Characteristics of granuloma formation and liver fibrosis in murine schistosomiasis mekongi: a morphological comparison between *Schistosoma mekongi* and *S. japonicum* infection

M. SHIMADA[1,2], M. KIRINOKI[1], K. SHIMIZU[3], N. KATO-HAYASHI[1], Y. CHIGUSA[1]*, V. KITIKOON[4], P. PONGSASAKULCHOTI[4] *and* H. MATSUDA[5]

[1] *Laboratory of Tropical Medicine and Parasitology, Dokkyo Medical University, Mibu, Shimotsuga, Tochigi 321-0293, Japan*
[2] *Department of Internal Medicine, Ashikaga Red Cross Hospital, Ashikaga, Tochigi 326-0808, Japan*
[3] *Department of Pathology, Ashikaga Red Cross Hospital, Ashikaga, Tochigi 326-0808, Japan*
[4] *Department of Social and Environmental Medicine, Mahidol University, 420/6 Rajvithi Road, Bangkok 10400, Thailand*
[5] *Institute of International Education and Research, Dokkyo Medical University, Mibu, Shimotsuga, Tochigi 321-0293, Japan*

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SUMMARY

A histopathological study was performed to clarify the characteristics of granuloma formation and liver fibrosis in *Schistosoma mekongi* infection in comparison with *S. japonicum* infection. Mice were exposed to *S. mekongi* (Laotian strain) and *S. japonicum* (Japanese strain) cercariae, and were dissected at 6, 8, 12, 16, and 20 weeks post-exposure. In the liver, granulomas in *S. mekongi* infection were cellular, initially organized with foam cells, and continuously appeared in the intralobular area, while granulomas in *S. japonicum* infection were fibrous and did not continuously appear in the intralobular area. Portal fibrosis was not seen in *S. mekongi* infection, but was commonly seen in *S. japonicum* infection in the later weeks. Granulomas in the small intestine were seen mainly in the submucosa with foam cells in *S. mekongi* infection and without foam cells in *S. japonicum* infection. The lung granulomas contained mainly histiocytes in both *S. mekongi* and *S. japonicum* infection. The absence of portal fibrosis in *S. mekongi* infection allows schistosome eggs to infiltrate into the intralobular area continuously, which can be what lies behind the ultrasonographic differences; the echogenic network pattern as was seen in *S. japonicum* infection, has not been noted in *S. mekongi* infection.

Key words: *Schistosoma mekongi*, granuloma formation, foam cells.

INTRODUCTION

Schistosoma mekongi infection has been an important public health problem in Laos and Cambodia along the Mekong River and its tributaries since the first case of schistosomiasis was reported in the Mekong River basin (Dupont *et al*. 1957). Its clinical manifestations in humans are hepatosplenomegaly and decompensation of portal hypertension (Urbani *et al*. 2002). Although portal fibrosis in human cases has been reported (Wittes *et al*. 1984; Monchy *et al*. 2006), infection with hepatitis B, liver flukes, or a history of alcohol consumption must be considered as well. Periportal thickening and portal enlargement were observed on sonography in Cambodia. Annual treatment with praziquantel improved sonographic patterns of liver fibrosis based on the Niamey classification of the World Health Organization in the year 2000, and reduced periportal thickening, but did not improve advanced liver fibrosis and portal hypertension (Urbani *et al*. 2002).

S. mekongi and *S. japonicum* are closely related phylogenetically, but differ in schistosome egg size (Sornmani, 1976; Voge *et al*. 1978) and in the starting point of egg deposition in mammalian hosts (Voge *et al*. 1978). In addition, the echogenic network pattern in the liver, a typical characteristic in *S. japonicum* infection, has not been noted in *S. mekongi* infection (Ohmae *et al*. 2004). Sonographic findings in the liver in *S. japonicum* infection were reported to correspond to histopathological findings (Murakami, 1986; Zilton and Cheever, 1993). However, there have been only limited studies of *S. mekongi* infection (Byram *et al*. 1978; Byram and Lichtenberg, 1980; Owhashi *et al*. 2005).

We therefore conducted this histopathological study of *S. mekongi* infection to clarify the characteristics of granuloma formation and liver fibrosis in comparison with *S. japonicum* infection.

* Corresponding author: Laboratory of Tropical Medicine and Parasitology, Dokkyo Medical University, 880 Kitakobayashi, Mibu, Shimotsuga, Tochigi 321-0293, Japan. Tel: +81 282 87 2134. Fax: +81 282 86 6431. E-mail: ychigusa@dokkyomed.ac.jp

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MATERIALS AND METHODS

Animals and infection

Female ICR mice, 4 weeks of age, were purchased from Japan SLC. Inc., Hamamatsu, Japan. *S. mekongi* (Laotian strain) cercariae were obtained from laboratory-infected *Neotricula aperta* gamma race by shedding. All of these snails were collected annually from the Mekong River located in Cambodia. *S. japonicum* (Japanese strain) cercariae were shed from laboratory-infected *Oncomelania nosophora* collected from Yamanashi Prefecture, Japan.

