DISTRIBUTION OF MAST CELLS IN RELATION TO *SCHISTOSOMA JAPONICUM* INDUCED LESIONS IN PIGS

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Abstract. The pathogenesis of schistosomiasis japonica has been extensively studied, however only little attention has been paid to the presence and localization of mast cells in relation to *Schistosoma japonicum* induced lesions. The aim of the present pilot study was to assess the parasitological and pathological responses in *S. japonicum* infected pigs with emphasis on the description of the distribution of mast cells in relation to lesions in the liver and cecum. Six pigs were exposed to 2,000 cercariae and examined 9 weeks post-infection. Three unexposed pigs of the same age served as helminth free controls. All infected pigs developed granulomatous hepatitis and typhlitis. In the liver, the degree of mast cell infiltration was higher in the infected pigs compared to the unexposed control group. This distinction could not be shown in the cecum. In both the liver and cecum, a mild to moderate number of mast cells were present within the granulomas. A significant relation was found between infection with *S. japonicum* and the mast cell infiltration in the liver. Due to their possible association with hepatic fibrosis, it seems as if they have some function in the fibrogenic process and thereby play a dual role in the pathogenesis of *S. japonicum*. In conclusion, the results show that mast cells are recruited to egg induced lesions in both the liver and the cecum.

INTRODUCTION

Schistosoma japonicum is the agent of an important zoonotic infection affecting both people and domestic as well as wild animals in endemic areas in Southeast Asia, mainly China and the Philippines (Fernandez *et al,* 1982; Ross *et al,* 1997; Jiang *et al,* 2002). Infection of a host results in granulomatous inflammation in the liver and intestine, and subsequent hepatic fibrosis following deposition of eggs in the mesenteric and portal veins (King, 2001). Since the pig is a natural host of *S. japonicum* and because of its anatomical, physiological and immunological similarities to man, it is considered an appropriate and relevant model for *S. japonicum* research (Willingham and Hurst 1996; Johansen *et al,* 2000). Recruitment of mast cells is a common feature of helminth infections (Lee *et al,* 1986). However the role, presence and localization of mast cells in relation to egg-induced lesions caused by *S. japonicum* have not received much attention in previous studies. Contrary to *S. japonicum* research, mast cells have received more attention in experimental studies conducted with *S. mansoni* (Weinstock and Boros, 1983a; Reis *et al,* 2001; De Jonge *et al,* 2002; Metwali *et al,* 2002). Also in a study of human liver biopsies, mast cells were abundant in livers from schistosome infected individuals compared to other granulomatous infections (Celasun *et al,* 1992). The aim of this pilot study was to evaluate the parasitological

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and pathological responses in *S. japonicum* infected pigs and to desrcibe the distribution of mast cells in relation to egg-induced lesions in the liver and cecum of infected pigs.

MATERIALS AND METHODS

Five castrated male and 4 female, specific pathogen free, Danish Landrace/Yorkshire/Duroc crossbred pigs, 6-8 weeks of age were allocated according to sex and weight into 2 groups, group A (n = 6) and B (n = 3). Group A were infected intramuscularly with 2,000 cercariae of a *S. japonicum* isolate from the Province of Anhui, People's Republic of China, according to the method described by Willingham *et al* (1996). These pigs were killed 9 weeks post-infection with pentobarbital intravenously and perfused according to the method described by Bøgh *et al* (1997) except no praziquantel was given prior to perfusion. Group B served as helminth free age controls. They were killed using a captive bolt pistol followed by exsanguination, and no perfusion was done. All pigs were housed in helminth free conditions and kept in pens with full concrete floors with straw bedding. They were fed a commercial pelleted dry feed and water was given *ad libitum*. The pigs used in this experiment were treated in accordance with the animal ethics laws of Denmark.

Worm numbers (male, female, immature, total) and tissue egg counts (TEC), from the liver and the cecum were determined as described by Bøgh *et al* (1996, 1997) and Giver *et al* (1999), respectively. The worm establishment percentage was calculated from each pig.

