-	-
	7	82	-	-	-	-
Group 4 (Infected)	8	82	90	24	26.67%	1:160
	9	82	100	28	28.00%	1:160
	10	82	95	25	26.32%	1:160

Table 2. Classification of histopathological data observed in liver sections obtained from hamsters infected with *Schistosoma haematobium*

Group	Hamster	P	PH	F	LPI	BP	E	MT	R
Group1 (Control)	1	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-
Group 2 (Infected)	3	+	++	+++	++	++	++	++	++
	4	+	++	++	++	+++	+++	++	++
	5	+	+++	+++	+++	+++	+++	+++	+++
Group 3 (Control)	6	-	-	-	-	-	-	-	-
	7	-	-	-	-	-	-	-	-
Group 4 (Infected)	8	+	++	++	++	++	++	+	+
	9	+	+++	+++	+++	++	++	+++	+++
	10	+	++	+++	+++	++	++	+++	+++

P, *S. haematobium* in histological section; PH, fibrous perihepatitis; F, lymphoid follicles; LPI, infiltration of lymphocytes and plasma cells; BP, bilharzial pigment; E, infiltration of eosinophils; MT, Masson Trichrome stain; R, Reticulin stain; -, absent; +, mild; ++, moderate; +++, severe.

The weight of the animals remained normal throughout the experiment.

Liver and fecal analysis, urinalysis, total adult worm burden and antibody production. Because Vuong et al.[13] reported that a mice *Mus musculus* infected with *S. haematobium* presented a squamous cell carcinoma of the urinary bladder, suggesting that the eggs from *S. haematobium* must cross the urothelium, while hamsters Mesocricetus displayed diverse lesions in digestive and genital tracts, liver and lungs, we searched the urine content, as well as the fecal content, for eggs to assert the single sex infection with males. All animals had their liver homogenate, feces and urine sediment negative for the presence of eggs as expected. In a time course of infections with *S. haematobium*, worm recovery was similar and remained constant in all groups. All worms were male. The antibody production was analyzed by IHA and found positive in all infected animals (**Table 1**).

Gross lesions. The livers from control groups showed no gross lesions on either the diaphragmatic or the visceral surface. Hamsters from groups 2 and 4 showed several tortuous whitish tracts. No macroscopic changes were observed in other organs. The weight of the organ remained normal throughout the experiment.

Microscopic lesions. No microscopic changes were seen in the spleen, lung, kidney, urinary bladder and gastrointestinal tract in samples obtained from all animals.

No liver histopathological changes were observed in hamsters from control groups. The liver histopathological findings observed in all groups are given in **Table 2**. Liver histopathology of animals infected with *S. haematobium* was characterized by dilated portal vein and marked periportal fibrosis (**Fig. 3B and D**). The number of liver flukes within dilated portal vein varies markedly and we did not observed any egg. **Figure 1B** shows a transverse section through a flat trematode worm within a dilated portal vein. Intestinal canal lined by simple columnar epithelium and viteline glands are seen.

Immunological worm reaction. Periworm granuloma-like formation occurred in all infected animals. The worms were lodged in portal veins of the liver (**Fig. 1B**). Granulomas of schistosome-infected hamsters are composed of numerous macrophages, eosinophilic granulocytes, lymphocytes and fibroblasts. Similar dominant cell types were characterizing the granulomatous-like reaction around the worms, thus closely resembling the hepatic granulomas induced by schistosome eggs although without the concentric layers (**Fig. 1B–D**).

