



Haplorchis taichui: Worm recovery rate and immune responses in infected rats (*Rattus norvegicus*)

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ABSTRACT

Worm recovery rate, mucosal mast cells (MMC)s, eosinophils and serum IgE concentration in rats were investigated after orally feeding 300 *Haplorchis taichui* metacercariae to male rats. The duodenal, jejunal and ileal tissue sections were stained with 1% alcian blue and 0.5% safranin-O for MMC count. Eosinophil count and the serum IgE concentration assay were measured from cardiac puncture blood. The average worm recovery rates were 20.00%, 13.00%, 0.67%, 1.67% and 0.00% on day 3, 7, 14, 21 and 28 post-infection (PI), respectively. The number of MMCs in the infected rats were significantly higher than in the controls ($P < 0.01$), reaching a peak on day 21 PI. They decreased thereafter, with the decline in worm recovery. Eosinophil count and Serum IgE concentration were also increased but not significantly higher than the controls. However, they showed a positive relationship to worm recovery. It could be concluded from the results that MMCs, eosinophils and IgE may play an important role in the expulsion of *H. taichui* from rat intestine. However, the mechanism by which the MMC result in the helminth expulsion still need to be understood, and it is recommended that other cells such as goblet cells be studied further.

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1. Introduction

Heterophyid fluke infections are an important public health problem in Southeast and Far-East Asia. They inhabit the intestinal mucosa, where they cause a mild inflammatory reaction with some necrosis (Chi et al., 1988), acute abdominal pain or appendicitis (Tantachumrun and Kliks, 1978). The eggs may also be deposited in the heart, brain, spinal cord and other organs. The lesions found in the myocardium led to heart failure in some patients (Africa and Garcia, 1935; Africa et al., 1936a,b). Among heterophyid infections in man, *Haplorchis taichui* was first documented from a human autopsy in Udonthani Province, Thailand (Manning et al., 1971). Later, various *Haplorchis* species have been reported in Taiwan, Laos PDR, Thailand and the Philippines (Ditrich et al., 1990; Giboda et al., 1991; Waikagul, 1991; Radomyos et al., 1994). A survey of metacercariae in fish showed that the *H. taichui* was the dominant species of *Heterophyidae* family (Waikagul, 1998). It is predictable that *H. taichui* infection will also become the most common species of intestinal fluke in Thailand in the near future.

In laboratory infections it was shown that the recovery of *H. taichui* from the gut of infected mice and chicks gradually

decreased with the increasing time (Sukontason et al., 2001; Kumchoo et al., 2003). Mechanisms such as inappropriate ecological niche and the host immune response have been considered to describe this phenomenon. Helminth infections in mice and humans usually induce Th₂ cell responses (Else et al., 1993; Pearlman et al., 1993; Jabara et al., 1988; Mossman, 1991). Activated Th₂ cells secrete cytokines; Interleukin (IL) 5, IL-4, IL-9 and IL-10, resulting in the induction of intestinal mastocytosis, blood and tissue eosinophilia, and immunoglobulin (Ig) secretion by B cells, particularly IgE and IgG₁ (Mossman and Coffman, 1989; Mossman, 1991; Finkelman et al., 1991; Thompson-Snipes et al., 1991). In *Nippostrongylus brasiliensis* infection, the characteristic immune responses of the host are eosinophilia, elevated serum IgE, mucosal mastocytosis and goblet cell hyperplasia (Rennick et al., 1990; Abe et al., 1993; Uchikawa et al., 1994; Chen et al., 1995). However, no information is available regarding of the host immune response to *H. taichui* infection.

2. Materials and methods

2.1. Collection of *H. taichui* metacercariae and infection to rats

The metacercariae of *H. taichui* were procured from *Henicorhynchus siamensis* is, collected from Mae-ngud Reservoir, by pepsin digestion method as previously described (Srisawangwong et al., 1997).

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The fish homogenate was filtered through a sieve mesh series, and the filtrate was rinsed repeatedly with normal saline. Finally, the metacercariae were isolated and counted under a dissecting stereomicroscope. The morphology of the *H. taichui* metacercariae was characterized according to the description of Yamaguti (1958) and Pearson and Ow-Yang (1982).

