RESEARCH NOTE

PHENOTYPICAL CHARACTERIZATION OF INTESTINAL *SCHISTOSOMA JAPONICUM* GRANULOMAS IN PIGS

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Abstract. The pig has been proposed as a model for human schistosomiasis japonica and the use of this animal model is increasing. The inflammatory response to schistosome infection in the liver and intestine of the pig shows morphological differences, and only the hepatic granulomas have been phenotypically characterized. The aim of the present study was to phenotypically characterize the cellular inflammatory response in the cecum by immunohistochemistry with particular reference to perioval granulomatous reactions in *Schistosoma japonicum* infected pigs. Six pigs were exposed to 2,000 cercariae and examined 9 weeks post-infection. Three uninfected pigs of the same age served as controls. Exposed pigs developed patent infections with the total number of worms between 6 and 110. Cecal granulomas were dominated by CD3 positive T-lymphocytes and IgG positive plasma cells. Despite the difference in the inflammatory response between the liver and the cecum, the results from this study indicate that the phenotypic cellular composition of cecal granulomas appears similar to what has previously been described in the liver.

INTRODUCTION

Schistosomiasis japonica, an important zoonotic disease, continues to be a serious public health and socioeconomic problem in endemic areas in Southeast Asia, mainly China and the Philippines (Fernandez *et al*, 1982; Ross *et al*, 1997; Jiang *et al*, 2002). Recognition of many biological similarities between pigs and humans has led to the use of pigs as a host model for human schistosomiasis japonica (Willingham and Hurst, 1996; Johansen *et al*, 2000). The cellular composition of the hepatic granuloma is well described in pigs, but not much is known of the intestinal lesions. This study was designed to investigate intestinal *Schistosoma japonicum* granulomas. To our knowledge, this is the first study to phenotypically characterize the cellular inflammatory response in the cecum with particular reference to perioval granulomatous reactions by immunohistochemistry.

MATERIALS AND METHODS

Nine helminth-naïve, specific pathogen free, Danish Landrace/Yorkshire/Duroc crossbred pigs (5 castrated males and 4 females) aged 6-8 weeks at the beginning of the experiment, were used. The pigs used in this study were treated in accordance with the animal ethics laws of Denmark. All pigs were kept in pens with full concrete floors with straw bedding and housed in helminth free conditions. They were fed a commercial pelleted dry feed and water was given *ad libitum*.

Six pigs were exposed to 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). Three uninfected pigs served as helminth free age controls. Nine weeks post-infection the exposed pigs were euthanized and perfused according to the method described by Bøgh *et al* (1997) except no praziquantel was given prior to

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perfusion. The unexposed pigs were euthanized by means of a captive bolt pistol and exsanguination, and perfusion was not done.

Worm numbers were determined as described by Bøgh *et al* (1997) and the worm establishment percentage was calculated for each pig. The cecal tissue egg counts (TEC) was done according to the method described by Giver *et al* (1999).

Gross lesions in the cecal mucosa were described and scored semi quantitatively based on the number of petechiae and the presence of nests. In this context, the word "nest" is used to describe an aggregation of several petechiae in a small area of the mucosa usually near an underlying worm pair in the vasculature.

For histological examination, tissue samples were collected from five predetermined sites in the cecum (Fig 1), and fixed for 24 hours in 10% neutral buffered formalin. The tissue was processed conventionally, embedded in paraffin and sectioned at 2-4 µm thickness. One section from the formalin fixed tissue was stained with haematoxylin and eosin (HE).

Immunohistochemical staining (Table 1) was done on cecal tissue to detect plasma cells expressing IgA, IgM, IgG and T-lymphocytes expressing CD3. The cecal tissue samples were serially sectioned onto Superfrost® Plus glass slides (Menzel-Gläser, Germany). As a positive tissue control, a section of a lymph node was used for each antibody.

Evaluation of slides was done on coded slides to blind the investigator to the identity and group affiliation of the pig. On cecal HE stained sections, the lesions and eggs were described and classified according to Hurst *et al* (2000b). Furthermore, the dominant cell type was noted and the superior cell density in the different layers of the cecum was scored semi-quantitatively from normal to severely increased. Finally, the cellular inflammatory response in the cecum was phenotypically characterized by immunohistochemical staining. The presence, degree and distribution of immunostained cells in relation to lesions were recorded.

