



Strain variation in the susceptibility and immune response to *Clonorchis sinensis* infection in mice

Md. Hafiz Uddin, Shunyu Li, Young Mee Bae, Min-Ho Choi, Sung-Tae Hong*

Department of Parasitology and Tropical Medicine, Institute of Endemic Diseases, Seoul National University College of Medicine, Seoul 110-799, Republic of Korea

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ABSTRACT

Mice have shown various susceptibility to infection by *Clonorchis sinensis*. To compare the intra-specific variation in the host–parasite relationship of *C. sinensis*, 6 strains of mice (ICR, BALB/c, C57BL/6, DDY, CBA/N, and C3H/HeN) with 3 different haplotypes were evaluated on their susceptibility. The worm recovery rate and immunological responses were observed after 4 and 8 weeks of infection with 30 metacercariae. The highest worm recovery rate was observed as 20.7% in the C3H/HeN strain after 4 weeks of infection along with histopathological changes. The rate was 10.0% in C57BL/6 mice after 8 weeks. ICR, BALB/c, and CBA/N showed elevated levels of IgE at both time points when compared to the rest of the strains. The serum IgG1 and IgG2a levels were elevated in most of the strains; however, the C57BL/6 strain showed a lower level of IgG2a that indicated the IgG1 predominance over IgG2a. The production of IL-4 after concanavalin-A stimulation of splenocytes slightly increased among the mouse strains except C3H/HeN after 4 or 8 weeks of infection, but each strain produced high levels of IFN- γ after 8 weeks, which implied mixed Th1/Th2 responses. ICR, DDY, CBA/N, and C3H/HeN strains showed a significantly increased level of IL-10 after 8 weeks as compared to C57BL/6. All of the strains showed an increased level of IL-13 and suggested fibrotic changes in the mice. In conclusion, mice are insusceptible to infection with *C. sinensis*; however, the C57BL/6, BALB/c and ICR strains are relatively susceptible after 8 weeks of infection among the six strains. Worm expulsion may be one of the causes of low susceptibility of C3H/HeN mice strain at the 8th week. Elevated IgE, IFN- γ , and IL-13 of infected mice suggest both Th1 and Th2 responses that may be related to the low host susceptibility.

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1. Introduction

Clonorchis sinensis, is a carcinogenic liver fluke infecting about 20 million people in endemic East Asia. *C. sinensis* infection in humans leads to cholangitis, bile duct obstructions, cholangiofibrosis, and cholangiocarcinoma [1–5]. Infections usually result from the consumption of raw cyprinoid fish containing *C. sinensis* metacercariae. Clonorchiasis is a major public health issue in Korea and is comparable to the problem of *Opisthorchis viverrini* infection in Thailand [6,7].

A number of animal models have been used to investigate the host–parasite interaction of *C. sinensis*. Among the in vivo models, hamsters serve as good candidates regarding the development of cholangiocarcinoma [8,9]. However, studies of the immune response and pathogenesis in hamsters have been limited because of the commercial unavailability of antibodies and other reagents against hamsters. The absence of gall-bladders in rats made them a non preferable host in previous studies [10]; however, mice may serve

both research purposes in having a gallbladder as well as commercially available antibodies and other reagents. Therefore, it is essential to develop a suitable and susceptible mouse model for *C. sinensis* in order to understand the mechanisms of carcinogenesis and the immune response.

Yoon et al. [11] compared two immunologically competent (BALB/c and FVB/NJ) and two immunodeficient (Nude and SCID) mice for the study of *C. sinensis* pathogenesis and described FVB/NJ as a suitable model for the host parasite relationship study. However, they suspected that the mouse haplotype could have some relation in regards to susceptibility. The relatively susceptible FVB/NJ mice have the H2q haplotype while BALB/c mice have the H2d haplotype that is resistant to *C. sinensis* infections.

We studied herein host susceptibility of mice through the evaluation of six mice strains of different haplotypes (H2q, H2d and H2k) for the infectivity of *C. sinensis*, worm recovery, pathological and immunological changes. Among the strains, ICR and DDY have at least two antigens controlled by H2q genes that were previously used by Kim et al. [12] to examine the egg production of *C. sinensis*. BALB/c and C57BL/6 mice have H2d haplotype. In addition, two strains of mice (CBA/N and C3H/HeN) with the haplotype H2k were also included in this study.

