

Experimental model in rats for study on transmission dynamics and evaluation of *Clonorchis sinensis* infection immunologically, morphologically, and pathologically

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Received: 18 August 2009 / Accepted: 26 August 2009
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Abstract This study aims to gain a better insight into the transmission patterns and immunologic profile of *Clonorchis sinensis* infection and make a headway on the pathogenesis regarding cholangiocarcinoma and hepatic lesions. Experimental models orally infected by *C. sinensis* metacercariae were constructed in rats. Immunological assays were performed to measure serum level of IgA, IgE, IgG1, IgG2a, IFN- γ , and IL-4. Infection parameters were assessed by worm recovery rate, eggs per gram faeces and worm size. Pathological sections with livers were managed with immunofluorescence, hematoxylin, eosin, and Masson's trichrome staining to evaluate the hepatic pathological changes. Interestingly, rats infected with only one *C. sinensis* metacercariae even gained a high worm recovery rate of 83.3% compared with rats infected with more metacercariae. Serological changes according to different infection doses indicated that immune response presented a tendency to Th2 type by expressing transient high level of IgG1, IL-4, and IgE. Hepatic tissues appeared inflammatory and fibrotic, revealed by different stainings. Intrahepatic bile ducts displayed cholangiectasis and proliferation with excreted/secreted antigen histologically located. *C. sinensis*, as a fish-borne zoonosis, presented novel transmission patterns which explained high infection

rate in endemic areas; infection rate of *C. sinensis* was frequency-dependent and dose-related. Humoral immunity played a prevalent role in resisting to *C. sinensis* based on the rat models. *C. sinensis* infection played an undoubted role in biliary and hepatic diseases.

Introduction

Human clonorchiasis, caused by a liver fluke *Clonorchis sinensis*, is a fish-borne zoonosis endemic predominantly in eastern Asian countries or regions, including China, Japan, southern Korea, and Vietnam (Rim 2005). High prevalence in endemic regions was mainly due to the increased consumption of raw or undercooked freshwater fish and shrimp with metacercariae of *C. sinensis* (Pan et al. 2000; Fang et al. 2000). It is currently estimated that based on epidemiological statistics, about 35 million human beings have been infected by *C. sinensis* globally, of whom 15 million are in China (Lun et al. 2005). There is evidence that the prevalence of this disease in people has been increasingly common over the past decades in both developed countries and nonendemic regions causing large socioeconomic loss as a result of people's unhealthy eating habit. Although clonorchiasis is not common in western countries, people can also be infected with liver fluke by eating frozen, dried, or pickled fresh-water fish containing *C. sinensis* metacercariae (MC) imported from epidemic areas (Zheng et al. 2005). The *C. sinensis* adults parasitize in bile ducts causing hepatic and biliary diseases. Heavy or long-term chronic infections often lead to pyogenic cholangitis, cholelithiasis, cholecystitis, pancreatitis, hepatic fibrosis, even cholangiocarcinoma, and liver cancer (Lin et al. 1987; Hou 1955; Yang et al. 2004; Choi et al. 2004; Watanapa and Watanapa 2002). Therefore, clonorchiasis

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has posed a threat to the public health in China. High prevalence in human beings of *C. sinensis*, to some extent, has improved awareness of clonorchiasis; however, major gaps between the fundamental knowledge and understanding of the specific transmission patterns of *C. sinensis* still exist. Epidemiological statistics indicates that 5 million people in Guangdong Province of China are infected (Pan et al. 2000). Some reports thought that (Finkelman et al. 1991) helminth infections were generally characterized by high levels of immunoglobulin E and eosinophils, induced by interleukin-4. These cytokines are produced by the helper T2 subset of CD4 + T cells, such as *Fasciola hepatica* (Waldvogel et al. 2004), but little was known about the immune profile of *C. sinensis* infection. To gain a better insight into the transmission mechanisms, we carried out an experimental models in rats to reveal key aspects regarding the transmission dynamics of *C. sinensis* and to determine the immune characteristics as well as to provide some considerable references for control of this important disease.