Statistical analysis was carried out using the F-test and two-tailed Student's *t*-test. Differences were considered statistically significant at p-values <0.05.

RESULTS

Patent infections with *S. japonicum* were established in all exposed pigs, with a total number of worms between 6 and 110. The individual variation in worm establishment rate varied with the lowest establishment rate being 0.3% and the highest 5.5%. The mean EPG (eggs per gram) in the cecum was [mean ± SD (standard deviation)] 1,898 ± 2,609. In all infected pigs, mild diarrhea was present at the start of egg excretion.

No gross lesions were seen in the cecum of the uninfected pigs. In the cecum of the infected pigs, a few to moderate numbers of disseminated petechial hemorrhages in the mucosa were observed, and in 3 pigs nest formation was evident.

On the histological examination, the overall degree of cell infiltration was significantly higher in the infected pigs compared to the uninfected pigs. The infected pigs showed a mild to moderate degree of diffuse infiltration with eosinophils and mononuclear cells in the lamina propria and the submucosa of the cecum, with eosinophils being the most dominant cell. Cecal granulomas were seen around apparently intact eggs or eggs in various stages of resorption in both mucosa and submucosa. When visible, eggs were usually found in clusters composed of two to seven. The majority of the eggs in the mucosa were free and often intact, without any tissue reaction and if a tissue reaction was present it was primarily as an acute inflammatory focus. In the submucosa, however, the response to the eggs was dominated by granuloma formation, mainly exudative-productive and mature-productive granulomas. In these submucosal granulomas most eggs were degenerated. Large worm granulomas were also found in the submucosa in a few pigs.

DISCUSSION

The patterns of immunostaining of IgM, IgG, and IgA positive plasma cells and CD3 positive

Table 1
Immunohistochemical staining.

Primary Ab	Specificity	Dilution	Negative control Ab	Pretreatment	Detection system	Chromogene
A100-104A[1]	Porcine IgG Fc fragment	1:7000	Purified goat IgG[3]	0.036% Protease[5], 5 min RT[5] 0.06 % H_2O_2, 15 min RT 5% normal rabbit serum[6]	PAP goat[7]	DAB[8]
A100-102A[1]	Porcine IgA	1:4000	Purified goat IgG[3]	0.036% Protease[5], 5 min RT 0.06 % H_2O_2, 15 min RT 5% normal rabbit serum[6]	PAP goat[7]	DAB[8]
A100-100A[1]	Porcine IgM-Mu chain	1:9000	Purified goat IgG[3]	0.036% Protease[5], 5 min RT 0.06 % H_2O_2, 15 min RT 5% normal rabbit serum[6]	PAP goat[7]	DAB[8]
Polyclonal A0452[2]	Human CD3º	1:2000	Normal rabbit Ig fraction[4]	0.018 % Protease[5], 5 min RT 1% Blocking reagent[9], 20 min RT	Envision/AP[10]	Fast Red[11]

In all immunohistochemical protocols, washing and dilution was carried out with TBS (Tris-buffered-saline, 0.05 M Tris, pH 7.6, 0.15 M NaCl) except primary antibodies and negative control antibodies that were diluted in TBS with 0.1% BSA (Bovine serum albumin, Sigma-Aldrich, Denmark A/S, A7906).
[1]Goat anti Porcine antibodies (Bethyl Laboratories Inc, Mongomery, TX, USA).
[2] Rabbit anti Human CD3º (DakoCytomation A/S, Denmark).
[3] I9140, Sigma-Aldrich A/S, Denmark, (1:56000).
[4] X0903, DakoCytomation A/S, Denmark (1:80000);
[5] Sigma P8038, Sigma-Aldrich A/S, Denmark; RT: room temperature.
[6] X0902, DakoCytomation A/S, Denmark.
[7] A three layer horseradish PAP (peroxidase anti-peroxidase) technique using Z0454 (1:200, 30 min RT) and B0157 (1:100, 30 min RT) (DakoCytomation A/S).
[8] DAB (3.3 diaminbenzedinetetrahydrochloride, Kem En Tec 4170, Denmark), 10 min RT. Counterstain; Mayers Hematoxylin (Bie & Berntsen, Denmark).
[9] Boehringer Ingelheim GmBH, Germany.
[10] Two-step alkaline phosphatase conjugated EnVision™method (DakoCytomation A/S, K4018).
[11] Fast Red (Kem En Tec, 4210), 10 min RT. Counterstain; Mayers Hematoxylin (Bie & Berntsen, Denmark).