Thirty male rats, aged between 4 and 6 weeks old, were purchased from the National Laboratory Animal Center, Mahidol University, Nakhon Pathom Province, Thailand. After acclimatizing for a week in the Animal House, Department of Biology, Faculty of Science, Chiang Mai University; a single inoculation of 300 active metacercariae of *H. taichui* was orally introduced into the rats ($n=25$) and the rest of the rats served as uninfected controls ($n=5$). The rats were fed a standard diet (C.P. 082) and water *ad libitum*. All experimental procedures were in accordance with Institutional regulations for Animal Care and Use (No. Re 001/07), Department of Biology, Faculty of Science, Chiang Mai University.

2.2. Worm recovery rate and mucosal mast cell (MMC) counts

Five rats from each infected group were sacrificed on day 3, 7, 14, 21 and 28 post-infection (PI), respectively. To observe the worm recovery rate, the duodenum, jejunum and ileum, from three infected rats, were removed and opened longitudinally. Each segment was placed in the Baermann's apparatus for 1.5 h at 37°C with periodically shaking (Chai et al., 1998). The free adult worms were collected and counted under a dissecting stereomicroscope. To evaluate the MMC count, 5–10 mm of duodenum, jejunum and ileum from both infected ($n=5$, in each group) and control rats ($n=1$, in each group) were removed and fixed in Carnoy's solution; dehydrated and embedded in paraffin. The sections, cut at 5 μ thickness, were stained with 1% alcian blue (pH 0.3) and counterstained with 0.5% safranin-O (pH 1.0) (Strobel et al., 1981). The numbers of MMC were counted in well-oriented sections using an eyepiece equipped with a graticule (10/100) at 10 $\times$ and objective lens at 40 $\times$. At least five fields of each tissue section were counted. The mean numbers of MMC/0.0625 mm² was expressed as mean and standard deviation and analysed using the Student *t*-test.

2.3. Eosinophil counts

At least 2.0 ml of blood from both infected and control rats were collected from the left ventricle for eosinophil count and serum IgE concentration. For eosinophil count, 20 μ l of EDTA-mixed blood was combined with Hinkelman's solution and gently agitated for 3 min. A direct eosinophil count was measured using an improved Neubauer hemocytometer.

2.4. Serum immunoglobulin E (IgE) concentration assays

The remaining blood from the cardiac puncture was centrifuged to obtain serum. IgE concentration measurements were performed using a rat IgE ELISA quantitation kit (Bethyl Laboratories, Inc.). Briefly, 96-well plates (NUNC) were coated with 10 μ g/ml of captured antibody (sheep anti-rat IgE, sigma) in carbonate buffer and then incubated at 4°C overnight. Plates were washed with PBS/Tween 20 (sigma) for 1–2 times. Standards and sera (1:50) were serially diluted with sample diluents (1% BSA/PBS, 0.05% Tween 20, pH 8.0) and the total 100 μ l were added to microwells. The plates were incubated at room temperature for 2 h, washed 4–5 times with PBS/Tween 20, pH 8.0. The detection antibodies (sheep anti-rat IgE labeled with horse radish peroxidase, Sigma; 1:2500) were diluted with 1% BSA/PBS, 0.05% Tween 20, pH 8.0 and 100 μ l was added and incubated at room temperature for 1 h. The plates were washed 4–5 times with PBS/Tween 20, pH 8.0. The enzyme reaction was started by adding a substrate (TMB, Sigma),

Table 1

Mean worm recovery rate in rats infected with *H. taichui*

	No. of rats	No. of Mc. infected	Mean numbers of worm recovery				
			Duodenum	Jejunum	Ileum	Total	%
Day 3	3	300	0.00	0.00	60.00 $\pm$ 9.17	60.00 $\pm$ 9.17	20.00
Day 7	3	300	0.00	0.00	39.00 $\pm$ 2.00	39.00 $\pm$ 2.00	13.00
Day 14	3	300	0.00	0.00	2.00 $\pm$ 1.15	2.00 $\pm$ 1.15	0.67
Day 21	3	300	0.00	0.00	5.00 $\pm$ 2.89	5.00 $\pm$ 2.89	1.67
Day 28	3	300	0.00	0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00
Total	15	1500	0.00	0.00	106.00 $\pm$ 3.59	106.00 $\pm$ 3.59	7.07