Materials and methods

Preparation of *C. Sinensis* metacercariae

C. sinensis MC were isolated from freshwater fish (*Pseudorasbora parva*), prepared from our ecologic pool constructed indoor at Sun Yat-Sen University in Guangzhou of China (Liang et al. 2009). Fishes were digested with acid pepsin solution (0.2% HCl, 0.6% pepsin, pH 2.0) and collected under stereomicroscope. The *C. sinensis* MC were kept in 0.1 M phosphate-buffered saline (PBS, pH 7.4) at 4°C until used.

Experimental animals

Six-week-old male Sprague–Dawley (SD) rats were purchased from the animal center of Sun Yat-Sen University and randomly divided into 19 groups according to the research design. Each group consisted of six rats and was kept in the clean animal cages until killed. The animal experiment was carried out under the international animal ethical standard.

Experimental schemes

The procedure of this experiment was designed as follows: Besides the control group, there were 18 experimental groups according to the study design. Each group of rats was orally administrated with different doses of fresh *C. sinensis* MC to imitate human's eating habit. Nine groups of rats were infected only one time with a dosage of 1, 3, 5,

10, 50, 100, 140, 500, and 1,000 of *C. sinensis* MC, respectively. Four groups of rats were infected four times at an interval of 1 day with 5, 15, 35, and 50 of *C. sinensis* MC, respectively, once a day. Five groups of rats were infected 14 times continuously with 1, 3, 10, 20, and 40 of *C. sinensis* MC, respectively, twice a day.

Antibody determinations and cytokines measurements

Serum samples were collected from both experimental rats and control rats at 0, 4, 8, and 16 weeks post primary infection. All serum samples were stored at −80°C until used for enzyme-linked immunosorbent assays (ELISA). The level of specific IgA, IgE, IgG1, and IgG2a in the serum was measured by ELISA coated with 0.5 µg excretory–secretory antigen (ESA) per well for one night at 4°C. After washing the ELISA plates with phosphate-buffered saline tween-20 (PBST, pH 7.4) three times, 1 min per time, blocking the plates with 5% skimmed milk for 2 h at 37°C, washing the ELISA plates with PBST three times again, and incubating the plates with the rats serum for 1 h at 37°C, the serum were diluted 1:400 for specific IgG1 (Bethyl, USA), IgG2a (Bethyl, USA), while 1:20 for specific IgA (Rockland, USA) and IgE (ARP, USA). Then, incubate the plates with the HRP conjugates for 1 h at 37°C, the HRP conjugates were diluted 1:5,000 for IgG1, IgG2a, and 1:1,000 for both IgA and IgE. ELISA kits (R&D, USA) were used to measure the serum level of IFN-γ and IL-4. The procedures were according to the manufacturer's instruction. Serum IFN-γ and IL-4 concentrations were obtained from the standard curve. The optical density was read at 450 nm using an ELISA reader (TECAN SUNRISE, Austria).

Morphological and histopathological observation

Fecal samples of rats were collected from the 25th day post primary infection. Eggs per gram feces (EPG) were calculated statistically according to Stoll's method at the eighth week. All rats were sacrificed with ether at the 16th week post primary infection. Adult worms were obtained and counted after dissection to make an appraisal of the maturity. Livers from both control group rats and experimental group rats were fixed with 10% formalin solution and routinely processed for pathological sections managed with immunofluorescence, hematoxylin and eosin (HE), as well as Masson's trichrome staining to make histopathologic study. The pathological sections of liver infected with 100 *C. sinensis* MC and liver infected with 1 *C. sinensis* MC as well as the normal liver were deparaffinized with 100%, 95%, 85%, and 75% alcohol, respectively. The sections were blocked with normal goat sera for one night at 4°C, then incubated with mouse antiESA serum for 1 h at

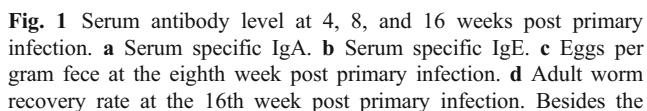
Statistical analysis

Results

Eighteen groups of rats were infected at different infection intervals and doses; no rats died of infection. Worm recovery rates were described in Fig. 1d. Interestingly, compared with other groups of rats, the highest mean worm recovery rate (83.3%) were distributed in rats, which were administrated totally with only one *C. sinensis* MC. Five rats of six were found with one big adult worm parasitizing in intrahepatic bile duct. Regardless of total infection