T-lymphocytes in control lymph nodes were consistent with results of other studies (Brown and Bowne, 1976; Tanimoto and Ohtsuki, 1996). The majority of submucosal granulomas did not display any IgM or IgA positive cells. However, in a few granulomas, a small number of IgM and IgA positive cells could be recognized in the periphery. Contrary to this, IgG positive cells were seen dispersed within most granulomas in moderate numbers. In addition, all granulomas contained high numbers of CD3 positive T-lymphocytes, mainly located in the peripheral zone of the granuloma, but occasionally a few intermingled with the epitheloid cells in the center near the egg (Fig 2).

The clinical, parasitological and pathological findings in the infected pigs are in accordance with results of 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).

In the present study, we found that submucosal granulomas in the cecum were dominated by CD3 positive T-lymphocytes and IgG positive plasma cells, while IgA and IgM positive plasma cells were infrequently detected, and only in very low numbers. These findings are similar to what has been reported in hepatic granulomas (Hurst *et al*, 2002). Still, many cells in the intestinal granulomas have yet to be identified. In studies
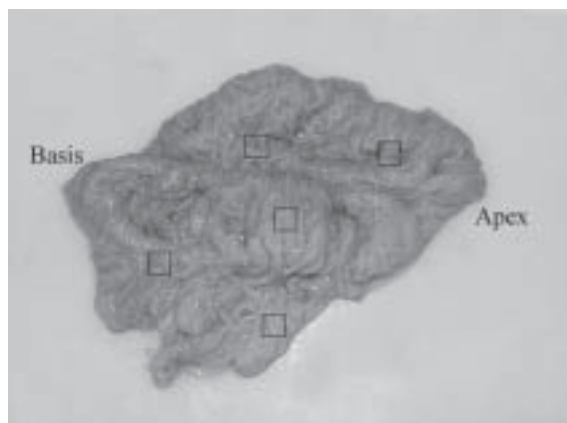
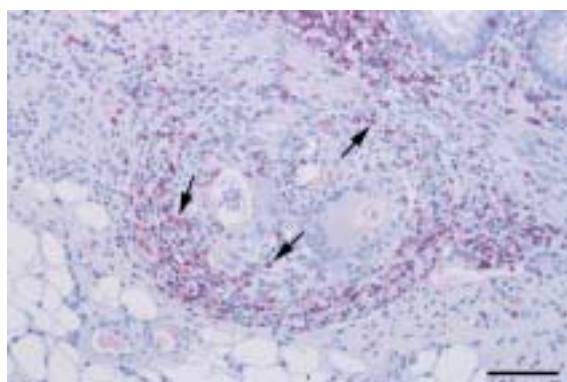Fig 1–Sampling sites in the cecum.

Fig 2–CD3 positive T-lymphocytes (arrows) in the periphery of a submucosal granuloma in the cecum. Positive stained cells show immunoreactivity with polyclonal anti-CD3°. Bar=100 μm.

of the inflammatory response in the intestine of *S. bovis* infected goats, it has been demonstrated that macrophages and epitheloid-like cells of granulomas expressed MHC Class II molecules, whereas multinucleated giant cells did not. B-lymphocytes were observed inconsistently scattered in the periphery of granulomas (Lindberg *et al*, 1999). Whether this also occurs in the intestine of *S. japonicum* infected pigs has yet to be determined. Furthermore the subsets of the CD3 positive T-lymphocytes remain to be identified in the submucosal granulomas of pigs.

As shown in this study and in the study by Hurst *et al* (2000b), intestinal reactions to eggs consist mainly of no reaction or as acute inflammatory foci. The intestinal granulomas located in the submucosa are more exudative than the hepatic granulomas. Despite this distinct difference in the inflammatory response between the liver and cecum, the phenotypic cellular composition of granulomas in the cecum appears similar to granulomas in the liver with T-lymphocytes being present in high numbers. T-lymphocytes are capable of producing a wide range of cytokines, thus having the ability to create different local cytokine environments in the particular organs. Oswald *et al* (2000) found increased IL-4 mRNA in both the liver and the intestine, whereas only IL-10 mRNA was increased in the cecum. This could be one of the explanations for the difference in reaction to *S. japonicum* eggs between the liver and the intestine and should be further explored.