* Corresponding author. Tel.: +82 2 7408343; fax: +82 2 7656142.
 E-mail address: hst@snu.ac.kr (S.-T. Hong).

2. Materials and methods

2.1. Preparation of *C. sinensis* metacercariae

Metacercariae of *C. sinensis* were collected from naturally infected fish *Pseudorasbora parva* caught in Shenyang, Liaoning Province, China. The metacercariae were isolated following the procedure described by Li et al. [13]. After collection, they were preserved in 1× PBS with antibiotics (penicillin 100 U/ml and streptomycin 100 µg/ml) and stored at 4 °C until use.

2.2. Animal and experimental design

Six strains (ICR, BALB/c, C57BL/6, DDY, CBA/N, and C3H/HeN) of five-week-old male mice were infected with 30 metacercariae of *C. sinensis* by gavage intragastric intubation in PBS and the control group received a dose of PBS only. Among the strains all were inbred except ICR and DDY, which are closed colony strains. Of the 20 mice in each strain, 14 were infected and 6 served as the uninfected control for each strain. The mice were maintained at the animal laboratory of the Seoul National University College of Medicine, provided with a sterilized commercial diet (Samtako Co., Seoul, Korea) and tap water ad libitum. Animal experiments were conducted according to the guidelines of Seoul National University Institutional Animal Care and Use Committee. A total of 10 mice from each strain were sacrificed at the 4th and the 8th week of infection, including the uninfected controls. Dead mice during the experiment were excluded from the study.

2.3. Blood sampling and immunoglobulin measurements

Blood was directly and immediately collected from the heart after anesthesia and kept at room temperature for 1 hour. Sera were collected by centrifugation at 3000 rpm at 4 °C for 10 min and stored at −20 °C until use. The serum IgG, IgG1, IgG2a and IgE level of each mouse was determined by sandwich ELISA according to the instructions of the manufacturer (Bethyl Lab, Montgomery, TX, USA).

2.4. Gross observation, worm recovery and histopathology

Each liver collected from the mice was photographed under a stereomicroscope for gross observation. Adult and juvenile worms were recovered from the liver using a combination of stereomicroscopic observations as well as a Baermann worm collection technique. Two livers from each strain were fixed in a 10% neutral phosphate buffered formalin, routinely processed, and stained with hematoxylin and eosin for the histopathological findings.

2.5. Culture of splenocytes

After anesthesia, each spleen was removed aseptically from mice for a cell culture. Spleen cells (SC) were harvested from the mouse strains as previously described [14]. All the mice from each strain were individually used for the SC culture. The spleen was homogenized and washed three times in a cold RPMI-1640 medium containing 2% fetal bovine serum (FBS) and the erythrocytes were removed with a RBC lysis buffer. The cell number was counted using a hemocytometer. Splenocytes (5×10^6 cells/ml, 24-well plate) were cultured in RPMI-1640 media supplemented with 10% FBS, 50 µM 2-mercaptoethanol and antibiotics (penicillin 100 U/ml and streptomycin 100 µg/ml) with or without 5 µg/ml concanavalin-A (Con-A) (Sigma, St. Louis, MO, USA). Cells were maintained at 37 °C in a 5% CO₂ atmosphere for 48 or 72 h. The supernatant was collected by centrifugation and stored at −70 °C until use.

2.6. Cytokine assay

A commercial sandwich ELISA was performed to measure the concentration of IL-4, IL-10, IL-13 and IFN-γ in SC culture supernatant according to the instructions of the manufacturer (eBioscience, San Diego, CA, USA). The values of the cytokine assay were expressed as an average from all the mice in a group.

2.7. Statistical analysis

The statistical difference was analyzed by Student's *t*-test using PASW Statistics, version 18.0 (SPSS Inc., Chicago, IL, USA). A *P* value of less than 0.05 was considered to indicate statistical significance.

3. Results

3.1. Gross observations

In the gross view, mice infected with *C. sinensis* showed the dilated extrahepatic bile duct compared with the uninfected control (Fig. 1). Severe dilation of the duct was observed in CBA/N and C3H/HeN mice reaching about 1 mm whereas it was similar to the uninfected controls in C57BL/6 and DDY strains.