Gross hepatic lesions on both the surface and the cut surface were noted. The degree of portal and septal fibrosis was graded as: no fibrosis, mild, moderate or severe fibrosis. Gross lesions in the cecal mucosa were scored semi quantitatively based on the presence and distribution of petechiae in the mucous membrane.

Tissue samples were collected from five predetermined sites in the liver (one sample from each lobe) and the cecum (scattered sites) for histological examination. Two samples were taken from each site and fixed for 24 hours in 10% neutral buffered formalin and Carnoy's fixative, respectively. The tissue was processed conventionally, embedded in paraffin and sectioned at 2-4 μm thickness. One section from all formalin fixed tissue was stained with hematoxylin and eosin (HE). Sections from the right and left lateral lobes of the liver were stained with van Gieson for collagen (Bradbury and Rae, 1996), PicroSirius Red for collagen and reticular fibers (Junquiera *et al,* 1979), Gordon and Sweets' stain (without gold-toning) for reticular fibers (Bradbury and Rae, 1996) and Luna's stain for eosinophils (Luna 1968). Selected sections from the cecum of each pig were stained with Luna's stain for eosinophils (Luna, 1968). All liver and cecal tissue samples fixed in Carnoy's fixative were sectioned and stained with Toluidine blue (pH 0.5) as described by Xu *et al* (1993).

Histological examinations

Evaluation of slides was done on coded slides to blind the investigator to the identity and group affiliation of the pig. Liver sections stained with HE were used for overall evaluation. The first 20 granulomas encountered, starting from the left lateral lobe, were classified according to developmental stage into exudative-productive, mature-productive, or involutional (Hurst *et al,* 2000b). Eggs present were classified as either intact (immature/mature) or non-intact. Presence of small mononuclear cell clusters and GALF (granuloma-associated lymphoid follicles) was noted. Distribution of eosinophils in the liver and their relation to lesions was examined in sections stained by Luna's stain for eosinophils. Assessment of septal fibrosis was done on sections stained with van Gieson according to the measuring method described by Johansen *et al* (2001). For comparison, PicroSirius Red was

also used to evaluate the distribution of fibrosis in granulomas, interlobular septa and portal areas using image analysis (see below). Sections stained with Gordon and Sweets' stain were used to describe the presence of reticular fibers. Using Toluidine blue stained liver sections, the mast cell infiltration of the non-parenchymal tissue was quantified by counting 10 randomly chosen areas using an ocular grid. A total area of 0.25 mm^2 was counted in each section. The mast cells location in the liver and their occurrence in relation to lesions and their principal location (central, peripheral or dispersed) within the granulomas were also recorded.

The number of granulomas and the number of egg clusters free of tissue reaction or present in acute exudative inflammatory foci were recorded in cecal HE stained sections. Granulomas displaying central eggs were classified according to developmental stage as described by Hurst *et al* (2000b). If possible, eggs were classified as described above. The degree of mast cell infiltration and their location in the different layers of the cecum was noted using Toluidine blue stained sections, and their occurrence in relation to granulomas was evaluated as in the liver. Presence of eosinophils was evaluated as described for the liver.

Image analysis

To evaluate the amount of hepatic fibrosis, sections stained with PicroSirius Red were analyzed. One image of each section was captured digitally by a digital camera (DP50, Olympus, Tokyo, Japan) using a 1 X objective and a green filter in a Vanox ABT3 microscope (Olympus, Tokyo, Japan). Collagen was analyzed by using a quantitative image analysis system (Image-Pro Plus ver. 5.0, Media Cybernetics, Maryland, USA).

Statistical analysis

Statistical analysis was carried out using variance analysis in a mixed model including a random effect for each pig. The outcomes and covariates were transformed by using log + 1. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Infections with *S. japonicum* were established in all exposed pigs. In all infected pigs, mild diarrhea was present at the start of egg excretion.