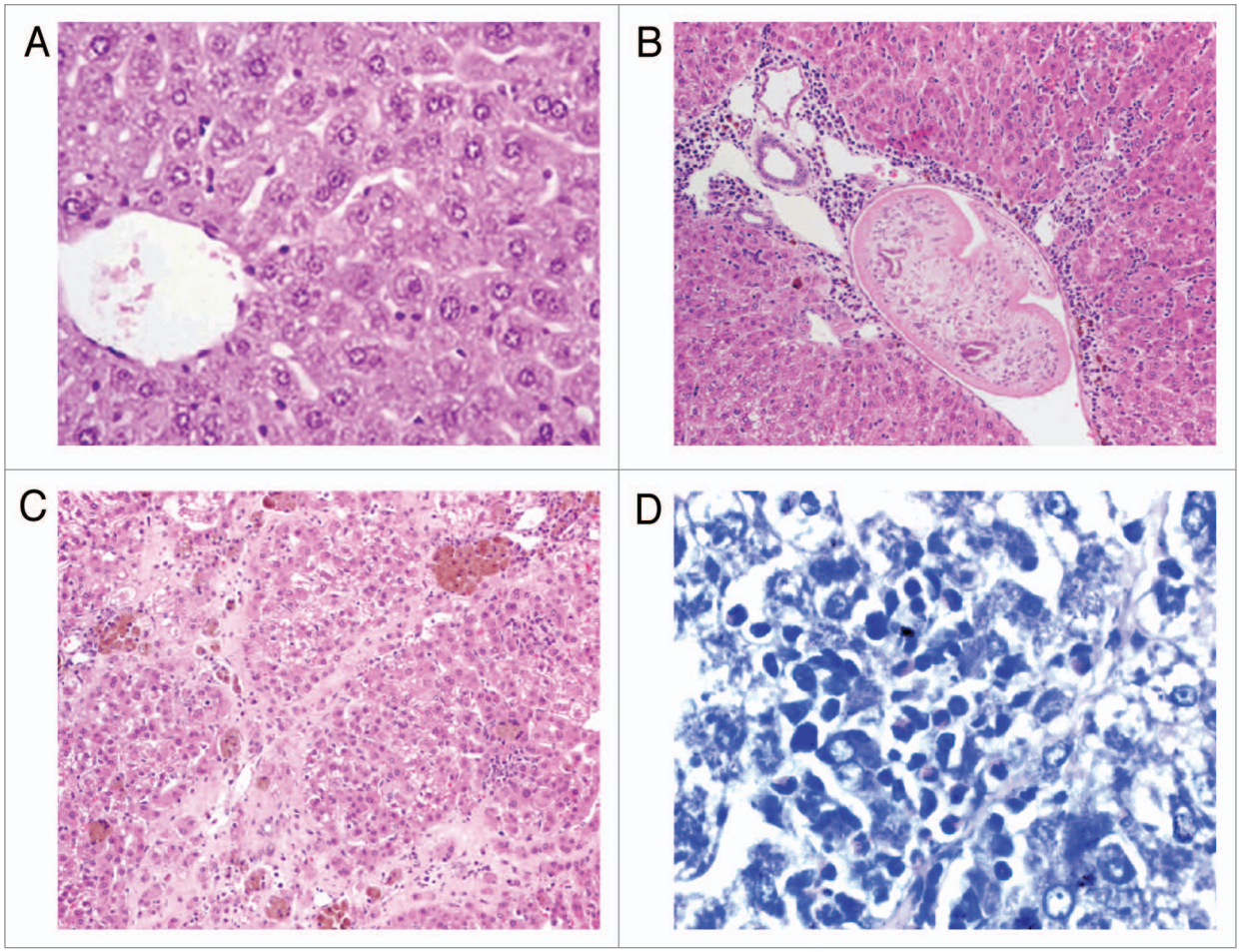

Figure 1. Histopathology of parasitic liver. (A) Control; (B) Liver section of *Schistosoma haematobium*-infected hamster, H&E, 400X; (C) Macrophages with abundant bilharzial pigment, H&E, 200X; (D) Eosinophiles infiltrate, Wright, 1000X.

Macrophages, fibroblasts and lymphocytes, and eosinophils were found to infiltrate along the outer layer of the hepatic induced granuloma-like. These histopathology appearances represent an early advanced feature of granuloma formation. Cellular infiltration was very apparent in the livers of infected animals. A slight to moderate inflammation was seen both within centrolobular vein and within portal areas (lymphocytes, plasma cells and macrophages with abundant bilharzial pigment) (**Fig. 1C**). Lymphoid follicles were often present in portal areas. These lesions were more severe in hamsters from group 4 than in those of group 2. Diffuse infiltration of eosinophils was often observed in areas of hepatic parenchyma and portal spaces (**Fig. 1D**).

The granules observed in the interior of macrophages were negative for hemosiderosis, bile granules or other type of infection as observed by the negative staining of PAS, Prussian Blue, Fouchet and Ziehl-Nielsen (**Fig. 2**).