3.2. Worm recovery

The higher numbers of worms were recovered from C3H/HeN (20.7%) and BALB/c (16.0%) mice than from other strains, whereas ICR showed no worm recovery at the 4th week of infection. At the 8th week, the worm recovery rate decreased in all of the strains, except the ICR and DDY. Highest worm recovery (10%) observed in C57BL/6 mouse strain followed by BALB/c (8.3%). Among the recovered worms, 18.18% were adults at the 4th week of infection and 10% at the 8th week of infection. The C57BL/6 strain demonstrated a similar recovery at both the 4th and the 8th week of infection; however, CBA/N revealed no infection at the 8th week (Table 1).

3.3. Histopathological findings

Liver sections indicated slight variation in the histopathological changes according to the strain and week of infection. At the 4th week, severe proliferation of the bile duct wall was observed in C3H/HeN and CBA/N strains whereas BALB/c, DDY and ICR showed moderate to mild proliferation. Severe inflammation was observed in C57BL/6 strain followed by BALB/c and C3H/HeN. However, at the 8th week, a little increase of severity observed in ICR and BALB/c and almost unchanged in other strains (Fig. 2).

3.4. Serum immunoglobulin level

Summarized levels of immunoglobulins in the mouse sera are shown in Fig. 3. The IgE production was somewhat higher in the ICR, BALB/c, and CBA/N than in the C57BL/6, DDY, and C3H/HeN. No significant differences were observed between the 4th and the 8th week of infection, except for the DDY ($P=0.049$) where the overall IgE production was very low. An increased IgG level was observed in all of the strains, compared to the uninfected control, and from the 4th week to the 8th week of infection this was significant for the C57BL/6 ($P=0.036$), DDY ($P<0.001$) and C3H/HeN ($P<0.001$). The IgG1 also increased significantly ($P<0.001$) compared to the control and a significant increase of IgG1 was found in CBA/N ($P<0.001$) and C3H/HeN ($P=0.002$) from the 4th to the 8th week of infection. IgG1 levels were almost equal in ICR and C57BL/6 mice. A slight difference of IgG2a level was observed among the strains. Infected C57BL/6 mice produced almost the same IgG2a as the uninfected control, which suggested IgG1 dominance over IgG2a. However, it showed a significant

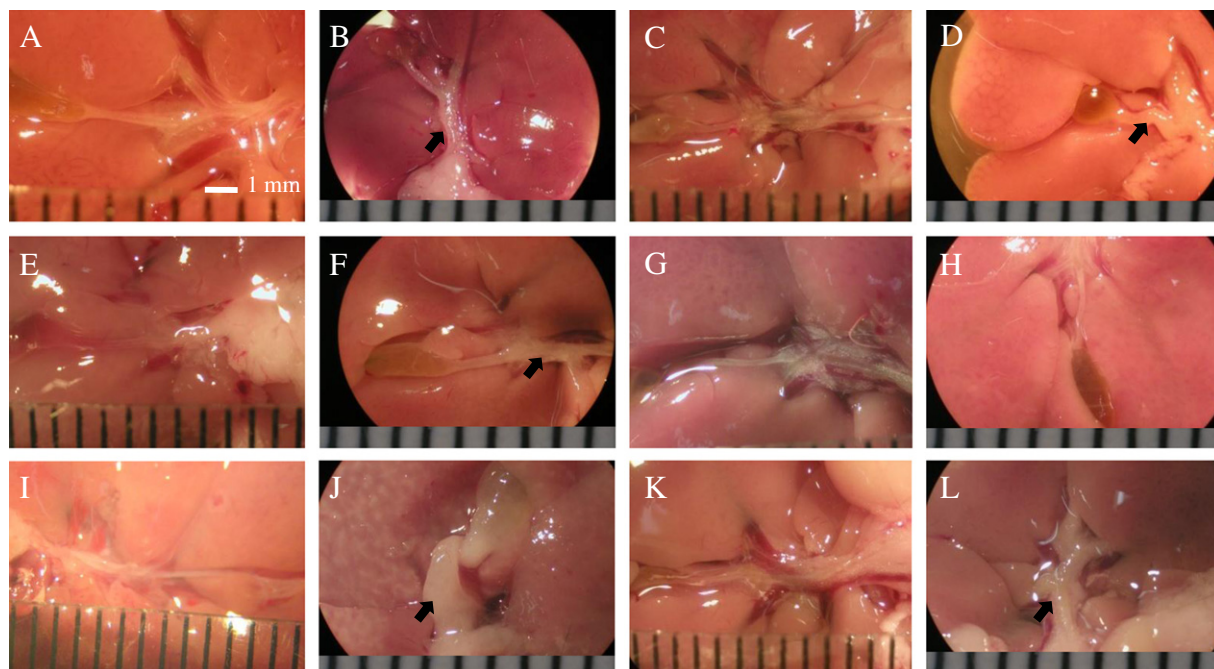


Fig. 1. Gross findings of the extrahepatic bile ducts of control and *C. sinensis* infected mice at the 4th week of infection according to strains. The dilated bile ducts were observed (arrows) in all strains except for the DDY mouse.