Immune responses

In general, serum antibody levels indicated that immune response was produced from primary infection and raised steadily during reinfection. On one hand, for instance, serum level of IgE of all infected rats was significantly higher than that of IgA ($p<0.01$) at the 8th week. On the other hand, IgE level rose rapidly and peaked at the 8th week; however, IgA rose slowly and lasted for a longer time. By ELISA (Fig. 2a, b), comparing to the control group, IFN- γ and IL-4 raised from primary infection, and both IFN- γ and IL-4 declined to a low level at the 16th week post primary infection; whereas, deducted with control values, IL-4 of most of the infected rats were higher than that of IFN- γ ($p<0.05$), especially for the altofrequent infection rats. What is more, IgG1 and IgG2a were detected at the 4th week, 8th week, and 16th week to



control group, each group of rats was administered with MC of *C. sinensis* at different doses and intervals; for example, 1×1 means one MC for one time, etc. Results are mean±SE for six rats per group; a value of $p<0.05$ was considered significant

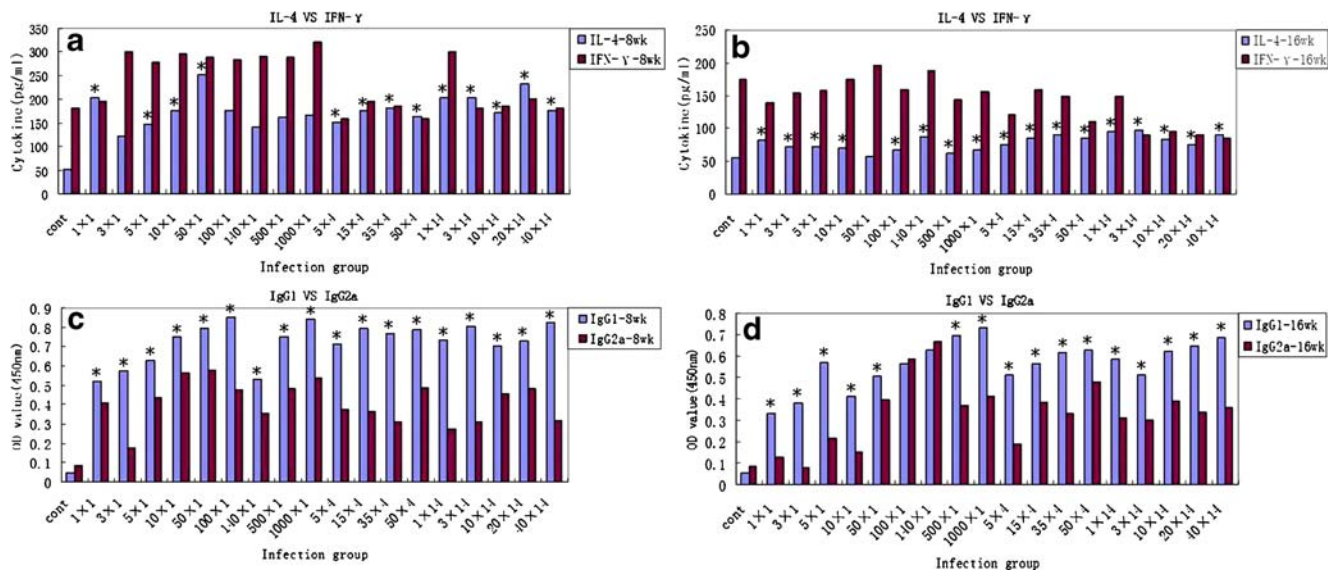


Fig. 2 Cytokine responses and immune type assessment of *C. sinensis* infection. **a** IL-4 vs IFN- γ at the eighth week. **b** IL-4 vs IFN- γ at the 16th week. **c** IgG1 vs IgG2a at the eighth week. **d** IgG1 vs IgG2a at the 16th week. Besides the control group, each group of

rats was administered with MC of *C. sinensis* at different doses and intervals; for example, 1×1 means one MC for one time, etc. Results are mean±SE for six rats per group; asterisk indicates statistical difference ($p<0.05$) between IL-4 and IFN- γ , IgG1, and IgG2a