Mc, metacercariae.

incubated in the dark at room temperature for 30 min and stopped by adding 100 μ l of 1 M H₂SO₄. The absorbance at 450/630 was measured by Anthos 2010 (Austria) microplate reader. The results were compared to the control groups and the values were expressed as mean $\pm$ SD.

3. Results

3.1. Worm recovery rate

The average worm recovery rate was 7.07% with the highest rate of 20.00% on day 3 PI. Recoveries remained relatively high until day 7 PI (13.00%) and decreased rapidly to 0.67% on day 14 PI, 1.67% on day 21 PI, disappearing by day 28 PI. Worms were only recovered from the ileum (Table 1). The incidence of *H. taichui* in the small intestines of rats was 60.00% (9/15) and the intensity ranged from 0 to 60.

3.2. MMC counts

The kinetics of MMC numbers is shown in Table 2 and Fig. 1. MMC numbers in the ileum were slightly higher than those in the duodenum and jejunum. The peak level of mastocytosis was observed on day 21 PI. The MMC number in the infected rats was significantly increased in all segments of the small intestine, through the whole period of observation as compared with uninfected controls. They had a tendency to decrease along with the decline in the worm numbers.

Table 2

Mean numbers of mucosal mast cells in small intestines of rats infected with *H. taichui*

Day PI	No. of rats	No. of Mc. infected	Mean no. of MMCs/0.0625 mm ² (mean ± SD)		
			Duodenum	Jejunum	Ileum
<i>Control</i>					
Day 3	1 [#]	0.00	17.80±0.57	17.10±0.14	17.65±0.78
Day 7	1 [#]	0.00	14.45±0.21	17.00±0.85	15.05±1.48
Day 14	1 [#]	0.00	17.85±0.78	17.55±0.49	17.10±0.99
Day 21	1 [#]	0.00	17.65±0.64	17.30±0.99	15.30±1.70
Day 28	1 [#]	0.00	16.55±0.07	15.50±0.42	15.35±0.07
<i>Infected</i>					
Day 3	5	300	29.40±0.14	30.80±1.13	29.70±1.70
Day 7	5	300	28.55±0.35	27.80±3.39	28.80±0.57
Day 14	5	300	31.85±1.91	32.60±2.26	37.20±1.70
Day 21	5	300	36.80±1.84	37.20±1.70	41.25±1.06
Day 28	5	300	32.50±0.71	22.40±3.11	28.50±0.71

Mc, metacercariae; 1[#], the shared data from other two experiments (*Stellantchasmus falcatus* and *Centrocestus caninus* infection groups) were used for increasing the effective number of control animals available for each experimental time to 3. As can be seen from the narrow SDs, there is little variation in uninfected animals.