Parasitology

Parasitological results are presented in Table 1. Individual variation in worm establishment was present within group A with the lowest establishment being 0.3 % and the highest 5.5 %. The mean EPG (eggs per gram) liver was [mean ± SD (standard deviation) = 396 ± 296] and in the cecum (1,898 ± 2,609).

Gross pathology

In the unexposed control group (group B), no lesions were observed in the liver and in the cecum. All infected pigs (group A) showed a multifocal granulomatous hepatitis with mild to moderate multifocal portal and septal fibrosis. In the cecum, mild to moderate disseminated petechial hemorrhages were seen in the mucosa and in 3 pigs nest formation with underlying worm pairs was evident.

Histopathology

All *S. japonicum* infected pigs showed perioval granulomas along with multifocal or diffuse septal and portal fibrosis in the liver. No free eggs or acute inflammatory foci were observed in the liver. Granulomas were mainly of the mature-productive type (70 - 100%) and primarily located in portal triads and interlobular septa (Fig 1A). Where eggs were displayed in the granulomas, they were usually single and non-intact. Yet, a small proportion of granulomas contained clusters of eggs or remnants. No heterophils were observed and necrosis was only visible centrally in very few granulomas. GALF were eccentrically located at the

Table 1
Parasitological results: The number of worms (male, female, immature and total) and tissue egg counts in liver and cecum of *Schistosoma japonicum* infected pigs.

Group A Pig number	Worms				Tissue egg counts/g	
	Males	Females	Immature	Total	Liver	Cecum
1	27	26	5	58	540	1,476
2	3	2	1	6	36	7
3	19	10	25	54	360	604
4	16	16	24	56	240	260
5	52	45	11	108	900	6,987
6	28	22	60	110	300	2,053

border of a few granulomas, and mainly seen in connection with mature-productive stage granulomas as single follicles (Fig 1B). In contrast, several GALFs were found surrounding large worm granulomas. In all pigs, a few scattered foci of small mononuclear cells were seen within liver lobules. The mean numbers of small mononuclear cell foci were in group A (6.7 ± 3.4) and group B (6.0 ± 3.0). No significant difference could be found between the groups.

The fibrosis in the liver was distributed multifocally or diffusely in the portal areas and interlobular septa without affecting the parenchymal pattern of the liver (Fig 1C and 1D). Compared to the uninfected pigs, the collagen fibers of the infected pigs had a more unorganized appearance. This was most evident in the PicroSirius Red stained sections. The reticular fibers were predominantly observed lining the interlobular septa and along the sinusoidal walls (Fig 1E). Peripheral concentric bands of fibrosis surrounded the granulomas at varying degrees. The fibrotic tissue consisted of a mixture of collagen and/or reticular fibers in various amounts (Fig 1F, 1G, and 1H). The total fibrosis score measured with the method according to Johansen *et al* (2001) was in group A (19.9 ± 9.5 μm) and in group B (9.5 ± 1.5 μm), and significant difference was seen between the groups ($p < 0.001$). The fibrosis measured with image analysis was in group A (12.7 ± 3.3 %) and in group B (5.2 ± 0.8 %) and likewise significant difference was seen between the groups ($p < 0.001$).

Cecal granulomas were seen around apparently intact eggs or eggs in various stages of re-absorption in both the mucosa and the submucosa. When visible, eggs were usually found in clusters composed of two to seven. The majority of eggs found in the mucosa were free eggs without any tissue reaction. If tissue reactions to eggs were present in the mucosa, it was primarily as acute inflammatory foci, whereas in the submucosa, the response to eggs was mainly characterized by exudative-productive and mature-productive granulomas. Most eggs in submucosal granulomas were degenerated while intact eggs characterized the findings in lamina propria (Fig 2A). In a few sections large worm granulomas were found in the submucosa, however no GALF were found. No remarkable fibrosis was seen in the cecum.

