MORPHOMETRICAL AND IMMUNOPATHOLOGICAL DISSECTION OF THE HEPATIC *SCHISTOSOMA HAEMATOBII* GRANULOMA IN THE MURINE HOST

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Summary :

This paper presents the results of a study aimed at identifying the immunopathological and morphometrical characteristics of liver granulomas induced by the parasite *Schistosoma haematobium*. The OF1 mouse is a susceptible host for experimental infection with the Egyptian NAMRU-3 strain of *S. haematobium*. Formation of liver granulomas started after 13-14 weeks of infection. These hepatic granulomas reached a maximum volume after 20 weeks of infection (MGV: $1.82 \pm 0.15 \times 10^7 \mu\text{m}^3$) and regressed thereafter ($1.55 \pm 0.16 \times 10^7 \mu\text{m}^3$ after 24 weeks). Strong cellular upregulation of the adhesion molecules ICAM-1, LFA-1, syndecan-1, VLA-4, but not VLA-6 is seen, both in acute and chronic granulomas. Finally, pathological differences exist between hepatic *S. mansoni* and *S. haematobium* granulomas particularly at the level of extracellular matrix deposition. *S. haematobium* induced peri-ovular deposition of mainly fibronectin and to a lesser extent type I and IV collagen.

KEY WORDS : *Schistosoma haematobium*, granuloma, fibrosis, adhesion molecules, morphometrics, mouse.

Résumé : DISSECTION MORPHOMÉTRIQUE ET IMMUNO-PATHOLOGIQUE DU GRANULOME HÉPATIQUE DE *SCHISTOSOMA HAEMATOBII* CHEZ LA SOURIS

Cette étude présente les résultats d'une investigation immuno-pathologique et morphométrique du granulome généré par le parasite *Schistosoma haematobium* dans le foie de la souris. La souris blanche OF1 est un hôte susceptible pour l'infection expérimentale de la souche Égyptienne NAMRU-3 de *S. haematobium*. La formation des granulomes dans le foie débute après 13-14 semaines d'infection. Les granulomes hépatiques atteignent un volume maximal après 20 semaines d'infection (MGV: $1,82 \pm 0,15 \times 10^7 \mu\text{m}^3$) et puis régressent progressivement dans le temps (MGV $1,55 \pm 0,16 \times 10^7 \mu\text{m}^3$ après 24 semaines). L'induction cellulaire forte des molécules adhésives ICAM-1, LFA-1, syndecan-1, VLA-4, mais pas de VLA-6 est observée dans le granulome aigu et chronique. Finalement, des différences pathologiques existent entre le granulome hépatique de *S. mansoni* et *S. haematobium*, particulièrement au niveau de l'accumulation des molécules de la matrice extracellulaire. *S. haematobium* induit une accumulation majeure péri-ovulaire de fibronectine et mineure de collagène types I et IV.

MOTS CLÉS : *Schistosoma haematobium*, granulome, fibrose, molécules d'adhésion, morphométrie, souris.

INTRODUCTION

The blood fluke *Schistosoma haematobium* is a human parasite, widely distributed in more than 50 countries of Africa and Asia (WHO, 1993). The clinically important lesions in humans infected with *S. haematobium* are generally confined to the urogenital system. In experimental animals, lesions of the urogenital tract are the exception, although they may occur in infected primates (Cheever, 1985). Only a limited number of experimental investigations or comparative studies with other

schistosome infections have been carried out with respect to the pathogenetic mechanisms of granuloma formation and fibrosis in *S. haematobium*-infected animals. Convenient experimental models for the study of tissular damage induced by *S. haematobium* are still a matter of research and debate and both infected primate (Sadun *et al.*, 1970; Cheever, 1985) and rodent (Imbert-Establet *et al.*, 1992; Vuong *et al.*, 1996) animals have been investigated. The murine model presents unique features in its host's cellular response towards antigenic *S. haematobium* determinants. Egg deposition in the liver with peri-ovular granuloma formation and hepatic fibrogenesis are characteristics of murine infection (Cheever *et al.*, 1983). Although *S. haematobium* eggs may frequently be found in the liver of man, hepatic fibrosis appears not to be a prominent feature of human infection (Cheever *et al.*, 1974). Hashem (1947) and Elwi & Attia (1962) observed 10-15% "bilharzial cirrhosis" induced by *S. haematobium* compared to 37-72% during *S. mansoni* infection. This interspecies discrepancy in host-parasite interaction may be a useful tool for the elucidation

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tion of the differential mechanisms underlying granulomogenesis and fibrogenesis in human and experimental schistosome infections.