Thirty ICR mice were anaesthetized for *S. mekongi* infection, and the same number of mice was anaesthetized for *S. japonicum* infection by subcutaneous injections of a mixed solution of ketamine (Veterinary Ketalar® 50; Sankyo Lifetech Co., Ltd, Tokyo, Japan) and xylazine (Xylazine®; Bayer Health Care, Germany). Each mouse was infected with 20 cercariae of *S. mekongi* or *S. japonicum* percutaneously through a shaven abdomen under anaesthesia using the cover-slip method (Maeda *et al*. 1982). When infected ICR mice died before autopsy, the same number of mice was infected to replace those which had died.

The care and use of all animals complied with the Rules on the Care and Use of Laboratory Animals of Dokkyo Medical University, and the Institutional Review Boards of the author's institution approved the study protocol.

Anatomy

From the *S. mekongi*- and *S. japonicum*-infected groups, 6 ICR mice each were chosen at random, euthanized, and dissected at 6, 8, 12, 16, and 20 weeks post-exposure. The adult schistosome worm pairs in the portal vein were picked up by forceps and counted. The chest and abdomen were opened, and the left liver lobe, the lung, and the small intestine were removed from the mice. The left liver lobe was cut into halves, the lung was cut at the section including the apex of the lung, and a few centimeters of the proximal and distal part of the small intestine were cut (Hirose *et al*. 2007). The organs were fixed in 20% formalin and embedded in paraffin. Sections of 5 μm thickness were cut and stained with haematoxylin-eosin and Masson's trichrome stain.

Liver granuloma area calculation

The areas (μm^2) of granulomas, which contained a single schistosome egg in a section of the liver, were calculated in each mouse. Eight continuous sections of one granuloma were checked to determine whether the granuloma was formed by a single egg and contained an intact or degenerated miracidium. The granulomas were observed with an Olympus AX-80 microscope, photographed with an Olympus DP 71 camera, uploaded by Olympus DP controller with DP manager, and the areas calculated with Win ROOF software package Ver.5.8.1 by tracing the border of granulomas.

Table 1. The number of schistosome worm pairs found in the portal vein

	S. mekongi		*S. japonicum*	
Weeks	No. of worm pairs*	min–max**	No. of worm pairs*	min–max**
6	2·0 ± 0·63	1–3	6·7 ± 2·07	3–9
8	2·2 ± 1·47	1–5	4·5 ± 3·02	1–10
12	3·4 ± 1·67	2–6	4·5 ± 1·64	2–6
16	3·0 ± 0·89	2–4	6·7 ± 1·41	5–8
20	3·3 ± 0·82	2–4	5·2 ± 1·94	4–9

* Mean number of worm pairs ± S.D.
** Minimal number–maximal number of worm pairs.

Statistical analysis

Two granulomas in each section were chosen at random for the statistical analysis of calculated liver granuloma areas (μm^2). The areas of granulomas were analysed by Steel-Dwass's multiple comparison test, and P values of less than 0·05 considered significant.

RESULTS

Schistosome worm burden and mouse survival

Three mice with 4 worm pairs survived until 20 weeks with *S. mekongi* infection. No mice died during the experimental period even when harbouring 9 worm pairs in the case of *S. japonicum* infection. Throughout the experimental period, the total number of schistosome worm pairs of *S. mekongi* was less than that of *S. japonicum* (Table 1).

Changes in relative liver weight

The liver weight per mouse body weight was observed for the definition of hepatomegaly during the experimental period. Hepatomegaly began to be seen at 8 weeks in *S. mekongi* infection, and reached the same level as in *S. japonicum* infection at 12 weeks. In contrast, hepatomegaly was already seen at 6 weeks in *S. japonicum* infection (Fig. 1).

Granuloma area in liver

Liver granulomas varied in size; some appeared very large in the early period and were smaller later in both *S. mekongi* and *S. japonicum* infection (Table 2). The granuloma area seemed to be largest at 12 weeks in

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Table 2. Granuloma size in the liver

Weeks	*S. mekongi* infection		*S. japonicum* infection	
	No. of granulomas measured	Area of granuloma* ($1000\,\mu m^2$)	No. of granulomas measured	Area of granuloma* ($1000\,\mu m^2$)
6	1	53·5 ± 0	11	103·7 ± 55·4
8	7	68·8 ± 109·3	26	116·6 ± 73·2
12	9	201·6 ± 135·3	23	52·4 ± 22·7
16	31	91·1 ± 53·1	48	35·5 ± 26·9
20	26	76·1 ± 47·7	53	16·9 ± 21·2

* Mean area of granulomas ± S.D.