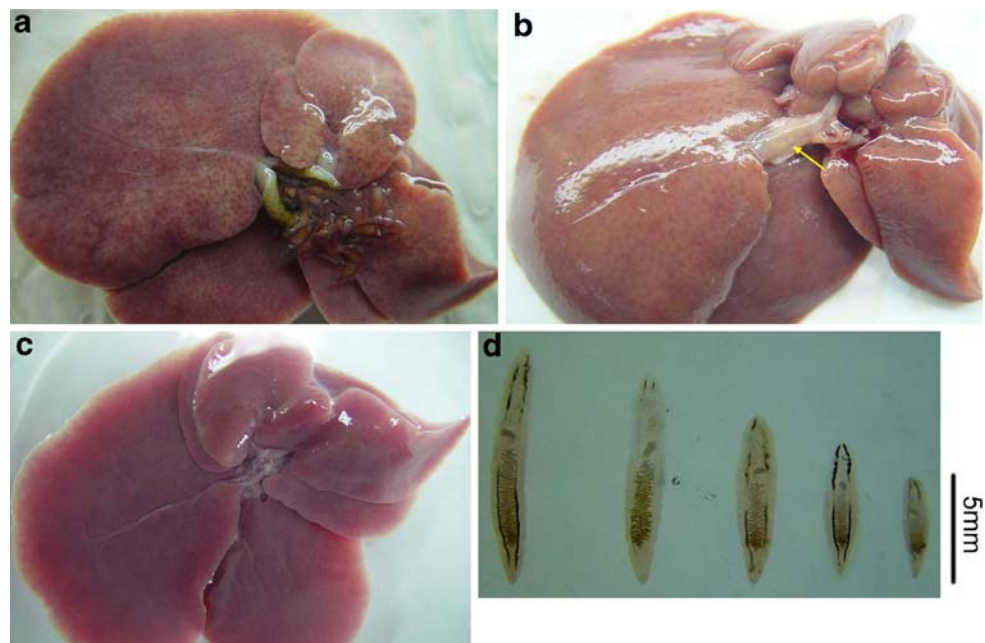
reevaluate the immune characteristic of *C. sinensis* infection. In accordance with the comparison of IL-4 and IFN- γ , IgG1 was significantly higher than that of IgG2a at the 4th week, 8th week, and 16th week ($p<0.005$).

Morphological observations and pathological changes

Morphologically (Fig. 3), to be surprised, Fig. 3a, b showed different number of *C. sinensis* adult worms parasitizing in extrahepatic bile duct and resulted in liver cirrhosis. With

the increase of adult worm numbers, the size of worms decreased regularly, that was to say, the maximal worm was obtained from dissected liver in which there was only one adult worm (1 MC), while the minimal worm was obtained from 1,000-MC rat. In Fig. 3d, from left to right, the worms were recovered from 1-MC rat, 50-MC rat, 100-MC rat, 500-MC rat, and 1,000-MC rat, respectively. The length of worm from 1-MC rat was 2 cm, yet to 1,000-MC rat, the length was 0.5 cm. By immunolocalization (Fig. 4), ESA was located in both intrahepatic and extrahepatic bile ducts.

Fig. 3 Morphological comparison of livers and *C. sinensis* adult worms. **a** Cirrhotic liver parasitized by 100 adult worms. **b** Liver with one worm obstructing extrahepatic bile duct (arrow in panel b). **c** Normal liver. **d** Five adult worms of different sizes are showed; from left to right are worms from 1-MC rat, 50-MC rat, 100-MC rat, 500-MC rat, and 1,000-MC rat, respectively