Granulomatous inflammation is one of the most complex cellular responses towards pathogenic antigens, requiring cellular interaction between inflammatory cells and activated endothelial cells and between immunocompetent cells mutually or with extracellular matrix proteins. Cell-cell interactions can be mediated by binding of intercellular adhesion molecule-1 (ICAM-1) with its reciprocal-receptor lymphocyte function-associated antigen-1 (LFA-1) (Springer, 1990) while the very late antigens (VLA) 4 and 6 can mediate cellular interaction with the matrix molecules fibronectin and laminin respectively (Shimizu *et al.*, 1990). Previously, several authors have pointed out the important role cellular adhesion molecules play in both the genesis and modulation of the *S. mansoni* granuloma (Lukacs *et al.*, 1994; Secor *et al.*, 1994; Langley & Boros, 1995; Ritter & McKerrow, 1996; Jacobs *et al.*, 1997a,b, 1998a,b).

The aim of this study was to perform a more in depth morphological investigation of the immunopathological characteristics of hepatic granulomogenesis and fibrogenesis in the *S. haematobium* infected murine host. Special attention was paid to the involvement of various adhesion molecules in granulomogenesis and differences in extracellular matrix deposition between *S. mansoni*- and *S. haematobium*-infected mice.

MATERIALS AND METHODS

EXPERIMENTAL *S. HAEMATOBIIUM* INFECTIONS

Swiss, male OF1 mice, six weeks of age, (Iffa Crdo, St. Germain sur L'Arbresle, France) were infected with 150 cercariae per mouse of the Egyptian NAMRU-3 (Cairo) strain of *S. haematobium* via the transcutaneous route. Mice were provided with water and food ad libitum. The animals were sacrificed after 13, 16, 20, and 24 weeks of infection respectively by an overdose of pentobarbital injected intraperitoneally. Mice were perfused (transected portal vein) with citrated phosphate buffered saline and the worms were collected.

Tissue samples were taken from the liver and snap frozen in liquid nitrogen or fixed in 4% formaldehyde for conventional histological processing. All animal experiments were conducted under supervision of an animal welfare officer according to the ethical guidelines of the institution.

MORPHOMETRY

Stereological volume-estimation using point-sampled intercepts has proven a useful and simple method for

estimating schistosome granuloma volume (Jacobs *et al.*, 1997b; Jacobs *et al.*, 1998a). This method offers the advantage of volume estimation without any assumption of the granuloma shape, location, or the mode of histological sectioning (Gundersen & Jensen, 1983). Using point-sampled intercepts Mean Granuloma Volume (MGV; Jacobs *et al.*, 1997b) was estimated. The practical methodology of MGV estimation was described previously (Jacobs *et al.*, 1997b). Single, non-confluent granulomas containing at least one ovum were measured. An average of 60 liver granulomas per animal were measured. Each experimental group consisted of six infected mice. An image analysis system, specifically designed for stereological measurements was used (Stereology 2.0, Kinetic Imaging Ltd., Liverpool, UK).

IMMUNOHISTOCHEMISTRY

Three conventional immunohistochemical techniques were applied for the immunodetection of antigens: the fluoro-isothiocyanate (FITC) fluorescence technique, the indirect immunoperoxidase (PEROX) and alkaline phosphatase/anti-alkaline phosphatases (APAAP) technique. Immunohistochemical testing was performed as follows. Five micrometer thick liver sections were cut, mounted on PLL-coated slides and air-dried. Polyclonal rabbit anti-mouse antibodies directed against type I collagen, type IV collagen and laminin (Institut Pasteur, Lyon, France, dilution 1:200) and fibronectin (Telios Pharmaceuticals Inc., San Diego, USA, dilution 1:200) and monoclonal rat anti-mouse antibodies directed against ICAM-1, LFA-1 (R & D Systems, Minneapolis, USA, dilution 1:100), F4/80 [macrophages], VLA-6, and CD40 [B-cells] (Serotec, Oxford, UK, respective dilutions 1:200, 1:50, and 1:50), Syndecan-1 (Pharmingen, San Diego, USA, dilution 1:320), and VLA-4 (Southern Biotechnologies, Birmingham, USA, dilution 1:30) were used. Primary antibodies were applied overnight at 4 °C. Afterwards, the slides were washed extensively and incubated with the secondary antibody [FITC labelled goat anti-rabbit IgG antibodies (Jackson ImmunoResearch Laboratories Inc., West Grove, USA), peroxidase conjugated goat anti-rat IgG antibodies (Rockland, Gilbertsville, USA) or rabbit anti-rat immunoglobulins (Dako, Glostrup, Denmark/APAAP)] and tertiary antibody (rat APAAP complex, Dako) according to the manufacturer's guidelines. The reaction product was visualized by incubation with diaminobenzidine tetrahydrochloride (Sigma/PEROX) or the Vectastain SK-5100 kit (Vector, Burlingame, USA/APAAP). Slides were counterstained with haematoxylin and mounted in DPX (Fluka Chemika-Biochemika, Buchs, Switzerland/ PEROX, APAAP) or mounted in PBS/glycerine (FITC).