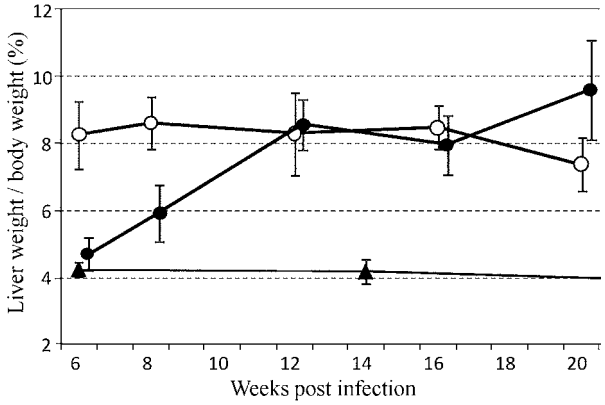

Fig. 1. Changes in relative liver weight (%) of ICR mice in *Schistosoma mekongi* and *S. japonicum* infections. Filled circles; mean ± S.D. of mice with *S. mekongi* infection. Open circles; mean ± S.D. of mice with *S. japonicum* infection. Filled triangles; mean of normal ICR mice quoted from CLEA JAPAN, INC.

S. mekongi infection and 8 weeks in *S. japonicum* infection.

Histopathology of granulomas in the liver

In *S. mekongi* infection, histiocytes and eosinophils were seen around schistosome eggs at 6 weeks. Eosinophil abscess-like granulomas appeared with histiocyte-oriented foam cells in the intralobular area at 8 weeks (Fig. 2A1 and A2), and increased in number at 12 weeks. In the granulomas in the intralobular area, epithelioid cells with eosinophils were seen around the schistosome eggs, which were surrounded by fibrous tissue with eosinophils at 12 weeks (Fig. 2B1 and B2). The fibrosis of granulomas in the intralobular area increased with a mixture of fibrous tissue and eosinophils at 16 weeks. Epithelioid cells around the egg became more like giant cells at 16 weeks (Fig. 2C1 and C2). All stages of granulomas were seen accompanying granulomas with foam cells in the intralobular area at 20 weeks (Fig. 2D1). Inflammatory cells, mainly lymphocytes, infiltrated in the periphery of the granulomas during the experimental period.

In *S. japonicum* infection, eosinophil abscess-like granulomas, which accompanied necrosis of parenchyma cells, were seen in the intralobular area at 6 weeks. In the intralobular area, the granulomas consisted of histiocytes, eosinophils, and gradually increasing fibrous tissue at 8 weeks (Fig. 3A). Granulomas became more fibrous by 16 weeks (Fig. 3B). Lymphocytes and eosinophils infiltrated in the periphery of the granulomas during the experimental period. New granulation was not seen in the intralobular area at 20 weeks, and degenerated eggs were observed to be scattered in normal looking parenchyma (Table 3).

Periportal lesions

In *S. mekongi* infection, inflammatory cells, mainly lymphocytes, infiltrated into the periportal area during the experimental period, and no portal fibrosis was seen even at 20 weeks (Fig. 2D2). In *S. japonicum* infection, inflammatory cells, mainly lymphocytes, infiltrated into the periportal area at 6 weeks. Eosinophil infiltration was increased at 12 weeks. Portal fibrosis was seen at 16 weeks and 20 weeks (Fig. 3C1) along with predominance of eosinophils and fibrous tissue (Fig. 3C2).

Small intestine lesions

Schistosome eggs were deposited in the capillary vessels of the lamina propria mucosa in the early period of *S. mekongi* and *S. japonicum* infection with little inflammatory cell infiltration. Schistosome eggs formed granulomas mainly in the submucosa, accompanied by destruction of the muscularis mucosa (Fig. 4A) after 12 weeks in *S. mekongi* and after 8 weeks in *S. japonicum* infection. *S. mekongi* granulomas contained foam cells, neutrophils, and fewer eosinophils than in liver granulomas (Fig. 4B). *S. japonicum* granulomas were also seen in the submucosa, but did not contain foam cells.