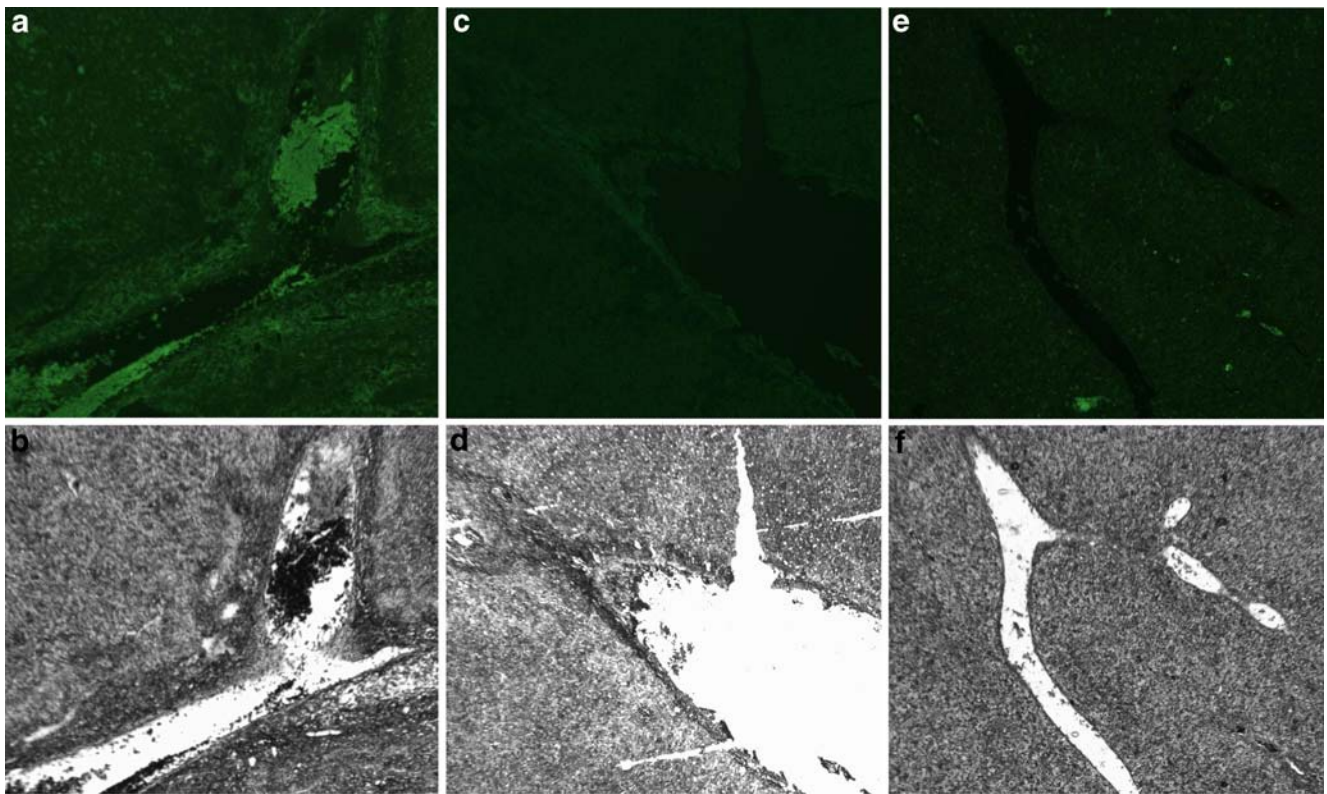


Fig. 4 Immunolocalization of ESA of *C. sinensis* in infected livers. **a** ESA was located in 100-worm liver along the wall of bile ducts ($\times 40$, fluorescent light). **b** ESA was located in liver infected with 100 MC ($\times 40$, white light). **c** ESA was located in liver infected with 1 MC

($\times 40$, fluorescent light). **d** ESA was located in liver infected with 1 MC ($\times 40$, white light). **e** Normal intrahepatic bile duct ($\times 40$, fluorescent light). **f** Normal intrahepatic bile duct ($\times 40$, white light)

ESA of *C. sinensis* distributed along the wall of bile ducts with mixture of ESA, and bile depositing at the end of bile ducts (Fig. 4a, b). In contrast to the normal, all infected intrahepatic bile ducts presented dilated and asperous. Even one adult worm parasitized in the liver induced cholan-

giectasis, as revealed by immunofluorescence (Fig. 4c, d). We managed pathological sections of infected livers with hematoxylin and eosin to assess the pathological changes caused by *C. sinensis* (Fig. 5a, b, d). In response to *C. sinensis* infection, large inflammatory cells penetrated from