The density of mast cells in the non-parenchymal tissue of the liver was significantly higher in group A (270.1 ± 48.1 cells/0.25 mm^2) compared to group B (68.3 ± 21.1 cells/0.25 mm^2) ($p < 0.001$). Mast cells showed a high degree of pleomorphism and granules were evident in the cytoplasm of most cells. They were mainly located in portal and septal

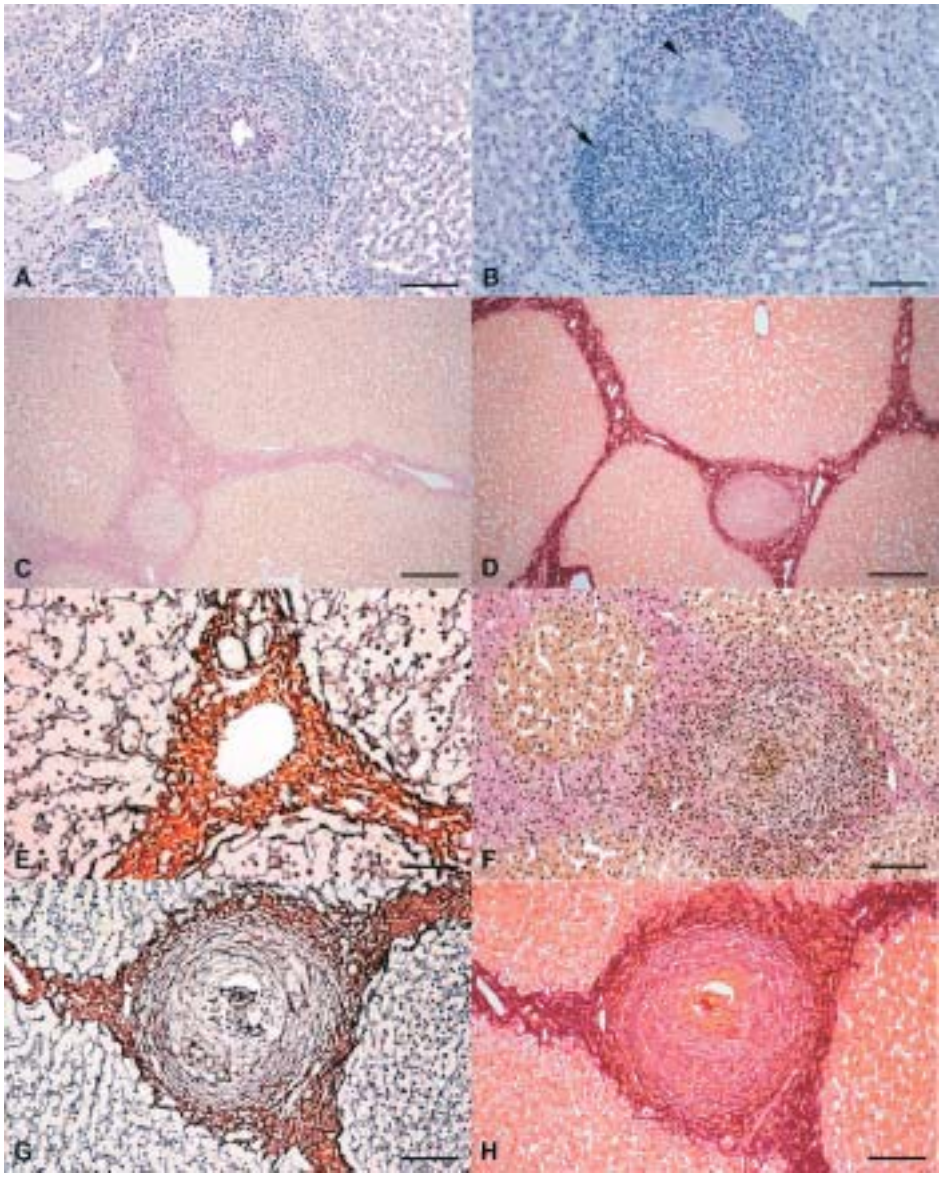

Fig 1–Hepatic granulomas and fibrosis in *S. japonicum* infected pigs. A: A portal area with a mature-productive granuloma with a central placed egg and numerous giant cells, epitheloid cells and eosinophils. Hematoxylin and Eosin (HE). Bar = 100 μm. B: A mature-productive granuloma with granuloma-associated lymphoid follicle (arrow). Multinucleated giant cells (arrowhead). HE. Bar = 95 μm. C: Liver septa with increased septal fibrosis (red collagen fibers) and moderate cellular infiltration. A granuloma can also be seen. Van Gieson stain (VG). Bar = 255 μm. D: Fibrosis of hepatic septa and portal areas. Notice the apparently disorganized red extracellular matrix fibers. A granuloma can also be seen. PicroSirius Red (PSR). Bar = 210 μm. E: Reticular fibers (black) line the hepatic septa and sinusoids. Collagen fibers are golden. Gordon & Sweets' stain without gold-toning (GS). Bar = 45 μm. F, G and H: Fibrosis in a hepatic granuloma. Bar = 90 μm. F is the VG stain showing collagen deposition in the periphery of the granuloma, G is a GS staining displaying reticulin creating a meshwork within the granuloma, and H shows the PSR stain, where both collagen and reticular fibers are stained.