($P=0.009$) increase between the 4th and the 8th week of infection in all strains except the C57BL/6.

3.5. Level of cytokines in spleen cell culture supernatant

Fig. 4 monitored cytokines in the spleen cell culture supernatant stimulated with Con-A. At the 4th week of infection, both BALB/c and C57BL/6 showed an increased amount of IL-4 as compared to other strains, while in the 8th week it increased in BALB/c but decreased in the C57BL/6. An increasing tendency was observed in ICR and CBA/N from the 4th to the 8th week of infection, but this was not significant (ICR, $P=0.435$; CBA/N, $P=0.067$). IFN- γ production was high in all strains at the 8th week of infection, but in the 4th week it was low in ICR, BALB/c and C3H/HeN strains. A significant increase of IFN- γ was observed in ICR ($P=0.023$), C57BL/6 ($P=0.017$), CBA/N ($P=0.001$) and C3H/HeN ($P=0.020$). The level of IL-10 increased in all strains in the 8th week except in C57BL/6 and CBA/N mice. It significantly decreased in C57BL/6 mice ($P=0.025$). In the 8th week of infection, ICR, DDY, and C3H/HeN mice showed higher levels of IL-10 than other strains. IL-13 increased in all of the strains except C3H/HeN from the 4th to the 8th week of infection, but it was significant only in C57BL/6 mice ($P=0.040$).

4. Discussion

The gross view of the liver indicated evidence of *C. sinensis* infection in most of the strains. Dilated bile ducts were marked in most of the strains, except the DDY strain. There were no cysts or tumor-like masses in all strains of mice, whereas cystic dilatation of the intrahepatic bile duct was noticed in FVB mice [10,11]. Instead, the liver had small white spots, especially in the median lobe in most of the strains. Those spots were supposedly made by inflammation and edema around the infected bile duct. Only the DDY mice had no abnormal gross view though they were infected by a few worms. They were recorded as a good strain for *C. sinensis* [12].

The worm recovery rate of *C. sinensis* in mice varies greatly by their strains. The worm recovery rates of some mice strain in the present study were similar with the previous rate of 22.3% [15]. Yoon et al. [11] found a higher recovery rate (6.7% to 33.3%) in FVB mice than that in BALB/c, nude and SCID mice. Sohn et al. [16] observed $17.6\pm 13\%$ recovery rate in FVB mice. Recovery of the worm at the 8th week divided the mice strain in two groups. The 1st group including ICR, BALB/c, C57BL/6 showed a recovery of 5% to 10% where as other 3 strains (DDY, CBA/N, C3H/HeN) showed less than 1% worm recovery. As a whole, the recovery rate in mice is not as high as that in rats, rabbits, and other experimental mammals. This low recovery rate in

Table 1

Infection and recovery rate of *C. sinensis* worms among the mouse strains at the 4th and the 8th week of infection. The highest recovery rate was shown in C3H/HeN mice after 4 weeks of infection and by C57BL/6 after 8 weeks of infection. No worms were recovered in ICR mice at the 4th week and CBA/N at the 8th week of infection. Mc, metacercaria; A, adult; J, juvenile.