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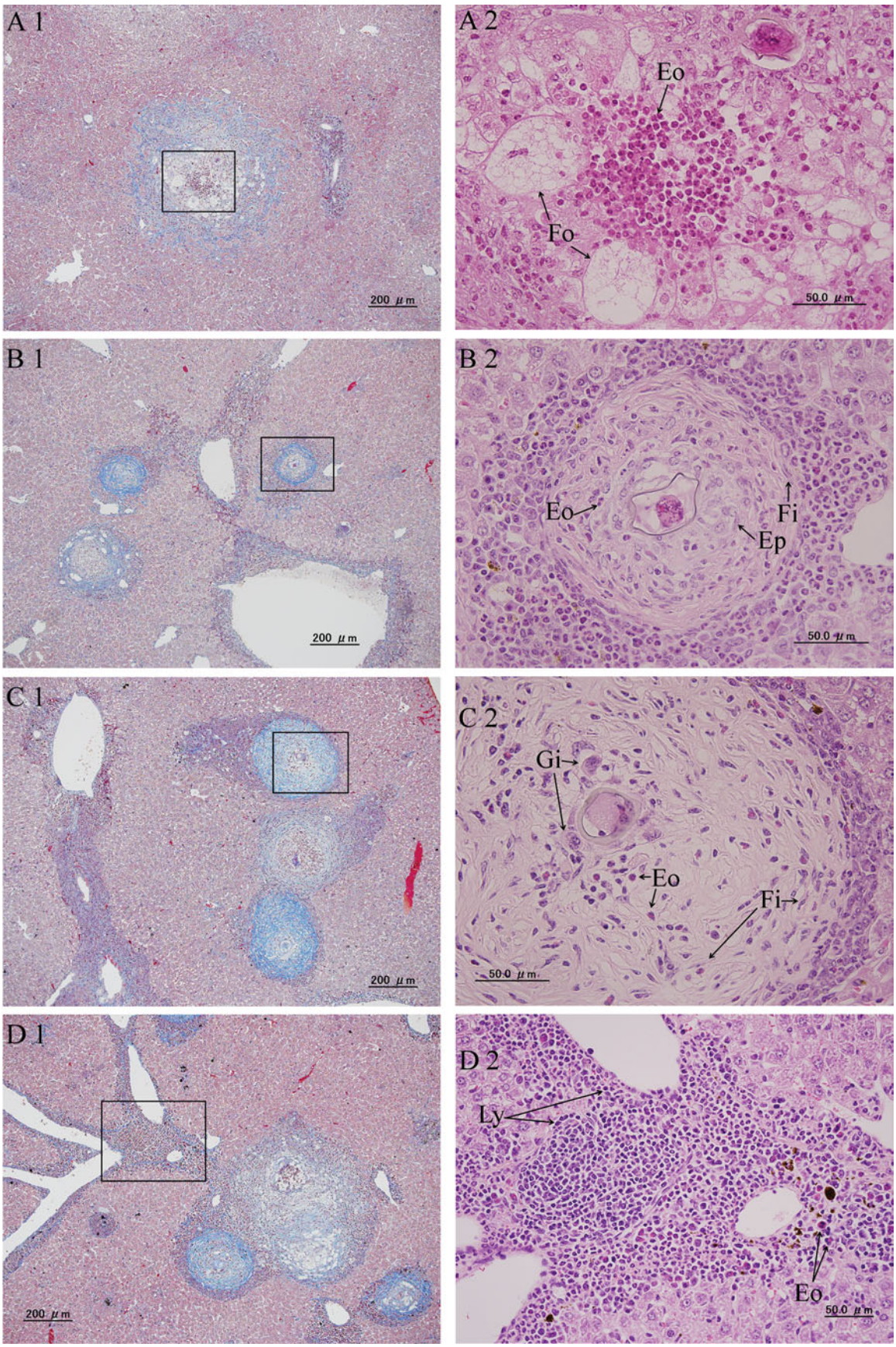

Fig. 2. Histopathology of the liver in *Schistosoma mekongi* infection. (A1) A granuloma in the intralobular area at 8 weeks (Masson's trichrome stain). (A2) The square shown in A1 contains foam cells (Fo) and eosinophils (Eo) (H&E stain). (B1) Granulomas in the intralobular area at 12 weeks (Masson's trichrome stain). (B2) The square shown in B1 contains epithelioid cells (Ep) with eosinophils (Eo), and surrounding fibrous tissue (Fi) with eosinophils (Eo)