Hepatic fibrosis. Schistosomiasis lead to a pathobiochemical reaction termed liver fibrosis. Thus, histological examination of a liver biopsy is essential for a diagnosis of liver fibrosis. All infected animals showed well-developed fibrosis. **Figure 3A** represents normal liver Masson Trichrome stain. The liver section showed in **Figure 3B** represents liver fibrosis, with portal-portal fibrous bridging and fibrous septa, connecting portal areas to each other's and lobule centers, hepatocytes were separated by blue colored collagen sheath. The aminopeptide of type III procollagen is the most widely used parameter. The reticulin technique was used to identify procollagen type III. In **Figure 3C** we may observe the normal trabecular pattern in the hamster liver sections stained for reticulin, which outlines the walls of sinusoids. Plates of hepatocytes in the normal adult liver are mostly one cell thick. In animals infected with *S. haematobium* we observe thin fibrous septa which tend to from bridges between damaged centrolobular zones (**Fig. 3D**).

Discussion

This study produced a very surprising finding: the consistent development of granulomatous-like immune reaction and fibrosis caused by adult worms of *S. haematobium,* characteristic of schistosome eggs. This detailed morphological study of hepatic lesions that occur in the hamster model of schistosomiasis is a contribution to understanding the pathogenesis of hepatic schistosomiasis. In the present work histopathological and histochemical studies were performed on the different organs. Histopathology