Fig. 1. – Extracellular matrix proteins. Deposition of fine, granular type I collagen (A) and relative abundant type IV collagen (B) is seen around deposited *S. haematobium* eggs. Abundant deposition of broad bands of fibronectin (C) is observed. Deposition of laminin (D) in *S. haematobium* granulomas was poor, except for encroached blood vessels at the periphery of the expanding granuloma (FITC stain, bar = 50 μ m).

RESULTS

PARASITOLOGY

All mice were infected, but carried relatively low numbers of *S. haematobium* worms. An average of 8.8 worms (range 5-11) per mouse with a predominance of male worms was seen. The mean worm recovery did not alter significantly over the period 16-24 weeks.

The liver weight/total weight and spleen weight/total weight ratios were higher at 20 weeks when compared to 16 weeks [liver: 0.054 $\pm$ 0.008 and 0.044 $\pm$ 0.003 respectively ($P = 0.05$) and spleen: 0.0067 $\pm$ 0.0017 and 0.0043 $\pm$ 0.0006 respectively ($P = 0.04$)].

HISTOPATHOLOGY

Hepatic egg deposition and starting granuloma formation were first observed after 13-14 weeks of infection. Clusters of *S. haematobium* eggs were seen in many granulomas. Granulomas were composed of abundant eosinophilic granulocytes. In addition, immunohistochemically, many granuloma cells were immunoreactive with the F4/80 antibody, indicating their macrophage nature. CD40 positive B-cells were seen in the *S. haematobium* granuloma, located mainly at the periphery of the granuloma (Fig. 1,H). Areas of parenchymal necrosis were sometimes present. Tissue extracellular matrix (ECM) deposition was mainly granuloma-associated, while portal fibrosis was rather limited. Schistosomal pigment was present in parenchymal Kupffer cells and granuloma macrophages. A moderate degree of portal inflammation, consisting of eosinophilic granulocytes and macrophages, was present.

EXTRACELLULAR MATRIX PROTEINS

Collagen I immunoreactivity in the liver granuloma was sparse and could only be observed as fine, granular strands around deposited ova (Fig. 1,A). Relatively abundant deposition of the basement membrane com-

	Sinusoids	Granuloma	Blood vessels
Collagen type I	+++	+	+++
Collagen type IV	-/+	++	++
Fibronectin	+++	+++	+++
Laminin	+	-/+	+++

- : no reactivity; + : weak reactivity; ++ : moderate reactivity; +++ : strong reactivity.

Table I. - Immunoreactivity for ECM molecules in hepatic *S. haematobium* granulomas 24 weeks post-infection.

ponent type IV collagen was seen in the granuloma, mainly at the periphery (Fig. 1,B). Fibronectin was the dominant ECM protein deposited in *S. haematobium* granulomas. Deposition of thick, homogeneous bundles of fibronectin was observed (Fig. 1,C). Little or no deposition of laminin was seen in liver granulomas. Some laminin immunoreactivity could be seen at the periphery of the granuloma, representing blood vessels encroached by the expanding granuloma (Fig. 1,D). No qualitative differences were immunohistochemically observed over the time period 16-24 weeks post-infection. Negative controls with an unrelated polyclonal antibody were performed in all cases and did not show any immunoreactivity. These results, as well as the constitutional expression of these antigens in the liver are summarised in Table I.