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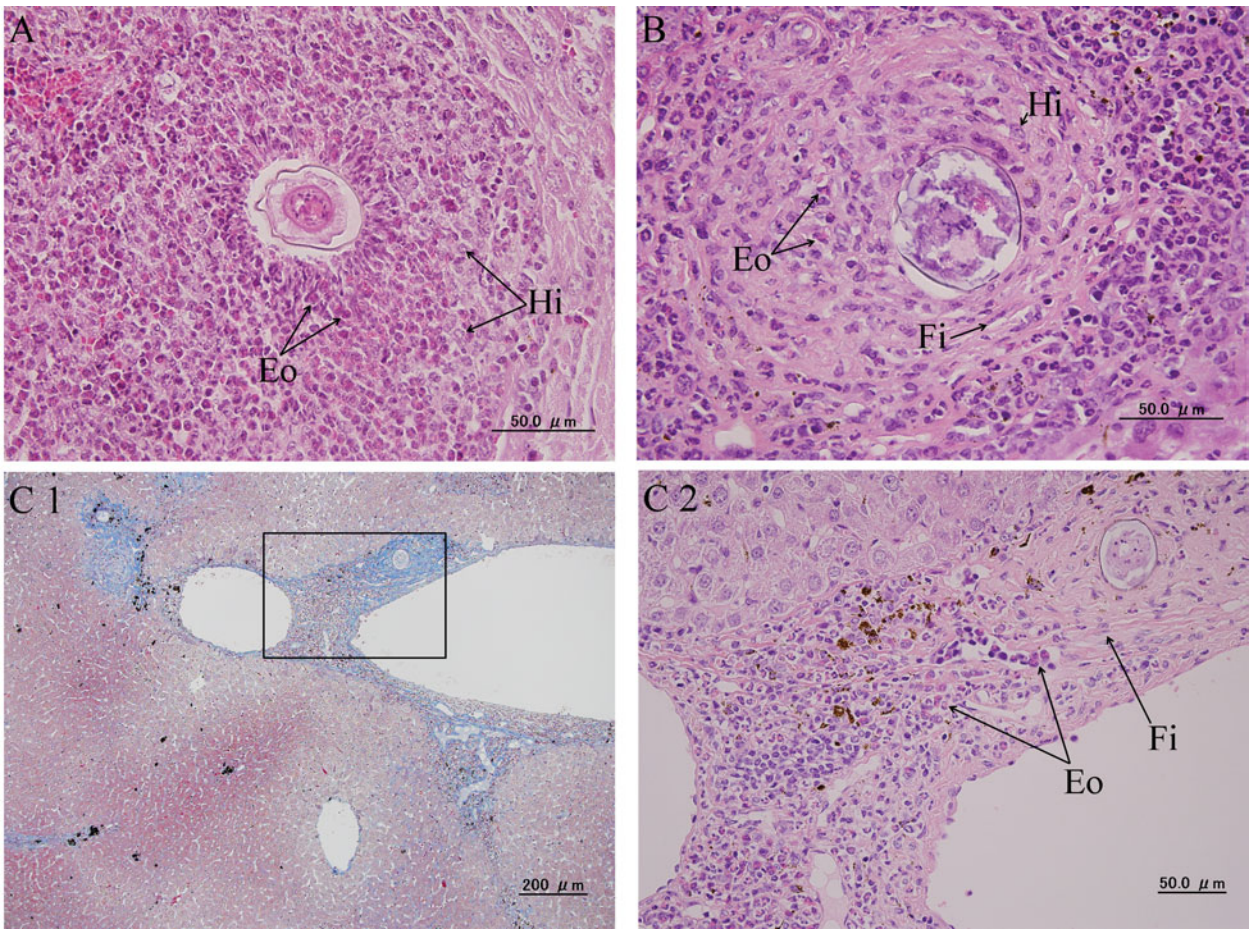

Fig. 3. Histopathology of the liver in *Schistosoma japonicum* infection. (A) Histiocytes (Hi) and eosinophils (Eo) in a granuloma at 8 weeks (H&E stain). (B) Fibrous tissue (Fi), histiocytes (Hi), and eosinophils (Eo) in a granuloma at 16 weeks (H&E stain). (C1) Portal fibrosis contained a schistosome egg at 20 weeks (Masson's trichrome stain). (C2) The square shown in C1 contains eosinophils (Eo) and fibrous tissue (Fi) around a schistosome egg (H&E stain).

A large number of schistosome eggs, which contained live miracidia, were located in the lamina propria mucosa with inflammatory cells, mainly neutrophils, and were likely to be released into the lumen with destroyed villi in the later period in both types of infection. The small intestine with *S. mekongi* infection at 20 weeks is shown in Fig. 4A.

Lung lesions

The number of granulomas, which contained a schistosome egg, was counted in a single section of the lung. In *S. mekongi* infection, 2 granulomas were seen in one case along with an intact section of single schistosome worm at 16 weeks. One schistosome worm was seen without a granuloma in one case at 16 weeks. Three cases with 1–3 granulomas were seen at 20 weeks. In *S. japonicum* infection, 2 granulomas were seen in one case at 12 weeks. One case had 3 granulomas and the other had 5 granulomas with male and female schistosome worms at 16 weeks. Three cases had 1–3 granulomas at 20 weeks. Granulomas in both types of infection consisted mainly of histiocytes and lymphocytes. Inflammatory cells infiltrated, but no fibrosis was seen in the alveolar wall (Fig. 5).