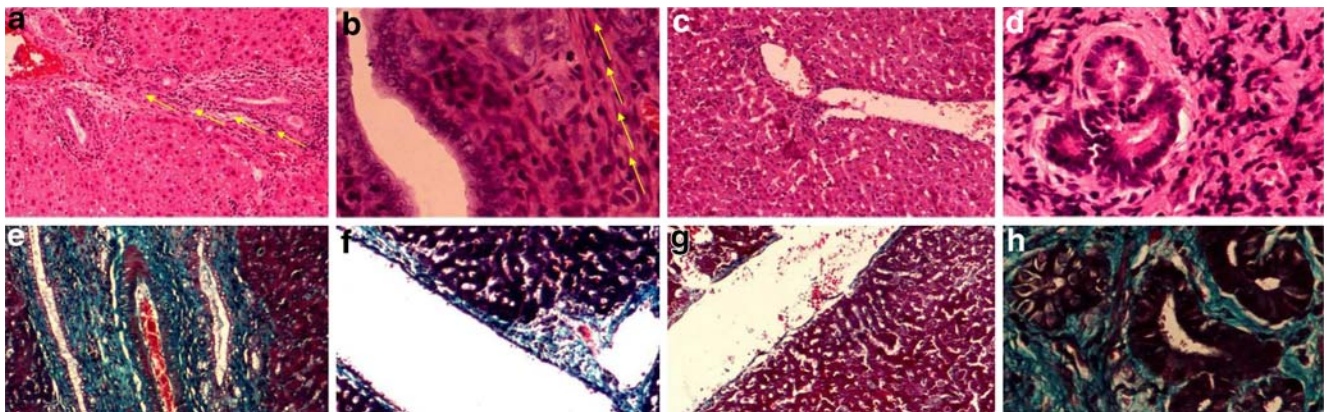


Fig. 5 Pathological changes of infected liver. **a** Large infiltrative inflammatory cells ($\times 100$, H&E). **b** Activated stellate cells rearranged in a line ($\times 400$, H&E). **c** Normal bile duct ($\times 100$, H&E). **d** Magnified adenomatoid hyperplasia ($\times 400$, H&E). **e** Fibrotic changes of intrahepatic bile duct infected with 100 MC ($\times 100$, Masson's trichrome staining). **f** Fibrotic changes of intrahepatic bile duct infected with 1

MC ($\times 100$, Masson's trichrome staining). **g** Normal bile duct ($\times 100$, Masson's trichrome staining). **h** Magnified adenomatoid hyperplasia ($\times 400$, Masson's trichrome staining). Arrows in **a** and **b** presented the rearranged stellate cells. Collagen fibers were showed as *bluish-green* in Masson's trichrome staining

blood vessels and aggregated nearby bile ducts (Fig. 5a). Furthermore, adenomatoid hyperplasia emerged around dilated bile ducts with inflammatory cells infiltrating extensively (Fig. 5d). Accompanying with inflammatory cells, the most noticeable phenomenon was when activated stellate cells rearranged in a line (arrows in Fig. 5a, b). Besides hematoxylin and eosin, to evaluate the fibrotic changes resulted from *C. sinensis* infection, we managed the pathological sections with Masson's trichrome staining (Fig. 5e, f, g, h). Both biliary ducts and liver parenchyma proved to be fibrotic proportionally with the increase of infection dosage (Fig. 5e, f). Compared with normal one (Fig. 5g), collagen fibers in infected livers proliferated along the wall of dilated biliary ducts. Moreover, the same results as hematoxylin and eosin, adenomatoid hyperplasia was found to surround the dilated biliary ducts with collagen fibers infiltrated (Fig. 5h).

Discussion

In the present study, to evaluate the infection of *C. sinensis*, we constructed the animal model in rats. The most striking result we acquired was that the worm recovery rate achieved 83.3% in rats which infected with only one *C. sinensis* metacercariae. Furthermore, to certify the high infection rate, we reinfected ten SD rats with only one *C. sinensis* metacercariae each. Nine rats were successfully found with one adult worm in extrahepatic bile duct and with pathological changes (infection rate was 90%, data not published). The infection rate will be higher for human beings than rats, for rats were known to highly resist *C. sinensis* during reinfection (Sohn et al. 2006; Zhang et al. 2008); whereas, human develops immunotolerance to *C. sinensis*. Up to now, little was reported about the least infection dose of pathogens. The result from the present study will innovate our viewpoint that intake of only one *C. sinensis* MC will also result in high infection rate. This interesting phenomenon distinguished human clonorchiasis with other parasites, or even any pathogens reported before in invading host, and powerfully explained the high infection rate of *C. sinensis* in human beings of epidemic areas. Considering worm recovery rate, given the same infection dose, regardless of resistance, the results from our experiment suggested that infection frequency correlated more than infection dose to worm recovery rate and hepatic lesions; that is to say, the more frequent people consume freshwater fish, the higher risk people will contract *C. sinensis*. Unclear immunotolerance and immune escape have hindered deep research on the prevention of *C. sinensis*. Increasing consumption of freshwater fish will make the prevention of *C. sinensis* more burdensome. As for the disproportionate worm recovery rate and infection