Strains	4 weeks after infection						8 weeks after infection					
	No. examined (infected)	No. of Mc ingested	Recovered worms			Recovery rate (%)	No. examined (infected)	No. of Mc ingested	Recovered worms			Recovery rate (%)
			A	J	Total				A	J	Total	
ICR	5 (0)	150	0	0	0	0.0	4 (3)	120	1	5	6	5.0
BALB/c	5 (4)	150	7	17	24	16.0	4 (4)	120	0	10	10	8.3
C57BL/6	5 (4)	150	2	12	14	9.3	4 (3)	120	2	10	12	10.0
DDY	5 (4)	150	2	15	17	11.3	5 (1)	150	0	1	1	0.67
CBA/N	4 (4)	120	5	8	13	8.3	4 (0)	120	0	0	0	0.0
C3H/HeN	5 (5)	150	2	29	31	20.7	4 (1)	120	0	1	1	0.83

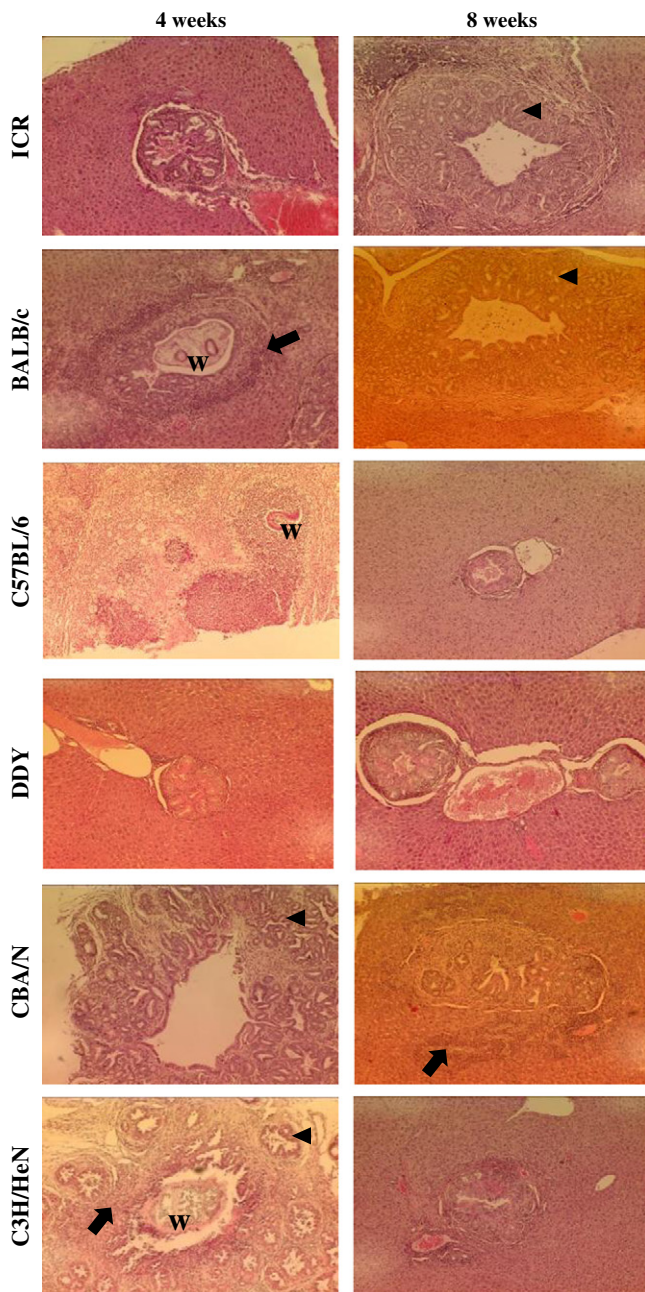


Fig. 2. Liver sections of all six strains with hematoxylin–eosin staining at the 4th and 8th week of infection ($\times 100$). W, worm; arrows, infiltration of inflammatory cells; arrow heads, extensive proliferation of the bile ducts. Histopathological changes are prominent in most strains at the 4th week of infection. At the 8th week, an increased severity of histopathology was observed in ICR and BALB/c but decreased in CBA/N and C3H/HeN, and remained the same in the other 4 strains.

mice suggests that the mouse is not a suitable host of *C. sinensis* regardless of its strain.

Another parameter for host susceptibility is development of worms. The present study showed that most of the worms recovered from mice were immature after 4 or 8 weeks. Only 18.18% and 10% of the recovered worms were mature adults after 4 and 8 weeks, respectively. The mature adults were recovered from most strains of mice after 4 weeks; however, only 3 adults survived in 2 strains of mice after 8 weeks. The finding means that most of the infected worms are unable to survive in mice although they arrive at the bile duct. In mice, a small proportion of the worms (under 20% of the infected) grow to mature adults, and furthermore, a small proportion of them (less than 20% of adult worms in 4 weeks) survive until

8 weeks. The same phenomenon was demonstrated in the resistant rats that were re-infected or super-infected [17,18]. Only a few worms survived in re-infected or super-infected rats and the super-infected worms were underdeveloped. Our findings in mice were very similar with those in the resistant rats. The data of low recovery rates and underdevelopment of worms suggest that it is hard for *C. sinensis* to survive and develop in mice regardless of strains, except for FVB mice [10,11].