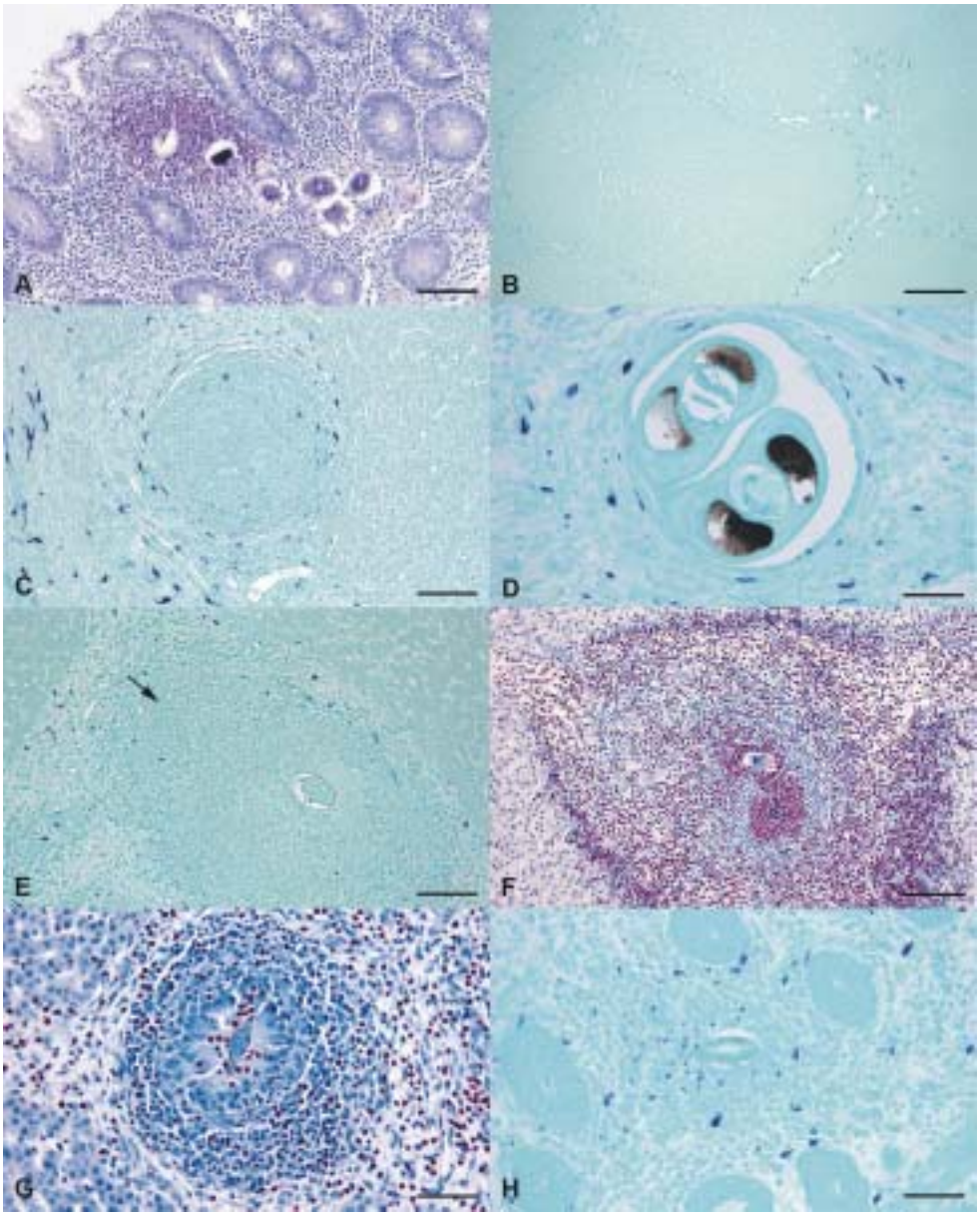

Fig 2–Cecal lesions, as well as mast cells and eosinophils in the liver and cecum of *S. japonicum* infected pigs. A: Free eggs and an acute inflammatory focus in the cecal mucosa. Hematoxylin and Eosin (HE). Bar = 100 μm. B: Metachromatic mast cells distributed in the fibrotic hepatic septa. Toluidine blue (pH 0.5) with 0.12 % Light green as counterstain (Tol). Bar = 95 μm. C: Scattered mast cells in a hepatic granuloma in a portal area. Tol. Bar = 50 μm. D: Cecal venule containing female worm(s). Notice eggs in the uterus. Mast cells surround the venule. Tol. Bar = 60 μm. E: Mast cells in a hepatic granuloma with a GALF (arrow). Tol. Bar = 90 μm. F: An exudative-productive granuloma in the liver with a severe eosinophil infiltration dispersed throughout the granuloma. Luna's stain for eosinophils (Luna). Bar = 100 μm. G: A mature-productive granuloma with a moderate number of eosinophils. The eosinophils located in the center and periphery of the granuloma. Luna. Bar = 65 μm. H: A free egg in the lamina propria of the cecum surrounded by mast cells. Tol. Bar = 60 μm.

areas (Fig 2B). A mild to moderate degree of mast cell infiltration was found in the majority of granulomas (Fig 2C). The mast cells were mainly located in the periphery but occasionally they were found dispersed within the granuloma. No significant association was found between hepatic fibrosis and the density of mast cells when infection status was included in the analysis regardless of the method used to estimate hepatic fibrosis (Method from Johansen *et al,* 2001: $p = 0.555$; The image analysis method: $p = 0.399$). Mast cells were also seen around a worm in a cecal venule (Fig 2D). There was neither significant association between the density of mast cells in the liver and the number of worms collected from pigs in group A ($p = 0.801$) nor the TEC in the liver ($p = 0.558$). A few mast cells were noted in GALF associated to egg granulomas (Fig 2E). A mild to severe infiltration with eosinophils was observed with either a diffuse or multifocal distribution in the interlobular septa, portal areas and in the granulomas. The location of the eosinophils in the granulomas varied greatly according to the stage of the granuloma (Fig 2F and 2G). Comparing the Toluidine blue stained sections with the sections stained with Luna's stain for eosinophils, the presence and location of mast cells and eosinophils seem to overlap. Both cell types were few in the hepatic parenchyma. However, the density of eosinophils in granulomas, portal areas and interlobular septa of the liver was much higher than the observed density of the mast cells. In the granulomas, the eosinophils were found to have a more unrestricted location than the mast cells.