dose, nutrition limitation, and space restriction may explain the reason. Many adult worms died before we inspected the rats (sacrificed at the 16th week). Maybe serological assays (detected at the 8th week) and EPG (calculated at the 8th week) can reveal the truth. Pathological changes showed that livers with only one worm were inflammatory and swollen. However, it was no doubt that rats infected with 1,000 *C. sinensis* MC confronted much more than rats infected with only one *C. sinensis* MC for pathogenic substances produced by adult worms still induced subsequent devastating injury, though worms died during parasitism.

IL-4 is an inducer of Th2 type responses and can influence on the differentiation of Th0 cells into Th2 cells by inhibiting the differentiation into Th1 cells (Pearce and Reiner 1995). In the present study, when deducted the basal production, elevated IL-4 prevailed over elevated IFN- γ during the infection procedure. The more frequent to infect, the much IFN- γ was suppressed. Moreover, in our results, IgG1 as the representative Th2 type antibody subclass predominated significantly to IgG2a during infection. The results we gained suggested that *C. sinensis* infection induced a compound immune response which polarized to Th2 type immune response. This was similar to a study on mice infected with *C. Sinensis* (Choi et al. 2003), Th2 type immune response was provoked by overexpressing the IL-4 production. It was demonstrated by antibody titers, especially IgA and IgE that humoral immunity played a key role in resisting infection with *C. sinensis*; however, both IgA and IgE descended rapidly 2 months post primary infection, while IgG increased progressively and maintained for 4 months at a high level. In other words, as reported (Quan et al. 2004), serum IgG predominantly correlated to defending *C. sinensis* infection. What was proved in the present study confirmed immune types of *C. sinensis* infection and proposed inducing humoral immunity as a new protocol to prevent *C. sinensis* infection.

In addition, *C. sinensis* infection also resulted in hepatic fibrosis, which was revealed by pathological changes. So far, little is known about the pathogenesis of *C. sinensis* infection. Besides the key role in hepatic fibrosis and extracellular matrix remodeling, hepatic stellate cells (HSC) contributed to the progression of liver carcinogenesis (Luigi et al. 2009). In the present study, we evaluated the fibrotic changes with Masson's trichrome staining which was known as a method to reveal collagen fibers. Pathologically, HSC were activated by inflammatory cells which induced by *C. sinensis* infection. Masson staining markedly showed proliferation of collagen fibers both along bile ducts and liver parenchyma. As is reported, other than HBV and HCV infection, *C. sinensis* infection also contributed to hepatic fibrosis (Hu et al. 2009), liver cirrhosis, and even liver cancer (Chuang et al. 2008; Shin et al. 1996). Furthermore, the modeling results have confirmed the hypothesis that *C.*

sinensis can lead to pyogenic cholangitis, cholelithiasis, cholecystitis, and cholangiocarcinoma.

In conclusion, in the present study, we have constructed the *C. sinensis* infection model and revealed the novel transmission dynamics systematically. Besides other fish-borne parasites, as was shown in the modeling study, considering the high worm recovery rate of one *C. sinensis* metacercariae infection, *C. sinensis* has posed a new threat to both human health and fish farming. Further researches need to be done on the pathogenesis of *C. sinensis*. Clinically, radiologic devices have provided availability for diagnosis of *C. sinensis* (Park and Son 2008; Choi and Hong 2007), but to bear the brunt, how to prevent the helminth infection occupies the most significant position. As we have reported, with advances in molecular biology and medicine, recombination protein obtained from genetic engineering has provided effective tools in prevention and diagnosis of *C. sinensis* (Zhou et al. 2008). However, for the poorly understood pathogenesis, more work should be carried out on the immunologic mechanism underlying the *C. sinensis* infection.

Acknowledgment This work was supported by the National Natural Science Foundation grant funded by National Natural Science Foundation of China (no.30671831) and the National High Technology Research and Development Program of China (973 program; no.2010CB530000). We would like to thank Doctor Yili Huang at Pathology Department in Cancer Center of Sun Yat-sen University for the pathological work in this article.

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