ADHESION MOLECULES

Cellular upregulation in the hepatic granuloma of the adhesion molecule ICAM-1 (Fig. 2,E) and its counter receptor LFA-1 (Fig. 2,F) was seen, both at the early (16-20 weeks) and late (24 weeks) stage of infection. Upregulation of the β integrin adhesion molecule VLA-4, but not of VLA-6 was seen in hepatic *S. haematobium* granulomas. Granuloma-associated VLA-6 immunoreactivity was only observed in blood vessels encroached by the expanding granuloma. A subpopulation of syndecan-1 immunoreactive antigen-stimulated B-cells

	Sinusoidal endothelium	Kupffer cells	Portal tract inflammatory cells	Hepatocytes	Granuloma inflammatory cells	Blood vessels (EC)
ICAM-1	+++	+++	+++	-	+++	+++
LFA-1	-	+++	+++	-	+++	-
VLA-4	-	+	+++	-	+++	-
VLA-6	++	-	+	-	+	+++
Syndecan-1	++	++	-	-	++	-
F4/80	-	+++	+++	-	+++	-
CD40	-	-	-	-	++	-

- : no reactivity; + : weak reactivity; ++ : moderate reactivity; +++ : strong reactivity; (EC) : endothelial cells.

Table II. - Immunoreactivity for various cell surface molecules in liver tissue and *S. haematobium* granulomas.

Fig. 2. – Adhesion molecules. Strong upregulation of ICAM-1 (E, peroxidase stain) and LFA-1 (F, APAAP stain) is seen in *S. haematobium* granulomas. A subpopulation of syndecan-1 immunoreactive cells was seen, located mainly at the inner part of the granuloma (G, peroxidase stain). These cells differed from unstimulated B-cells located mainly at the periphery of the granuloma (H, APAAP stain) (bar = 50 μ m).

Weeks post infection	MGV
16	1.61 +/- 0.30
20	1.82 +/- 0.15
24	1.55 +/- 0.16

Table III. – MGV and standard deviation ($10^7 \mu\text{m}^3$) of hepatic *S. haematobium* granulomas at various stages in time.

(Jacobs *et al.*, 1998b) was seen, located in the inner part of the granuloma, in the vicinity of deposited *S. haematobium* eggs (Fig. 2, G). These cells differed from unstimulated CD40 positive B-cells (Fig. 2, H). These results, as well as the constitutional expression of these adhesion molecules in the liver are summarized in Table II.

MORPHOMETRY

Granuloma volumes were estimated 16, 20, and 24 weeks after infection. Hepatic granuloma volume was maximal after 20 weeks of infection, yielding a mean granuloma volume of $1.82 \pm 0.15 \times 10^7 \mu\text{m}^3$. At the later stage of infection (24 weeks), hepatic granulomas slightly, but significantly, regressed in volume (MGV: $1.55 \pm 0.16 \times 10^7 \mu\text{m}^3$) ($P = 0.002$ compared to 20 weeks). Results are presented in Table III.

DISCUSSION

Although in our experiments the Swiss OF1 mice was a susceptible host for infection with *S. haematobium* with resultant peri-ovular granuloma formation and subsequent fibrosis, only low numbers of adult worms were recovered at the time of sacrifice. Our findings are consistent with the observations by Moore & Meleney (1954) and Cheever *et al.* (1983) indicating the mean worm return to be usually below 10% in mice. Agnew and co-workers (1988) however were able to demonstrate that CBA mice were highly permissive for infection with *S. haematobium*. Care however should be taken when interpreting these results since at least two confounders can be identified. Besides the different genetic backgrounds of the murine hosts used by various investigators, the genetic character of the different *S. haematobium* strains used – e.g. different geographic origin – should be taken into consideration (James & Webbe, 1973, 1975; Vera *et al.*, 1990). The tissue location of worms and eggs appears to be dependent on the type of host. Cheever (1985) reported that in primates *S. haematobium* worms were often found in the urinary tract venous plexuses, while in rodent species worms can be located mainly in the mesenteric veins or both in the mesenteric and urinary tract venous plexuses

(Vuong *et al.*, 1996). In our experiments, we detected worms only in the mesenteric veins and eggs were lodged mainly in the liver and intestine, but not in the bladder.