A mild to moderate degree of mast cell infiltration in the cecum was observed in all pigs (group A and B). In both groups, mast cells were found dispersed in the lamina propria between the crypts, and in some instances they were located around crypts. Few mast cells were located in the submucosa and even fewer in tunica muscularis. In the submucosa, mast cells were sporadically found surrounding vessels. In the infected pigs (group A) the infiltration of eosinophils in lamina propria and tunica muscularis was higher than in the uninfected pigs. In group A, the majority of free eggs in the lamina propria did not seem to attract mast cells or eosinophils. However, in a few instances an increased degree of infiltration of both mast cells and eosinophils was observed around eggs located in the lamina propria (Fig 2H). It was not possible to assess whether these eggs were intact or non-intact. In two pigs, free eggs were seen in the submucosa surrounded by a moderate number of mast cells. Some of the mast cells were very close to the egg and apparently attached to the eggshell. Equivalent to the liver, mast cells were found in the majority of submucosal granulomas with the same degree of infiltration and distribution as in hepatic granulomas.

DISCUSSION

Clinical, parasitological and pathological findings in group A are consistent with what has been reported in other studies with similar infection doses and duration of infection in pigs (Willingham *et al,* 1998; Johansen *et al,* 1998, 2000; Hurst *et al,* 2000a,b, 2002).

The occurrence of mast cells in relation to *S. japonicum* egg-induced lesions in either the liver or intestines has not received much attention. In various studies of *S. mansoni* infections, mast cells have been reported to occur in low numbers within schistosome granulomas (Moore *et al,* 1977; Epstein *et al,* 1979; Weinstock and Boros 1983a,b; Lenzi *et al,* 1987; Celasun *et al,* 1992). This finding is supported by the present study, where low to moderate numbers of mast cells are found in the *S. japonicum* granulomas in both the liver and cecum, which could indicate that they do play a role in the dynamic of the granuloma. The present study also showed that the density of mast cells in the liver was signifi-

cantly higher in *S. japonicum* infected pigs compared to uninfected pigs at 9 weeks post-infection. In a study by De Jonge *et al* (2002) it was found that after unisexual infection, no mastocytosis was observed indicating that mast cell recruitment is triggered by egg deposition and inflammation and not by circulating worm antigens. Likewise in the present study, no significant association was found between the burden of worms and the density of mast cells in the liver. However, no correlation between TEC in the liver and mast cell density was found either, which could suggest that the increased density of mast cells is correlated to infection but not to the exact number of eggs in the liver tissue. In a study by Oswald *et al* (2000) it was shown that, following experimental infection with *S. japonicum*, pigs developed a Th2 orientated immune response as characterized by an increased level of mRNA encoding for IL-4 and IL-10 in their liver and intestines. Similar findings have also been observed in other experimental animals (*eg* mice) (Xu *et al,* 1991; Metwali *et al,* 2002). This proposed orientation towards a Th2 cytokine response in schistosome infected pigs and mice could explain the presence and distribution of both mast cells and eosinophils in the present study. Still, the exact role of mast cells within granulomas remains to be clarified. Mast cells produce among others not only IL-4, a major Th2-cytokine, but also IL-5 which stimulates growth, differentiation and recruitment of eosinophils (Metwali *et al,* 2002; Paul, 2003). If eosinophil and mast cell functions are linked, a possible role of mast cells could be assistance in the egg destruction by eosinophils. This is however provided that egg destruction actually is one of the main functions of eosinophils, and this role of eosinophils in schistosomiasis is still greatly debated (James and Coley, 1978; de Brito *et al,* 1984; Reis and Andrade, 1987; Dent *et al,* 1997; Lenzi *et al,* 1997; Hanna *et al,* 2005).