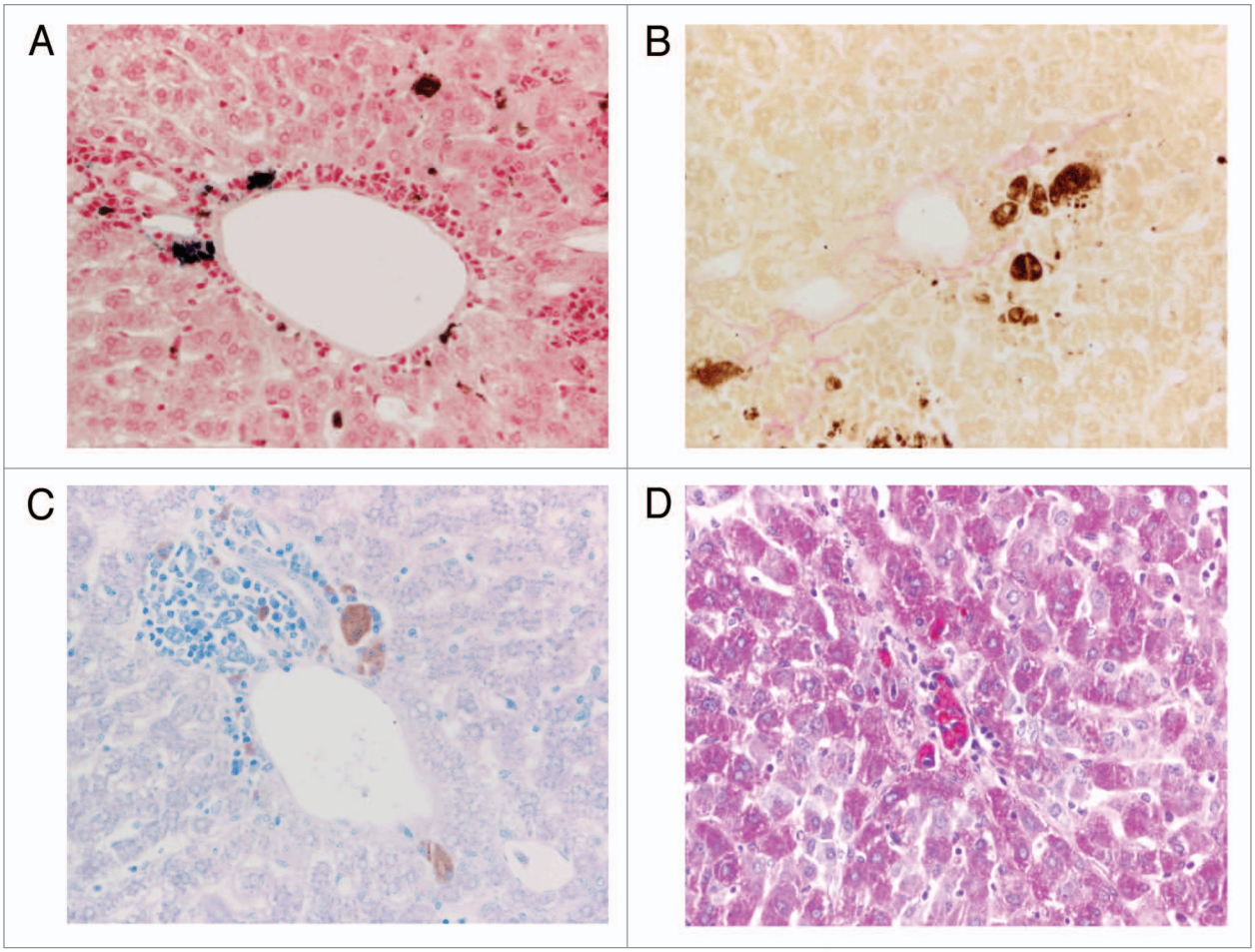

Figure 2. Macrophage granules. (A) Prussian blue, 400X; (B) Fouchet, 400X; (C) Ziehl-Nielsen, 400X; (D) PAS, 400X.

of liver sections from all infected hamsters revealed the presence of *S. haematobium* adult worms and fibrous perihepatitis. In addition, we recorded infiltration of eosinophils, plasma cells, macrophages with bilharzial pigment and fibroblasts. These features are the characterization of granulomas. Because these features, fibrosis and multinuclear cell formation are both hallmarks of granuloma development.[14] Our experimental single sex infection model revealed that adult worms had striking effects of granuloma-like development characterized by cellularity and/or cell composition (monocytes/macrophages, neutrophils and eosinophils) and fibrosis. In this experimental work, we did not observe *S. haematobium* eggs, this is explained by the inexistence of females during the experimental infection.

We have previously demonstrated that *S. haematobium* worm extract (Sh) has the potential to induce tumor development in a xenograft model, in which Sh-treated Chinese Hamster Ovary (CHO) cells formed tumors with similar phenotypes in all inoculated nude mice.[15] We have also demonstrated that Sh is likely to participate in a number of carcinogenesis mediated processes, such as increased cell proliferation and loss of p27, decreased apoptosis and increased expression of Bcl-2, and increased migration and invasion, all of which are processes needed for cancer cell survival,[16] as well as inducing dysplasia, a low grade intra-epithelial neoplasia, in the bladders of CD-1 mice instilled with Sh.[17] Although we do not yet fully understand the host-parasite interactions of *S. haematobium* adult worms, or the immunological mechanism in associated fibrosis, the present study revealed intriguing novel aspects. There is no report in the literature that associates *S. haematobium* adult worms to hepatic fibrosis. But in schistosomiasis mansoni Baki et al.[18] reported hepatic fibrosis and histopathological lesions in mice experimentally infected with male *Schistosoma mansoni*. These authors revealed that, from the 25th week post-infection, a diffuse fibrosis affected the main branches of the portal vascular system following the host inflammatory reaction, associated with the proliferation of myofibroblasts in situ.[19] An increase of fibrotic deposit occurred during chronic unisexual infection suggesting that antigenic substances secreted by adult schistosomes, in the absence of any eggs, might initiate periportal and perisinusoidal fibrous reaction, confirming the results in the present paper.[19] It is generally accepted that the main lesions in established and chronic infection are due not to the adult worms but to eggs that are trapped in the tissues. The eggs secrete proteolytic enzymes that provoke typical eosinophilic inflammatory and granulomatous reactions, which are progressively replaced by fibrotic deposits.[20] It is intriguing that the worms caused the alterations described above in liver

