FULL PAPER

Infectivity and Pathological Changes in Murine Clonorchiasis: Comparison in Immunocompetent and Immunodeficient Mice

Byung-II YOON1), Yang-Kyu CHOI2), Dae-Yong KIM1), Byung-Hwa HYUN2), Kyoung-Hwan JOO3), Han-Jong RIM3) and Jae-Hyun LEE4)

1)Department of Veterinary Pathology, College of Veterinary Medicine, Seoul National University, Suwon, 2)Korea Research Institute of Bioscience and Biotechnology, Taejon, 3)Department of Parasitology, College of Medicine, Korea University, Seoul and 4)Laboratory of Experimental Pathology, Korea Cancer Center Hospital, Seoul, Korea

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ABSTRACT. The main complications of clonorchiasis are periportal inflammation, biliary hyperplasia, periductal fibrosis, and subsequently the development of biliary tumors in the liver. This study was undertaken to compare the infectivity and histopathologic changes between immunocompetent FVB/NJ and BALB/cA strains, and immunodeficient severe combined immunodeficient (SCID) and athymic nude mice after the metacercariae of Clonorchis (C.) sinensis were infected. The experiment showed that C. sinensis was very infective in all strains studies, but the status of worm development, infectivity, recovery rate, and morphological changes of livers were very different in each strain. FVB/NJ mice showed more worm recovery than any other strain. Histopathologically the liver of FVB/NJ mice at 4 weeks postinfection showed marked cystic and fibrotic changes, in which C. sinensis was fully developed with ovum production, severe infiltration of inflammatory cells, mostly eosinophils, and high degrees of biliary hyperplasia. In SCID and nude mice, there were few foci of inflammatory cells even at 8 weeks postinfection in perportal areas of the liver, associated with no development into adult worm with ovum production. Fibrosis occurring at 4 weeks postinfection was highly correlated with inflammatory infiltration when each strain was compared. We suggest that massive infiltration of eosinophil and plasma cells caused by the infection might initiate cystic formation and fibrosis. These data demonstrate that the infection of C. sinensis might be related to pathologic consequences of inflammatory cell infiltration, cystic formation and fibrosis which might play a role in the defense mechanism against the parasitism in the liver of each strain. The FVB/NJ mouse model might be very helpful in elucidating the mechanism for human clonorchiasis.

Key words: Clonorchis sinensis, FVB, infectivity, SCID.

Clonorchis (C.) sinensis is a liver fluke parasites in the bile ducts of the human liver [19]. C. sinensis, when infected, causes cholangitis with marked eosinophil infiltration, biliary adenomatous hyperplasia, bile duct obstruction, and subsequently cholangiofibrosis [4]. It has also been known as an etiologic factor of cholangiocellular carcinoma [2, 11, 12]. The infection with C. sinensis results from eating raw fish contaminated with metacercariae. Like Opisthorchis viverrini infection in Thailand [17,18], C. sinensis infection still remains one of the major public health problems in Korea [19]. Although C. sinensis infection is a big concern, there has been little available data regarding the immunological mechanism and pathogenesis during C. sinensis infection. Hamsters have been widely used as an animal model since the liver provides a good environment for liver flukes and develops proliferative and inflammatory changes similar to those observed in humans [3, 13]. Hamiltons have also been used to study the mechanism of C. sinensis-associated biliary tumors [15, 16, 20]. However studying the immune response and immunopathogenesis related to C. sinensis infection in hamsters has been very limited due to a lack of commercially available antibodies or probes that are reactive to hamster. To overcome this limitation, a proper mouse model is definitively needed.

This study was performed to search for a more appropriate mouse model for studying the pathogenesis and host-parasite relationship of C. sinensis infection. We are particularly interested in studying the role of immune reaction and immunopathogenesis in C. sinensis infection. For that purpose, we chose two immunocompetent (BALB/cA and FVB/NJ) and two immunodeficient (BALB/cA-Hfh1I1and C.B-17-Prkdcscid) strains. We infected mice of each strain with C. sinensis metacercariae orally and compared the infectivity, recovery rate, and histopathological changes.

MATERIALS AND METHODS

Preparation of Clonorchis sinensis metacercariae: The metacercariae of C. sinensis were obtained from the flesh of Pseudorasbora parva captured in the Nakdong river basin in Korea. The whole flesh of the fish was removed and digested with artificial gastric juice for one hour at 37°C (0.6% pepsin in 0.7% HCl solution, pH 2.0). The digested content was then filtered with 0.147 mm diameter sieve and washed thoroughly several times with saline. Metacercariae were identified and selected under a stereoscopic microscope and kept at 4°C until inoculation.