Though the inflammatory reaction in the intestine and liver is granulomatous, the morphological pattern of response is not totally identical. This was also shown in the present study, where free eggs, acute inflammatory foci, and exudative granulomas dominated the cecal lesions contrary to the hepatic lesions. Another major difference in the inflammatory response between the two organs is the high degree of fibrosis seen in the liver but not in the intestine.

In this study, we found a higher degree of septal and portal fibrosis in infected pigs compared with uninfected regardless of the measuring method used. Considering that the PicroSirius Red stains more extracellular matrix components than the van Gieson stain and the image analysis is a very objective method, the image analysis of PicroSirius Red stained slides is probably a better method to measure the hepatic fibrosis. The higher degree of hepatic fibrosis in infected pigs is a well-known pathological consequence of *S. japonicum* infection (Hurst *et al,* 2000a,b; Johansen *et al,* 2000). The relationship between granulomatous inflammation, fibrosis and its persistence is complex and still unclear. In several other experimental studies for hepatic fibrosis the mast cell has been found in an increased number and has been described as a possible co-factor in the development of fibrosis (Peng *et al,* 1994; Ramos *et al,* 1994; Rioux *et al,* 1996; Armbrust *et al,* 1997). Mast cells secrete an extensive range of cytokines and mediators of inflammation *eg,* tumor necrosis factor alpha (TNF-α), heparin, and tryptase, which makes them capable players in the regulation of fibrosis (Brito and Borojevic, 1997; Gruber *et al,*1997; Benyon, 1999). Perhaps the organ-dependent difference in response concerning fibrosis can explain the findings that a higher degree of mast cell infiltration was found in the liver but not in the cecum of infected pigs compared to uninfected controls. In this study, however, no correlation was found between the degree of

hepatic fibrosis and the degree of mast cell infiltration. Therefore additional studies are necessary to further evaluate the connection between mast cells and hepatic fibrosis in pigs infected with *S. japonicum*. Furthermore, the present study only represents one time point post-infection, and it could be interesting to examine whether the mast cells have a bimodal pattern in *S. japonicum* infected pigs as seen in *S. mansoni* infected mice (Lenzi *et al,* 1987).

In addition, GALFs were found eccentrically located at the border of some hepatic granulomas in this study. Occurrence of GALF appears to be a unique feature of the granulomatous response in pigs to *S. japonicum* (Hurst *et al,* 2002). Studies have demonstrated that cells in these structures express CD21 antigen, which is normally expressed by mature B-cells and follicular dendritic cells (Hurst *et al,* 2002). In this study, however, only few mast cells were seen in the GALF associated with the granulomas and the relation between mast cell function and GALF are not known. Also the precise role for GALF is yet unrecognized. It has been hypothesized, that GALF could be a site for B-cell activity prior to the effector cell stage, and that plasma cells within granulomas may originate from these structures (Hurst *et al,* 2002). In this study, a row of multiple GALFs was found in the periphery of some hepatic worm granulomas. This could indicate that much antibody is needed in this situation, both to neutralize antigens released from the degenerating worm and possibly also to mediate antibody-dependent cell-mediated cytotoxicity by binding to Fc-receptors on local macrophages and eosinophils, supporting the hypothesis by Hurst *et al* (2002). Whether GALF occur in humans is not known, but they have been demonstrated in pigs and other animals in connection with other parasitic infections (Pérez *et al,* 2001).

In conclusion, the present study reveal that mast cells are distributed in both cecal and hepatic granulomas and in the interlobular septa and portal areas of the liver. This location of mast cells gives them potential to play a dual role in the pathogenesis of *S. japonicum ie* assisting in egg destruction and also some yet unknown function in the development of liver fibrosis.

ACKNOWLEDGEMENTS

To Laboratory of Pathology, Department of Veterinary Pathobiology, The Royal Veterinary and Agricultural University, Denmark and DBL–Institute of Health Research and Development, Denmark for assistance. To Novo Nordisk A/S, Department of Pharmacology Research, Denmark for technical support with the image analysis. To the Danish National Research Foundation for financial support of the study.

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