Histopathology of the liver sections after 4 weeks revealed bile duct proliferations in C3H/HeN, CBA/N, BALB/c, DDY and ICR and an infiltration of the inflammatory cells in C57BL/6, BALB/c and C3H/HeN strains. After 8 weeks, however, the pathological findings varied slightly according to the strain. Mice of CBA/N and C3H/HeN strains showed a reduced severity of proliferation from the 4th to the 8th week after infection, whereas the severity increased in ICR and BALB/c but remained the same in other strains (Fig. 2). More or less similar histopathological changes have been observed in previous studies [10]. The histopathological findings were not in perfect agreement with worm recovery or gross appearance and may originate from the fact that the mice are not susceptible to *C. sinensis*.

It is evident that IgE antibodies play an important role in worm rejection from rats after primary infection of *Schistosoma* [19–21]. In the present study, the highest worm recovery rate (10.0%) at the 8th week of infection was observed in C57BL/6 mice. The IgE level in C57BL/6 mice was significantly lower than that in ICR ($P=0.03$) and other strains. In contrast, CBA/N mice showed the lowest worm recovery in the 8th week, having a higher level (significant for DDY, $P=0.005$) of IgE in serum. In humans and rats, clonorchiasis and opisthorchiasis are also responsible for the elevated level of IgG and IgE, but not protective for re-infection [22–24] whereas Wang et al. [25] reported a decrease of IgE after 8 weeks in *C. sinensis* infected rats.

Serum IgG antibodies increased in almost all strains from the 4th week to the 8th week of infection in the present study while Zhang et al. [23] found a high plateau in the 4th week for infected rats. In *O. viverrini* infections, egg negative individuals of the endemic area showed high levels of IgG, IgA, and IgM in the sera and indicated a long-term persistence of IgG followed by the fluke infection [24]. Wang et al. [25] showed a high level of IgG in the rat sera infected with *C. sinensis* that was maintained for 4 months. Among the IgG subclasses, IgG1 increased in most of the strains, but IgG2a showed variability in production for the different strains at the 4th and 8th week of infection. In mice of C57BL/6, it seemed that IgG1 dominated IgG2a as found in rat clonorchiasis [25]; however, both IgG1 and IgG2a were elevated in other strains and suggested a mixed Th1/Th2 response [26].

Of cytokines, the level of IL-4 was higher in BALB/c and C57BL/6, moderate in ICR and DDY and lower in CBA/N and C3H/HeN at the 4th week of infection. However, after 8 weeks all the mice strain showed moderate level of IL-4 except BALB/c and C3H/HeN. The BALB/c mice showed little bit higher level of IL-4 which was previously described as a resistant strain for *C. sinensis* infection [10] whereas C3H/HeN demonstrated lower level of expression. This finding contradicted the relation between IL-4 production and the susceptibility of the mice as well as the protective role found in nematode infections [10,27]. A similar finding has also been observed in a rat model [28].

Mice infected with *Paragonimus westermani* showed a very low level of IFN- γ [29] but rats with clonorchiasis recognized a high level of IFN- γ [28]. However, in the present study, a high level of IFN- γ was detected in all strains from 4 to 8 weeks after *C. sinensis* infection. IL-10 level decreased in C57BL/6 and CBA/N, but in other strains it increased after the 8th week of infection which was observed in rat infected with *C. sinensis* [28]. In acute schistosomiasis, IL-10 was related to reduced pathology [30]. It is not clear whether the increased level of IL-10 influences the production of IFN- γ as observed in schistosome infections [31]. The high-level production of IFN- γ suggests mixed Th1/Th2 responses to *C. sinensis* infections in all