Using stereological volume estimation, we could demonstrate that OF1 mice mounted a maximal granulomatous response 20 weeks after infection, which corresponded to six-seven weeks of hepatic ovum deposition. These granulomas slightly, but significantly regressed in the course of infection. Similar data based on granuloma surface area or diameter measurements were also reported in *S. haematobium*-infected hamsters (Erickson *et al.*, 1974) or albino mice (Michael *et al.*, 1979). In the mouse, peak hepatic *S. mansoni*-induced granuloma formation occurs at approximately eight weeks of infection and decreases during chronic infection due to a mechanism called immune modulation (Colley, 1975). The data we present in this paper further provide evidence for the fact that the phenomenon of hepatic granuloma modulation can also be seen in *S. haematobium*-infected mice. The underlying mechanisms of granuloma modulation are currently incompletely understood. In *S. haematobium*-infected mice, the intensity of experimental infection appears to be one of the modulating factors of hepatic pathology (Cheever, 1986).

Our investigations further illustrate that in the murine host, *S. haematobium* induces marked peri-ovular, hepatic, granuloma-associated fibrosis. This feature seems to be species-specific since minimal fibrosis around liver-deposited *S. haematobium* eggs is seen in man, primates, and hamsters (Sadun *et al.*, 1970; von Lichtenberg *et al.*, 1973; Cheever, 1985). In contrast, significant hepatic fibrogenesis induced by *S. haematobium* was described in the murine host (Cheever *et al.*, 1983). Qualitative differences in hepatic ECM deposition exist between *S. haematobium* and *S. mansoni*-infected mice. In previous work we demonstrated immunohistochemically that *S. mansoni* egg antigens induced abundant deposition of both interstitial matrix proteins (type I collagen and fibronectin) and basement membrane components (type IV collagen and laminin) (Jacobs *et al.*, 1997b, 1998b). In contrast, in *S. haematobium* granulomas florid abundant deposition of fibronectin and type IV collagen was seen, while only little type I collagen and no laminin deposition was observed. Furthermore, whereas portal fibrosis can be seen in aged *S. mansoni* infections, particularly in mice carrying light infections (Andrade & Cheever, 1993) this was not the case in our experiments with *S. haematobium*. Clearly, antigenic moieties from different schistosome species induce different fibrogenic responses. These responses differ not only between the different hosts, but are also qualitatively distinct. This indicates that scar formation is a highly

dynamic tissular reaction pattern against harmful antigens and not a pathological "dead-end" (Wyler, 1992). Adhesion molecules constitute essential elements, not only in the genesis of the *S. mansoni* granuloma (Jacobs *et al.*, 1997a, b, 1998a) but also in the *S. haematobium* granuloma as demonstrated in this study. In *S. mansoni* infections, upregulation of ICAM-1 expression in the schistosomal granuloma is induced by egg-secreted antigens (Ritter *et al.*, 1994; Jacobs *et al.*, 1997a, b) and is mediated by tumour necrosis factor α (Lukacs *et al.*, 1994). T-lymphocyte responsiveness in murine schistosomiasis mansoni is dependent upon the adhesion molecules ICAM-1, LFA-1, and VLA-4 (Langley & Boros, 1995). Interaction between ICAM-1 and LFA-1 was proven to be the major interaction during *S. mansoni* granuloma formation (Ritter & McKerrow, 1996; Jacobs *et al.*, 1997a). After antigenic stimulation, B-cells acquire the cell surface molecule syndecan-1 (Sanderson *et al.*, 1989). In previous work, we demonstrated that in *S. mansoni* granulomas the syndecan-1 positive B-cells located near deposited ova became antigen-stimulated B-cells after presentation of schistosome egg antigen by antigen-presenting macrophages to egg-antigen-specific T-lymphocytes (Jacobs *et al.*, 1998a, b). In this work, we illustrated that in the *S. haematobium* granuloma a similar peripheral location of naive B-cells that differed from antigen-stimulated (syndecan-positive) B-cells was present and thus that B-cells were also involved in the genesis of the *S. haematobium* granuloma. From these observations we infer that the mouse is a suitable model for the study of hepatic *S. haematobium*-induced granuloma formation and fibrosis. In the murine host, granuloma formation is maximal after 20 weeks of infection and shows moderate modulation during aging of the infection. Specific adhesion molecules are involved in the granulomogenesis which results in ECM deposition that differs qualitatively from *S. mansoni* infection.

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