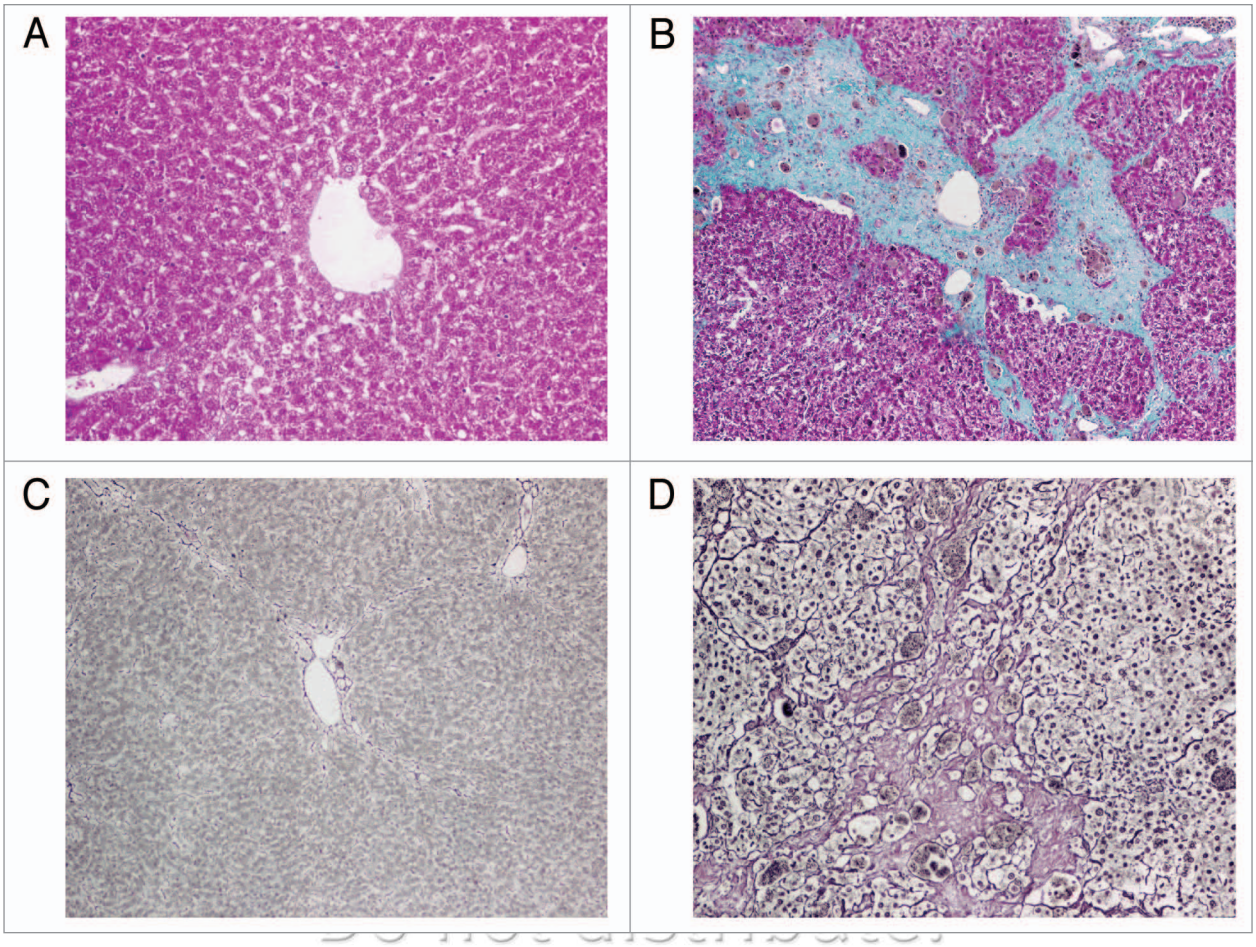

Figure 3. Liver fibrosis (A) Hamster liver section with normal Masson Trichrome stain, Trichrome stain, 200X; (B) Portal-portal fibrous bridging and fibrous septa, connecting portal areas to each others and lobule centers, Masson's trichrome stain was used. Collagen fibres show blue staining. Magnification 400X. (C) Normal reticulin pattern, reticulin stain, 100X; (D) black lines between hepatocytes, hamster with hepatic fibrosis, reticulin stain, 200X.

tissue similar to the ones described in egg granuloma-associated fibrosis. The most studied feature in the pathology of schistosomiasis is currently the immunogenicity of the egg antigens. Schistosome eggs express various glycosylated proteins and lipids that are able to induce humoral and cellular immune reactions.[21] Nevertheless, schistosome worms and eggs antigens share common molecules.[21,22]

We offer one possible explanation for the worm-induced granuloma-like and fibrosis in our model. In the course of an infection, it is established that the immune response progresses through at least three phases. In the first 3–5 weeks, during which the host is exposed to migrating immature parasites, the dominant response is T helper 1 (Th1)-like. As the parasites mature, mate and begin to produce eggs at weeks 5–6, the response alters markedly; the Th1 component decreases and this is associated with the emergence of a strong Th2 response. Immature dendritic cells (DCs) can acquire schistosome egg antigens and induce Th2 responses, but the process by which this occurs is unclear. Primarily, this response is induced by egg antigens.[23] It is possible that in the absence of eggs DCs acquire schistosome worm antigens instead and induce also a Th2 response. In accordance with our explanation it is known that established worms elicit an immune response which prevents continuous further accumulation of worms while themselves remaining invulnerable to immunological attack.[24] In accordance we found positive immune reaction detected by IHA in all infected animals.