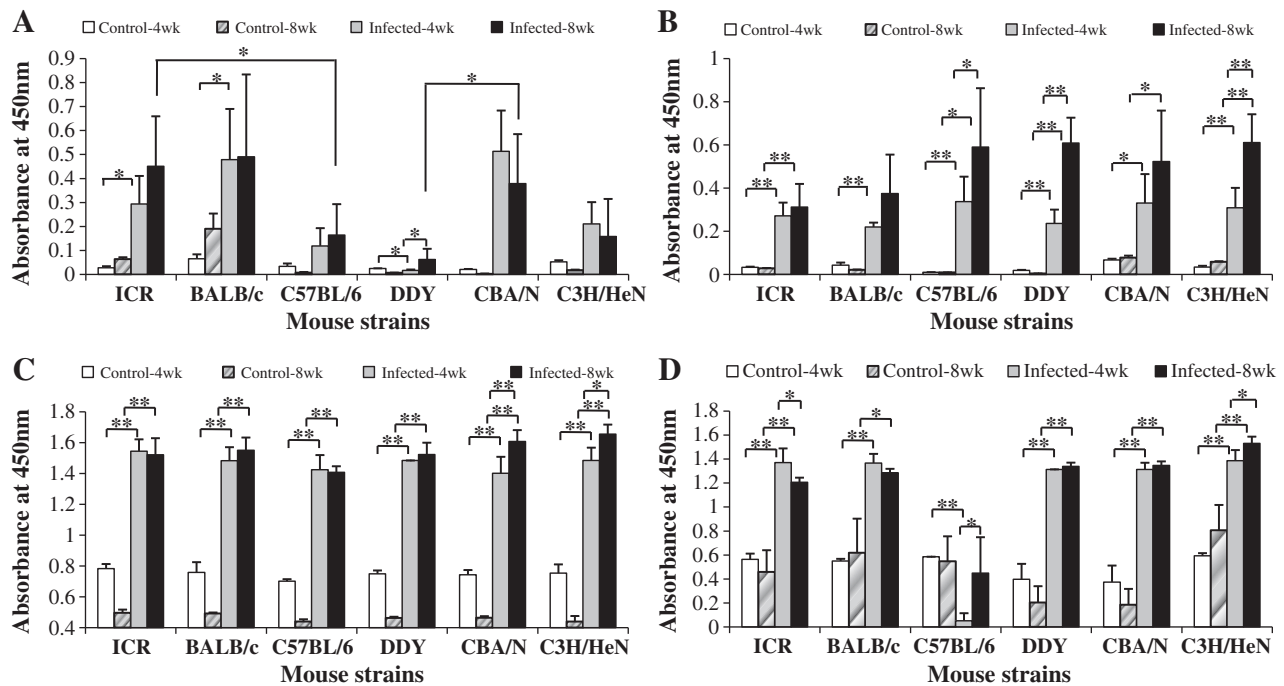


Fig. 3. Serum immunoglobulin (Ig) levels at the 4th and the 8th week of *C. sinensis* infection in the control and infected mice of different strains. The bars represent means $\pm$ SD. Blood was collected from the heart after anesthesia. Ratios of 1:400 sera dilution were used to measure IgE, IgG1 and IgG2a and 1:1000 sera dilution for the total IgG. (A) IgE, (B) IgG, (C) IgG1, and (D) IgG2a. An asterisk indicates a significant statistical difference (* P <0.05, ** P <0.01) between the groups. In the IgG1 and IgG2a, the difference between the control and infected was statistically significant, except C57BL/6 at the 8th week for IgG2a.

mouse strains. A multivariate analysis showed that IL-13 was strongly related to severe fibrosis in human and experimental schistosomiasis through the promotion of collagen production. Moreover, a decoy receptor IL-13Ra2, which blocks the IL-13 was found to reduce the fibrosis in mouse model [32]. Collagen I mRNA increased significantly in *Fasciola hepatica* infected rats [33]. In the present study, all of the strains produced IL-13 at a high level at the 8th week of infection

suggesting fibrotic changes. On the other hand, a relation with periductular fibrosis and IL-6 were observed by Sripa et al. [34] in *O. viverrini* infection in human. IFN- γ and IL-13 are produced mainly in the clonorchiasis of mice and this cytokine reaction may be one of factors that make the mice less susceptible.

Choi et al. [10] demonstrated that the FVB strain mice with an H2q haplotype were more susceptible over BALB/c with the H2d haplotype.