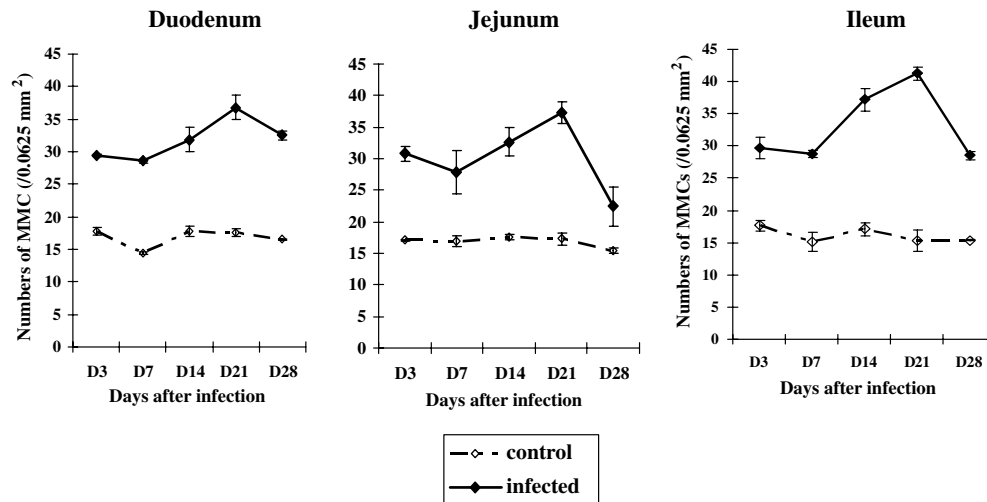


Fig. 1. The kinetics of the MMC numbers in different sites of small intestine of the rat infected with *H. taichui*. The mean numbers of MMC in all infected rats were significant higher than those of the controls ($P < 0.01$) and reached a peak level on day 21 PI. They decreased afterwards.

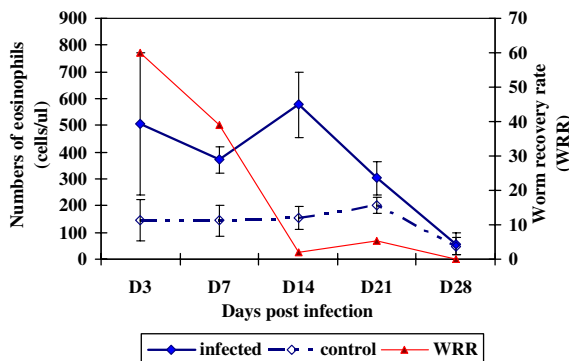


Fig. 2. Correlations between the mean worm recovery rate (WRR) and mean numbers of eosinophils in rats infected with *H. taichui*. The numbers of eosinophils reached a peak level on day 14 PI, and gradually decreased afterward. However, the mean values of eosinophil numbers in infected rats were within the normal range.

3.3. Eosinophil counts

Eosinophil counts in rats experimentally infected with *H. taichui* were higher than those of controls, and reached the peak levels on day 14 PI in when the number of worms was low; after that the counts gradually decreased along with the decrease in worm recovery. Although the counts were increased, they still were within the normal range (Fig. 2).

3.4. Serum IgE concentration assays

Serum IgE concentrations in rats infected with *H. taichui* were slightly higher than controls; however, there was no significant difference. The serum IgE concentration was increased on day 3 PI and reached a peak level on day 14 PI; thereafter gradually decreased along with the falling number of worms (Fig. 3).

4. Discussion

H. taichui was only found in the ileum. A high yield of adult worms could be obtained in the first week. A similar finding was observed in mice infected with *H. taichui* (Sukontason et al., 2001) while with chick infection, a high yield of *H. taichui* adult worms could be obtained until day 16 PI (Kumchoo et al., 2003). However,

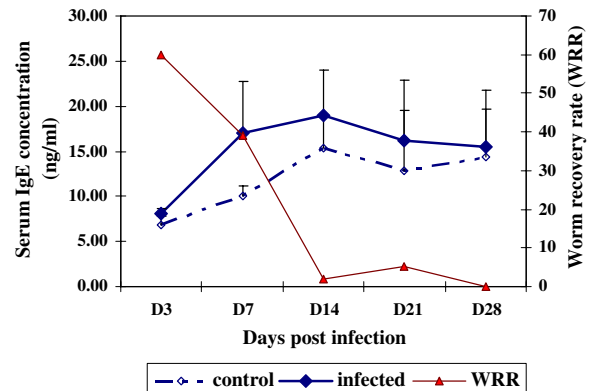


Fig. 3. Correlation between mean worm recovery rates (WRR) and means serum IgE concentration (ng/ml) in rats infected with *H. taichui*. Strong serum IgE concentration was observed on days 14 PI where the worm recovery rate was abruptly decreased. However, there was no significant difference as compared to the controls.

the worm recovery rate in chicks was observed to be lower after day 14 PI. This phenomenon corresponds to previous reports of other heterophyid fluke infections such as *Metagonimus yokogawai* (Chai, 1979), *Centrocestus armatus* (Hong et al., 1989) and *Stellantchasmus falcatus* (Wongsawad et al., 1998). The authors suggested that an inappropriate ecological niche, shorter life-span and reduced size of mature worms were the major causes for this. However, it is generally known that the parasites show a tendency to decrease if the suitability of the host is very low. Our results suggested that the rat is not a suitable host for *H. taichui* development because it can only survive in rat intestine for a week.