In contrast, Moloney and collaborators,[25] demonstrated that schistosome female worms from single sex infections induced no overt pathology in the host. Male worm burdens from these single sex infections induced distended hepatic portal veins and marked deposition of pigment in the livers of infected mice but induced neither hepatic lesions nor immune cell infiltrate. On the other hand, in agreement with our work, Jacobs et al.[6] demonstrated the positive modulation of hepatic *S. mansoni* egg antigen-induced granuloma formation and periparticulate fibrosis by living adult *S. haematobium* worms, resulting in more severe liver pathology. These researchers demonstrated the complex interactions that exist between parasite and host. Once more, the implication of the schistosome worm as a *primum movens* in the genesis and modulation of fibrosis has been demonstrated and therefore Schistosome worms deserve our full attention in the unraveling of the mechanisms underlying schistosome infection and pathology.[6]

The similarity in immune response seen in mice infected with *S. haematobium* worms compared to the immune response caused by eggs might provide important clues regarding the nature of the disease. While the use of egg-induced granulomas are the most common model to study fibrosis, to the best of our knowledge, experimental data using worms to study hepatic fibrosis have not been published. In the present study we employed single sex adult worms' infection of *S. haematobium* to clarify the stage specific roles of adult worms. Specifically, we looked for similarities of *S. haematobium* adult worm induced hepatic granuloma-like development and known egg-induced granuloma. The results of this study strongly indicate crucial roles for adult worms of *S. haematobium* in chronic stage of hepatic granuloma formation, especially in regards to fibrosis and the inflammatory cell types present.

In this in vivo study we demonstrated the positive modulation of hepatic fibrosis and hepatitis by living adult *S. haematobium* worms alone. These results reinforce the applicability of hamsters as a suitable model of fibrosis and hepatitis and provide new insights into the response of experimental models to *Schistosoma haematobium* infection.

Material and Methods

Animals. Eight-week-old female golden hamsters (LVG/SYR) were provided by Charles River (Barcelona, Spain). Animals spent one week being acclimated under routine laboratory conditions before starting the experiments. They did not receive any treatment prior to the study. Hamsters were kept in separated cages and fed standard balanced food and water ad libitum. All the animals were raised and maintained at the National Institute of Health (Porto, Portugal) in rooms with controlled temperature (22 ± 2°C) and humidity (55% ± 10%) and continuous air renovation. Animals were housed in a 12 h light/12 h dark cycle (8 am–8 pm). All animal experiments were performed in accordance with the National (DL 129/92; DL 197/96; P 1131/97) and European Convention for the Protection of Animals used for Experimental and Other Scientific Purposes and related European Legislation (OJ L 222, 24.8.1999).

Parasites. *S. haematobium* (Angolan strain) life cycle was maintained through successive passages in laboratory-raised *Bulinus truncatus* as invertebrate hosts and golden hamsters as vertebrate hosts. Cercariae of *S. haematobium* were obtained from infected snails by the use of artificial light.

Experimental infection. Ten hamsters were divided into four groups. Groups 1 and 3 (n = 2) were control groups. Groups 2 (n = 3) and 4 (n = 3) were infected with *S. haematobium*. Groups 1 and 2 were sacrificed 26 weeks after infection; group 3 and 4 were sacrificed 82 weeks after infection. Hamsters were experimentally infected by transcutaneous route with approximately 100 cercariae of *S. haematobium*. For the duration of the study, the hamster's state of health was monitored daily.

Animal's liver, stool and urine analysis. For the stool and urine collection animals were kept in single mouse metabolic cages (Tecniplast, Buguggiate, Italy) for 24 hours. The animals were acclimatized for 12 hours per day, 2 days before the 24 hours assay. This cage allows absolute and immediate separation of feces and urine. After the 24 hours assay feces and urine was collected and analyzed for the presence of eggs. The livers were removed from freshly killed mice. The organs were digested in 4% potassium hydroxide at 56°C and centrifuged (900 g) for 5 min. For oogram performance, duplicate 100 μL aliquots of the digest were placed on a glass slide and eggs were searched by light microscopy (100X).