Animals: Male mice of four strains were used in the experiment: BALB/cA, FVB/NJ, SCID (severe combined immunodeficient, C.B-17-Prkdcscid), and congenitally athymic nude (BALB/cA-Hfh1I1) mice. They were all six...
to seven weeks of age (20–30 g) at the commencement of the study. Mice were inbred and obtained from the specific pathogen-free facility at Korea Research Institute of Bioscience and Biotechnology (KIRIBB). All experiments were performed in accordance with Korea Welfare Guidelines. The mice were maintained in a temperature (21 ± 1°C)- and humidity (55 ± 5%)-controlled barrier system with a 12 hr light/dark cycle at KIRIBB. All animals had free access to a commercial basal diet and tap water ad libitum. After one week acclimation period, 16 mice from each strain were orally given 15 metacercariae of *C. sinensis* in saline. As a control, 8 mice from each strain received the vehicle only. Eight mice from each strain were sacrificed by cervical dislocation at 4 weeks postinfection and the remaining 8 mice were also killed at 8 weeks postinfection.

Histopathology: After the examination of gross findings, the livers were removed and 3 to 5 transverse sections per lobe were made from each animal and fixed in 10% phosphatebuffered neutral formalin. After fixation, the specimens were routinely processed, embedded in paraffin and stained with hematoxylin and eosin (H&E) for histopathological examination.

Infectivity and recovery rate of worms: After thorough gross examination, the livers were crushed thoroughly with glass plates and then the number of worms was counted under a dissecting microscope. The worms were fixed with alcohol-formal-acetic acid fixative and stained with acetocarmine to determine the developmental stages of the worms on the basis of genital organs and eggs. Infectivity and recovery rate of worms were determined by directly counting the adult and juvenile worms. The recovery rate of worms in each group of animals is expressed as a percentage as the ratio of total number of recovered worms to metacercariae administered.

Evaluation of periductal fibrosis and eosinophilic infiltration: To investigate the relationship between periductal fibrosis and eosinophilic infiltration, periductal fibrosis was graded by measuring the thickness of fibrosis surrounding hyperplastic bile ducts using a square millimeter grid and the number of infiltrating eosinophils was counted under ×400 light microscopy.

RESULTS

Gross and histopathology: Grossly 1 to 3 whitish cysts were observed only in FVB/NJ mice but not in mice of the other 3 strains at 4 and 8 weeks postinfection. The size of adult and juvenile worms was 0.5 cm to 1.0 cm and 0.2 cm in length, respectively (Fig. 1).

Table 1 shows the frequency of the histopathologic changes in livers of four strains of mice at 4 and 8 weeks postinfection. The cysts noted in the livers of FVB/NJ mice were lined by a single layer of flat to cuboidal epithelial cells and contained either fully matured or juvenile worms (Fig. 2A). The liver of the FVB strain showed bile duct hyperplasia and periductal fibrosis, with more prominent degree of hyperplasia and fibrosis in FVB mice as compared with the other 3 strains studied (Fig. 2B). In addition, infiltration of inflammatory cells including mainly eosinophils (Fig. 2C) was noted. The degree of eosinophilic infiltration was most pronounced in the FVB/NJ and BALB/cA strains but was slight to mild in nude and SCID mice. Lymphocyte and plasma cell response was the strongest in FVB/NJ mice. In the case of FVB/NJ and BALB/cA mice, the inflammation extended into the perportal area causing hepatocellular degeneration or necrosis.

Infectivity and worm recovery rates: *C. sinensis* was recovered from all strains and mutants of mice infected. But there were differences in infectivity, developmental stages and recovery rate of worms among each strain. The number of mice having worms grossly or microscopically found in the livers was the higher in FVB/NJ mice than any other strains (Table 2). Both adult and juvenile worms were observed in FVB/NJ mice, whereas in the other strains, only juvenile worms were observed at 4 weeks postinfection. The number of FVB/NJ mouse with adult worms at 4 and 8 weeks postinfection was 3 mice and 5 mice respectively. In the case of BALB/cA mice, adult worms were observed in only 1 mouse at 8 weeks postinfection.

Range of worm recovery rates was also the highest in FVB/NJ mice (6.7 to 33.3%). The most number of recovered worms was 5 worms in FVB/NJ mice. The adult worms had eggs in their uteri along with developed ovaries and dendritic testes that are morphological characteristics of *C. sinensis*. In contrast, in nude and SCID mice any parasite worms could not be found grossly but undeveloped worms were observed by microscope in the bile ducts in the liver sections.

The relationship between periductal fibrosis and eosinophilic infiltration: Figures 3 and 4 showed the close relationship between fibrosis and eosinophil infiltration in each strain. The periductal fibrosis was even more prominent in the liver of FVB/NJ and BALB/cA mice than in the liver of SCID and nude mice.

![Fig. 1. The liver shows 2 cysts of 5 × 6 mm in size in median and left lobes of an FVB mouse 4 weeks after *Clonorchis sinensis* infection. The cysts (arrows) are filled with adult worms and fluid.](image-url)
DISCUSSION

*C. sinensis* is a liver fluke that parasitizes in the biliary tree after the metacercariae of the worm had been ingested orally into human and animals [19]. The pathology of *C. sinensis* infection has experimentally been investigated in rats [6, 9], hamsters [14, 15] and rabbits [7, 13]. It is still unclear whether *C. sinensis* is infective and developed in the mouse or not, although there is a little available data dealing with egg production of *C. sinensis* [10]. They only reported that there were intraspecific variations by strains of mice in biologic incubation period, fertile period of *C. sinensis* [10]. Therefore the present study was carried out to compare the infectivity, recovery rate of *C. sinensis* as well as the histopathology of the host after infection of FVB/NJ, BALB/cA, SCID and nude mice with the metacercariae of *C. sinensis*.