DISCUSSION

Byram and Lichtenberg (1980) reported that mice harbouring 4 or more worm pairs of *S. mekongi* died prior to 10 weeks, and also reported that

(H&E stain). (C1) Granulomas in the intralobular area at 16 weeks (Masson's trichrome stain). (C2) The square shown in C1 contains a mixture of fibrous tissue (Fi) and eosinophils (Eo). Giant cells (Gi) surrounded the schistosome egg (H&E stain). (D1) Periportal inflammatory cell infiltration and granulomas in the intralobular area at 20 weeks (Masson's trichrome stain). (D2) The square shown in D1 contains periportal lymphocyte (Ly) infiltration with fewer eosinophils (Eo) than in the *S. japonicum* infection (H&E stain).

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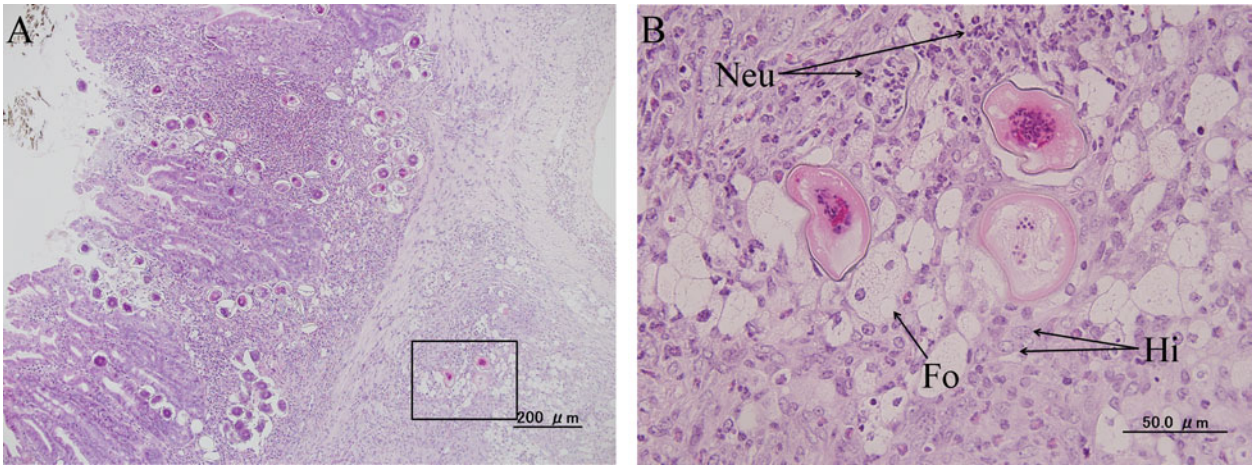

Fig. 4. Histopathology of the small intestine in *Schistosoma mekongi* infection at 20 weeks. (A) Granulomas in submucosa (H&E stain). (B) The square shown in A contains foam cells (Fo), histiocytes (Hi), neutrophils (Neu), and fewer eosinophils than in liver granulomas (H&E stain).

Table 3. Inflammatory cell infiltration of liver granulomas in the intralobular area

		Weeks after infection				
		6	8	12	16	20
S. mekongi	histiocytes	+	+	+	+	+
	foam cells	–	+	+	+	+
	epithelioid cells	–	–	+	+	+
	giant cells	–	–	–	+	+
	eosinophils	+	+	+	+	+
	fibrous tissue	–	–	+	+	+
	lymphocytes*	+	+	+	+	+
S. japonicum	histiocytes	–	+	+	+	–**
	foam cells	–	–	–	–	–**
	epithelioid cells	–	–	–	–	–**
	giant cells	–	–	–	–	–**
	eosinophils	+	+	+	+	–**
	fibrous tissue	–	+	+	+	–**
	lymphocytes*	+	+	+	+	–**

* Lymphocytes were seen in the periphery of granulomas in both types of infection.
** At 20 weeks with *S. japonicum* infection: no newly formed granulomas were observed in the intralobular area.

hepatomegaly increased towards 25 weeks in ICR mice with exposure to 5–10 cercariae. In this study, 7 mice with 2–6 worm pairs unexpectedly died before 15 weeks post-exposure with *S. mekongi* infection (data not shown). Throughout the experimental period, the total number of schistosome worm pairs of *S. mekongi* was less than that of *S. japonicum*. However *S. mekongi* worm burden proved fatal in some mice, indicating that infection with *S. mekongi* was more virulent than that with *S. japonicum*. Histopathological findings, which included continuous attack of *S. mekongi* eggs into the intralobular area of the liver, with accumulating damage to the liver parenchyma, may explain the higher virulence of