Serum antibody determination by indirect haemagglutination (IHA). Sera were obtained from blood samples collected from infected animals at euthanasia. Mice were individually tested for the quantitative detection of the antibodies present in their sera. Determination of antibodies specific for Schistosoma was performed as previously described.[27] The IHA schistosomiasis kit obtained from Fumouze Laboratories (Levallois-Perret, France) was used according to the instructions of the manufacturer. Briefly, the test procedure was as follows. Fifty microliters of a 1:20 initial dilution of each serum was subjected to further twofold serial dilutions, and 10 μl of sheep red blood cells sensitized with *S. mansoni* immature worm antigens was added to each diluted sample. Positive and negative control sera and nonsensitized red blood cells were included in each test as controls for naturally occurring antibodies. After incubation for 2 h at room temperature the titer in the test serum was recorded as one dilution before that which yielded a clear, sharp dark spot similar to those in the negative control wells. Titers were expressed as reciprocal values. All sera were tested in duplicate. The results were evaluated with a cutoff titer of 1:160 as recommended by the manufacturer.

Total immature worm recovery. The immature worm burden was determined by total blood perfusion and the numbers of worms determined. Immature worms of *S. haematobium* were recovered at 26 and 82 weeks after challenge by perfusion of the hepatic portal system via the aorta with citrated saline. Hamsters were perfused under anesthesia through the heart with citrated phosphate-buffered saline and the worms were recovered. Worm burdens were estimated after portal perfusion through an incised portal vein of infected mice euthanized by an anesthetic overdose.

Macroscopy. At the time of perfusion, complete necropsies were carefully conducted. After opening the abdomen, all organs were examined macroscopically for any changes; the organs were collected, weighed and immersed in 10% phosphate buffered formalin. Representative fragments of all organs were fixed in buffered formalin 10%. Tissue sections (2 μm) were stained with each of the following: haematoxylin and eosin (HE); Wright for eosinophiles, Ziehl-Nielsen, PAS, Fouchet and Prussian Blue for macrophage granules and reticulin and Masson trichrome for collagen.

Masson's trichrome. Masson's trichrome staining protocol is used to stain collagen fibers. Liver sections were deparaffinized and rehydrated through 100%, 95% and 70% alcohol. Then were washed in distilled water and stained in Weigert's iron haematoxylin working solution for 10 minutes. After sections were rinsed in running warm tap water for 10 minutes and washed in distilled water. Sections were after stained in Biebrich scarlet-acid fuchsin solution for 15 minutes and washed in distilled water,

differentiated in phosphomolybdic-phosphotungstic acid solution for 15 minutes. Sections were then transferred directly (without rinse) to aniline blue solution and stained for 5–10 minutes. Rinsed briefly in distilled water and differentiated in 1% acetic acid solution for 2–5 minutes. Later were washed in distilled water and dehydrated through 95% ethyl alcohol, absolute ethyl alcohol and clear in xylene. Finally were mounted with resinous mounting medium.

Reticulin. The reticulin technique was based on the following technique: liver sections were deparaffinized in xylene then took through alcohols to water. Sections were oxidized in acidified potassium permanganate during 3 minutes, rinsed in distilled water and decolorized with 2% oxalic acid for 1 minute. Sections were rinsed again in distilled water, placed in mordant in 4% iron aluminium for 10 minutes, and rinsed in distilled water. Sections were impregnated in ammoniacal silver solution for 11 seconds and quickly rinsed in distilled water. Sections were immediately reduced with 10% aqueous formalin for 2 minutes and washed in running tap water for 2 minutes. Sections were toned in 0.2% gold chloride for 2 minutes and rinsed in distilled water, fixed with 2% aqueous sodium thiosulphate (hypo) for 2 minutes and washed in tap water for 2 minutes. Sections were counterstained with neutral red for 2 minutes, dehydrated, cleared and mounted.

Histological analysis. A histological study was conducted on H&E-stained tissue sections to evaluate the following parameters: presence of *S. haematobium* immature worms in histological section; fibrous perihepatitis, lymphoid follicles, infiltration of lymphocytes and plasma cells; and the presence of bilharzial pigment. Wright-stained tissue sections were used to evaluate infiltration of eosinophils. Masson Trichrome and Reticulin stains were evaluated. To assess the severity of hepatic lesions, two researchers evaluated tissue sections independently, as follows: -, absent; +, mild; ++, moderate; +++, severe.

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