All strains of mice used in the study were infected with metacercariae of *C. sinensis*, but the development of the worm was very different in each strain. *C. sinensis* developed at 4 or 8 weeks postinfection into adult worm with egg production in the immunocompetent mice of FVB/NJ and BALB/cA, but not in the immunodeficient mice of SCID and nude strains. Over 37% of FVB/NJ mice infected with metacercariae had adult worms at 4 and 8 week postinfection. We could find adult worm in only one liver of the BALB/cA strain at 8 week postinfection but none in the SCID and nude strains. The cysts that contained worms along with clear fluid were observed only in the liver of FVB/NJ mice. We observed degenerating or calcified worms in the bile ducts of SCID and nude mice. It seemed that the worms could not sustain life in livers of SCID and nude mice [10]. *C. sinensis* was well infective and developed in the liver of FVB/NJ in which cystic formation and fibrosis was formed, but was poor in the liver of immunodeficient mice without cystic formation and fibrosis. These results suggested that the FVB/NJ strain could be a compar-

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<th>4 weeks Cysts</th>
<th>FVB/NJ</th>
<th>BALB/cA</th>
<th>nude</th>
<th>SCID</th>
<th>FVB/NJ</th>
<th>BALB/cA</th>
<th>nude</th>
<th>SCID</th>
<th>8 weeks Cysts</th>
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<td>Bile duct hyperplasia ++</td>
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<td>Eosinophilic infiltration +++</td>
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<td>Lymphocyte and plasma cell infiltration +</td>
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<td>Hepatic necrosis associated with inflammation ++</td>
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±, slight; +, mild; ++, moderate; ++++, severe.

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Fig. 2. Histopathology of a live of the FVB strains infected with *Clonorchis sinensis*. A; three adult worms are existed in dilated bile duct shown in a cyst in the liver of Fig. 1, × 40. B; periporal fibrosis and severely infiltrated inflammation are shown, × 100. C; periporal inflammation in the live of the FVB consists mostly of eosinophils, × 400.
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Table 2. Comparison of infectivity, worm burden and development of *C. sinensis* among the mouse strains on gross and histopathological examination

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<th>4 weeks</th>
<th>8 weeks</th>
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<td></td>
<td>Infectivity</td>
<td>Recovery rate</td>
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<td>FVB/NJ</td>
<td>8/8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.7–33.3</td>
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<td>BALB/cA</td>
<td>8/8</td>
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<td>Nude</td>
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<td>SCID</td>
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<td>6.7</td>
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<sup>a</sup> Number of mice harboring worms/Number of mice examined.

<sup>b</sup> Percentage of recovered worms/Number of infected metacercaria.

Fig. 3  Biliary fibrosis portal areas of mice livers at 4 weeks after infection with *C. sinensis*. Thickness of fibrosis measured by square millimeter grid.

Fig. 4  Eosinophilic infiltration in portal areas of mice livers at 4 weeks after infection with *C. sinensis*.

Suitively suitable host of *C. sinensis* among four strains used in the experiment.

Eosinophilic infiltration was closely related to various inflammatory fibrotic lesions. Pericysts in the livers of FVB/NJ were infiltrated with a lot of eosinophils and lymphocytes. The infection of *C. sinensis* might be related to pathologic consequences of inflammatory infiltration and of forming cystic formation and fibrosis in the FVB/NJ strain. Fibrosis in the BALB/cA strain was much less than in the FVB/NJ strain suggesting that antigenic substances secreted from adult worms of *C. sinensis* or eggs might immunopathologically initiate periportal fibrous reaction [1]. *C. sinensis* was not well developed in the BALB/cA strain as compared to the FVB/NJ strain. This data showed that the infectivity and development of *C. sinensis* seemed not only to involve the immune system relating to T and B cells shown as the study about *Opisthorchis viverrini* [21], but also to any unknown pathogenic mechanism shown in this study. Flavell et al. reported that T cell deprivation in an experiment of *Opisthorchis viverrini* infection has appeared to have no effect on worm establishment or egg production [7]. The liver of SCID and nude mice showed some infiltration of eosinophils. It was estimated that eosinophils seen in T cell-deficient strains might be infiltrated depending not on T cell-derived cytokines, but on another system like parasite-derived eosinophil chemotactic factor (ECF-P) [8]. Bile duct hyperplasia was found to be severe in the FVB/NJ strain but was similar between BALB/cA and immunodeficient mice. Bile duct hyperplasia seemed to be related to the development of the worms because the lesion was more prominent in FVB strains with adult worms than SCID and nude mice without adult worms. This pathological finding seemed to be a defense mechanism against the infection.

These data suggest that the FVB/NJ mouse model might be very helpful in elucidating the mechanism for human clonorchiasis and demonstrate that the infection of *C. sinensis* is related to pathologic consequences of inflammatory cell infiltration, cystic formation and fibrosis.

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REFERENCES