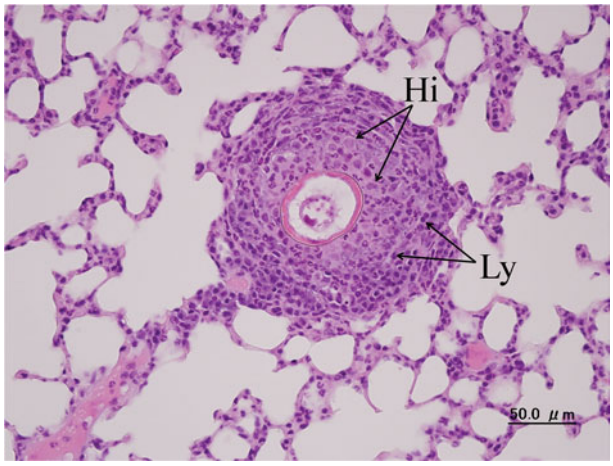

Fig. 5. Histopathology of the lung in *Schistosoma mekongi* infection at 20 weeks. A granuloma with histiocytes (Hi) and lymphocytes (Ly) (H&E stain).

S. mekongi infection compared with *S. japonicum* infection.

When mice were used as an animal model, hepatomegaly with *S. japonicum* infection was reported to usually appear by 6 weeks (Warren and Berry, 1972; Warren *et al.* 1978), while that with *S. mekongi* infection was reported to appear at 8 weeks (Byram and Lichtenberg, 1980). These findings were consistent with our results. The delayed hepatomegaly in *S. mekongi* infection might be related to the delayed egg deposition of schistosome worms when compared to that in *S. japonicum* infection (Voge *et al.* 1978).

In our study, hepatomegaly associated with harbouring of 5 pairs in *S. mekongi* infection was less than that with harbouring of 1 pair in *S. japonicum* infection at 8 weeks, showing that schistosome egg deposition and granulation had more effect on hepatomegaly than the number of schistosome worms. In *S. mekongi* infection, the maximal

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granuloma was reported to appear at 8 weeks (Byram and Lichtenberg, 1980). In this study, 1 granuloma showed a relatively large area at 8 weeks in comparison to the other 6 granulomas, thus resulting in a high standard deviation at 8 weeks in *S. mekongi* infection. Statistically speaking, the largest granuloma was seen at 12 weeks in *S. mekongi* infection in this study. Granulomas containing foam cells tended to be larger than the granulomas without foam cells in *S. mekongi* infection, and large granulomas with foam cells increased in number at 12 weeks, which might explain why the largest granulomas could be seen at 12 weeks. The largest size of the granuloma area in *S. japonicum* infection was reported to appear at 6 weeks in previous studies (Warren *et al*. 1978; Owhashi *et al*. 1996), but it was observed at 8 weeks in this study.

Kupffer cells in the liver are reported to play an important role in granuloma formation; they are transformed into epithelioid cells and giant cells in mice by injection of glucan, an insoluble polysaccharide (Naito and Takahashi, 1991; Takahashi *et al*. 1994). Histiocytes seen in *S. mekongi* infection were also transformed into epithelioid cells and giant cells after presenting as foam cells. The reaction of histiocytes to glucan and *S. mekongi* eggs was not similar to that of histiocytes to *S. japonicum* eggs. The foamy appearance of histiocytes, which contain droplets in the cytoplasm through the process of receptor-mediated endocytosis, accounts for foam cells (Brown and Goldstein, 1983). The foam cells detected in this study were probably the same cells as the vacuolocytic macrophages previously reported in *S. mekongi* infection (Byram and Lichtenberg, 1980). Although the droplets in foam cells are usually cholesteryl ester stored in the cytoplasm, the droplets appearing in *S. mekongi* infection were reported to have only scattered lipid droplets (Byram and Lichtenberg, 1980). The foam cells appearing in *S. mekongi* infection might be formed not only by the uptake of lipid released from necrotized hepatocytes but also that of substances related to the *S. mekongi* egg itself.

The eosinophil reaction to *S. mekongi* eggs was reported to be intense from the earliest time of egg deposition in mice (Byram *et al*. 1978; Byram and Lichtenberg, 1980), and also reported to be intense to *S. japonicum* eggs with more fibrous reaction at 8 weeks (Warren *et al*. 1975, 1978; Owhashi *et al*. 1996). In our study, the eosinophil reaction was intense in the liver in both *S. mekongi* and *S. japonicum* infection, and the neutrophilic reaction was more predominant in lesions in the small intestine in *S. mekongi* infection.