Additionally, *H. taichui* infection in rat can cause intestinal mastocytosis, elevated serum IgE concentration and eosinophilia. Our findings correspond to those of Ruitenber et al. (1979) and Jarrett and Miller (1982) suggested that the three immunologic hallmarks of intestinal nematode infections are eosinophilia, intestinal mastocytosis and elevated IgE production. Our study demonstrated that increased numbers of MMC were found in all parts of the small intestine of infected rats, particularly in the ileum where the worms were found. Peak intestinal mastocytosis was seen on day 21 PI when the worm recovery rate had decreased considerably. The association between the decline in worm recovery and

increased numbers of MMC was positively correlated, indicating that this may be involved in the process of worm expulsion from the rat intestine in addition to the environmental niche concept. Miller (1984) stated that the increase of the mast cell numbers in the mucosa is the hallmark of the host response to parasitic helminth infections in the intestine. It has also been reported to be an important effector cell in expelling intestinal trematode infections such as *Fibricola seoulensis* (Kho et al., 1990), *M. yokogawai* (Chai et al., 1993) and *Echinostoma hortense* (Kim et al., 2000). It is therefore strongly suggested that MMCs may be involved in the expulsion process of flukes from the rat intestine. The number of mucosal mast cells have also been shown to increase during acute and chronic phases of experimental *Schistosoma mansoni* infections in mice (Kermanizadeh et al., 2001). However, there is evidence that the effector cells for the expulsion of *N. brasiliensis* are not mast cells but goblet cells (Nawa and Korenaga, 1983). In mice infected with *Echinostoma trivolvis*, the expulsion of the worm was also associated with goblet cell hyperplasia (Fujino et al., 1993).

The numbers of eosinophils and serum IgE concentrations increased during the first week of infection and remained relatively high until the worms disappeared. However, strong serum IgE responses were still observed after day 14 PI when the worm recovery rate was very low, suggesting it to be a secondary response to *H. taichui*. It is thought that the increased numbers of eosinophils and elevated serum IgE be involved in the expulsion of *H. taichui* from the rat small intestine. Killing of helminth by eosinophils through antibody-dependent cellular cytotoxicity (ADCC) (Butterworth, 1984) is an attractive and widely cited mechanism for resistance to parasitic worms. Previously, in rats infected with *N. brasiliensis* it was demonstrated that IgE antibodies may play a secondary role by inducing immediate hypersensitivity reaction which aids IgG in killing the worms (Murray et al., 1971). Increased serum IgE levels were also previously reported in paragonimiasis, fascioliasis and hookworm infections (Kojima et al., 1972). Visser et al. (1995) reported that travelers from non-endemic areas experiencing the Schistosoma infection for the first time, showed both elevated IgE and eosinophilia. A positive correlation between IgE levels and protection was also reported in human infected with *S. mansoni* and *S. haematobium* (Dunne et al., 1992; Hagan, 1993). Also, the increased serum IgE levels, peripheral eosinophilia, intestinal goblet cell hyperplasia and intestinal mastocytosis have been observed in intestinal nematode infections (Nawa et al., 1994). However, the ablation of eosinophils by antibody-mediated neutralization of IL-5, the cytokine that promotes eosinophil production, has no effect on the level of *S. mansoni* (Abbas et al., 1996) or gastrointestinal nematodes (Finkelman, 1997) that establish during a primary infection. From the above studies, we may conclude that the effector mechanism for the expulsion of the intestinal helminths may be different depending on the physiobiological properties of both parasite and host species.

In conclusion, the intestinal mastocytosis, eosinophilia and elevated serum IgE may be involved in the expulsion of *H. taichui* from the rat intestine. However, further study of the roles of MMCs and other effector cells such as goblet cells on the helminth expulsion are recommended.

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