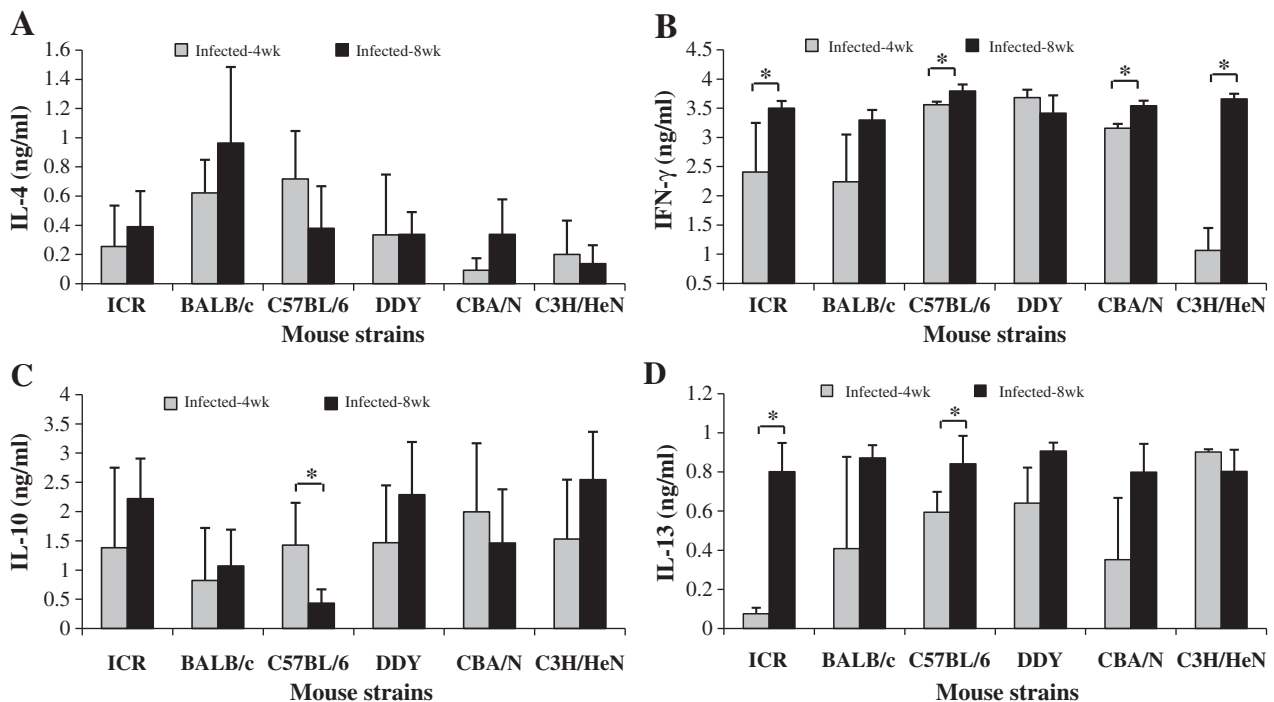


Fig. 4. Levels of cytokines in the culture supernatant of spleen cells stimulated with 5 μ g/ml concanavalin-A at the 4th and the 8th week of *C. sinensis* infection by mouse strains. (A) IL-4, (B) IFN- γ , (C) IL-10, and (D) IL-13. An asterisk indicates a significant statistical difference (* P <0.05) between the 4th and the 8th week of infection.

They suspected that the susceptibility and resistance may have some relation with the genetic differences of mice. In the present study, strains with the H2d haplotype showed a moderate worm recovery rate, both at the 4th and the 8th week of infection; however, the strains with H2k haplotype showed a moderate to high worm recovery at the 4th week and a very low to no worm recovery at the 8th week of infection. ICR and DDY mice strain which includes 2 antigen controlled by H2q genes [35] revealed variability in worm recovery and histopathology. Beside worm recovery, IgG2a and IL-10 increased and IL-4 decreased in H2k and H2q haplotype mice compared to H2d. Though H-2 is an important factor related with infection, in the present study no such relation was established which is in agreement [36] and disagreement [37–39] with different studies.

In conclusion, mice are insusceptible to infection with *C. sinensis*, however, C57BL/6, BALB/c and ICR strains are relatively susceptible after 8 weeks of infection compared to DDY, CBA/N and C3H/HeN. Removal of young worms after infection may be responsible for low recovery in C3H/HeN strain at the 8th week. The elevated IgE, IFN- γ and IL-13 of infected mice may induce both Th1 and Th2 responses, which may be related with low host susceptibility.

Acknowledgments

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