At 8 weeks in both types of infection, granulomas were seen in the intralobular area not in the periportal area in mice. It is not the smaller egg volume (Voge *et al*. 1978) but the non-fibrous portal reaction to eggs that allowed *S. mekongi* eggs to continuously infiltrate into the intralobular area and form granulomas. In *S. japonicum* infection, portal fibrosis containing schistosome eggs could be induced by increasing infiltration of eosinophils into the periportal area. Portal fibrosis may block schistosome eggs from infiltrating into the intralobular area. In *S. mekongi* infection, on the other hand, lymphocytes were predominant until 20 weeks in the periportal area, and portal fibrosis was not seen even at 20 weeks. Instead of the delayed egg deposition, the low rate of eosinophil infiltration into the periportal area explains the lack of portal fibrosis in *S. mekongi* infection. In *S. mekongi* infection, the continuous granuloma formation in the intralobular area and the non-fibrous portal reaction to eggs are strikingly different from *S. japonicum* infection. These morphological changes in the periportal area appeared to be associated with the induction of portal hypertension by periportal thickening and portal enlargement in *S. mekongi* infection (Urbani *et al*. 2002) and pipestem fibrosis in *S. japonicum* infection (Warren and Berry, 1972). Portal inflammatory cell infiltration, mainly by lymphocytes, is also seen in human cases of *S. mekongi* infection (Wittes *et al*. 1984; Monchy *et al*. 2006).

In the small intestine, schistosome eggs were seen initially in capillary vessels in the lamina propria mucosa, which were connected to the portal vein, from where the schistosome worms came to deposit eggs. *S. mekongi* granulomas were seen mostly in the submucosa as previously reported (Byram and Lichtenberg, 1980), contained fewer eosinophils than the liver granulomas, and contained more foam cells which might have been directly induced by schistosome eggs. Granulomas hardened the submucosa, which aided release of live eggs into the lumen. Schistosome eggs in the lamina propria mucosa, surrounded mainly by neutrophils, were likely to be released into the lumen with destroyed villi. Release of a large number of live eggs into the lumen is effective in protecting hosts from accumulation of schistosome eggs in the body, and also enabled parasites to survive.

Minimal fibrosis of the lung in *S. japonicum* infection has been reported in mice (Hirata *et al*. 1993) and in rabbits (Cheever *et al*. 1980). In our study, no fibrosis was seen in the alveolar walls either in *S. mekongi* or *S. japonicum* infection. Multiple lung granulomas, which contained degenerated eggs, were observed in both types of infection at 16 and 20 weeks. Eggs in the liver located close to the central vein in *S. mekongi* infection possibly reached the lung, but eggs trapped in the portal wall in *S. japonicum* infection could not reach the lung. Schistosome eggs laid in the portal vein might be able to reach the lung through collateral vessels when liver fibrosis was advanced, but lung granulomas could be seen without portal fibrosis along with schistosome worms in the lung. Although schistosome worms

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were unexpectedly seen in the lungs both in *S. mekongi* and *S. japonicum* infection, schistosome worms in the lungs were previously reported in human cases of *S. haematobium* infection (Buchanan and Gelfand, 1970), in laboratory-infected rabbits with *S. japonicum* infection (Inokuchi *et al*. 1979), and in field rats in Leyte, Philippines with *S. japonicum* infection (Oshima *et al*. 1978). Although we also reported the presence of *S. mekongi* worms in the lungs of laboratory-infected ICR mice, the question remained how and when the schistosome worms came to reside in the lungs. The possibility of direct egg laying in the lungs in schistosome infection should be mentioned, since the lung granuloma formation appeared to be more common than previously thought and could be seen with or without portal fibrosis in both *S. mekongi* and *S. japonicum* infection.

In conclusion, schistosome eggs flowing from the portal vein to the hepatic lobule impair liver parenchyma cells by inducing eosinophil abscess-like granulomas and cellular or fibrous granulomas with histiocytes. Generally, granulation gradually decreased when miracidia in the eggs degenerated with regeneration of the liver parenchyma. The granulation in the liver, the intestine, and the lung might be one of the protective measures taken by hosts against schistosomiases, with induction of degeneration of miracidia in the eggs. The advanced portal fibrosis resembled pipe-stem fibrosis which has not been seen in *S. mekongi* infection, is effective in preventing eggs from infiltrating into the intralobular area before impairment of the liver parenchyma. The absence of portal fibrosis in *S. mekongi* infection allows schistosome eggs to infiltrate continuously into the intralobular area, and this could be what lies behind the ultrasonographic difference; the echogenic network pattern as was seen in *S. japonicum* infection, has not been noted in *S. mekongi* infection.

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