In conclusion, we found that cecal granulomas were dominated by CD3 positive T-lymphocytes and IgG positive plasma cells, which are equivalent to hepatic granulomas.

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REFERENCES

Brown PJ, Bourne FJ. Distribution of immunoglobulin-containing cells in the alimentary tract, spleen, and mesenteric lymph node of the pig demonstrated by peroxidase-conjugated antisera to porcine immunoglobulins G, A, and M. *Am J Vet Res* 1976; 37: 9-13.

Bøgh HO, Willingham AL, Johansen MV, Eriksen L, Christensen NØ. Recovery of *Schistosoma japonicum* from experimentally infected pigs by perfusion of liver and mesenteric veins. *Acta Vet Scand* 1997; 38: 147-56.

Fernandez TJ, Petilla JrT, Banez B. An epidemiological study on *Schistosoma japonicum* in domestic animals in Leyte, Philippines. *Southeast Asian J Trop Med Public Health* 1982; 13: 575-79.

Giver H, Johansen MV, Christensen NØ, Nansen P.

Schistosoma japonicum in the pig: A new technique for estimation of intestinal tissue egg counts. *Southeast Asian J Trop Med Public Health* 1999; 30: 664-69.

Hurst MH, Shi YE, Lindberg R. Pathology and course of natural *Schistosoma japonicum* infections in pigs: results of a field study in Hubei Province, China. *Ann Trop Med Parasitol* 2000a; 94: 461-77.

Hurst MH, Willingham AL, Lindberg R. Tissue responses in experimental Schistosomiasis japonica in the pig: A histopathologic study of different stages of single low- or high-dose infections. *Am J Trop Med Hyg* 2000b; 62: 45-56.

Hurst MH, Willingham AL, Lindberg R. Experimental schistosomiasis japonica in the pig: immunohistology of the hepatic egg granuloma. *Parasite Immunol* 2002; 24: 151-59.

Jiang Q-W, Wang L-Y, Guo J-G, Chen M-G, Zhou X-N, Engels D. Morbidity control of schistosomiasis in China. *Acta Trop* 2002; 82: 115-25.

Johansen MV. Effect of praziquantel treatment on experimental porcine *Schistosoma japonicum* infection. *Parasitology* 1998; 116: 519-24.

Johansen MV, Bøgh HO, Nansen P, Christensen NØ. *Schistosoma japonicum* infection in the pig as a model for human schistosomiasis japonica. *Acta Trop* 2000; 76: 85-99.

Lindberg R, Johansen MV, Nilsson C, Nansen P. An immunohistological study of phenotypic characteristics of cells of the inflammatory response in the intestine of *Schistosoma bovis*-infected goats. *Parasitology* 1999; 118: 91-99.

Oswald IP, Dozois CM, Barlagne R, Fournout S, Johansen MV, Bøgh HO. Cytokine mRNA expression in pigs infected with *Schistosoma japonicum*. *Parasitology* 2000; 122: 299-307.

Ross AGP, Li YS, Sleigh AC, McManus DP. Schistosomiasis control in the People's Republic of China. *Parasitol Today* 1997; 13: 152-55.

Tanimoto T, Ohtsuki Y. Evaluation of antibodies reactive with porcine lymphocytes and lymphoma cells in formalin-fixed, paraffin-embedded, antigen-retrieved tissue sections. *Am J Vet Res* 1996; 57: 853-59.

Willingham AL, Bøgh HO, Vennervald BJ, *et al.* Worm establishment and egg production of *Schistosoma japonicum* in pigs infected by percutaneous methods or intramuscular injection. *Vet Parasitol* 1996; 61: 157-63.

Willingham III AL, Hurst M. The pig as a unique host model for *Schistosoma japonicum* infection. *Parasitol Today* 1996; 12: 132-34.

Willingham AL, Hurst M, Bøgh HO, *et al. Schistosoma japonicum* in the pig: The host-parasite relationship as influenced by the intensity and duration of the infection. *Am J Trop Med Hyg* 1998; 58: 248-56.