

Resistance to reinfection in rats induced by irradiated metacercariae of *Clonorchis sinensis*

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A study was made to observe the association between the resistance to reinfection induced by irradiated metacercariae (MC) of *Clonorchis sinensis* and antigen specific Th1- and Th2-type cytokine productions in rats. Rats were infected with 20 MC of *C. sinensis*, previously exposed to a single dose of gamma irradiation, which varied from 0 to 100 Gy. All of them, single dose of 12 Gy showed higher IgG antibody titer with lowest worm recovery. Thus, 50 MC were used to challenge infection in rats previously infected with 20 MC irradiated at 12 Gy and the highest resistance to challenge infection was observed. The results of lymphocyte proliferation with specific antigen, ES Ag were shown no difference of proliferative responses as compared with primary and challenge infection at 12 Gy irradiation dose. In the case of cytokines production were observed that interferon (IFN- γ) and interleukin (IL-2) were significantly enhanced, while IL-4 and IL-10 was almost unchanged to make comparison between primary and secondary infection at 12 Gy irradiation dose. In conclusion, the single dose of 12 Gy could be adopted for induction of the highest resistance to challenge infection. Up-regulation of Th1 type cytokines, IFN- γ and IL-2 may be affected to develop vaccine by irradiated MC.

Key words: irradiation - metacercariae - reinfection - interferon- γ - interleukin-2

Liver fluke infection caused by *Clonorchis sinensis* is still an important public health problem in many countries of the West Pacific area. It is estimated that about 30 million people are infected annually with *C. sinensis* or *Opisthorchis viverrini* by eating uncooked fresh water fish in Southeastern Asia (Rim et al. 1981, Akai et al. 1994). Studies about effects of irradiation on infective stage of *C. sinensis* have been reported (Lee et al. 1989, Song et al. 1992, Duan et al. 1993), and reports show that suitable dose of irradiation on isolated metacercariae (MC) or unisolated MC in fish can prevent infections. There are evidences from immunological studies that man and animals produce antibodies and cellular immunity after *C. sinensis* infections (Choi & Park 1987, Choi et al. 1990, Hagan et al. 1991, Akai et al. 1994, Quan et al. 2000, 2002). To the best of our knowledge no study was carried out on the association between the effects of gamma irradiation and immune responses in *clonorchiasis*. However, study on *Schistosoma mansoni* showed that high levels of protection have been achieved in mice against *S. mansoni* infection using gamma irradiated cercarial vaccines (Dean 1983, Coulson 1997). Also Th1- or Th2-type cytokines were reported to associate with protective immunity against various parasites (Stevenson & Tam 1993, Cetre et al. 1999,

Koyama et al. 1999). In the present study, *C. sinensis* MC were first treated with different dose of gamma irradiation to select the suitable dose for the study of immune responses. Then resistance to reinfection induced by irradiated MC of *C. sinensis* was evaluated and an association between resistance and Th1- and Th2-type cytokines were observed.

MATERIALS AND METHODS

Parasites and experimental animals - Sprague-Dawley (SD) rats (female, 8 wk old) were purchased (Samyook animal center, Osan-shi, Kyonggi-do, South Korea) and used for whole experimental process. Parasites *C. sinensis* MC collected from *Pseudorasbora parva* were used for infection of rats and rabbits. Male white rabbits (New Zealand white) about 1.5 to 2 kg were used to recover adult *C. sinensis* for collection of *C. sinensis* excretory-secretory antigen (ES Ag). All procedures involving animals and their care were in conformity with institutional guidelines that comply with national and international laws and policies.

Irradiation - MC were dispensed into each micro tube (1.5 ml) containing 200 μ l saline. The MC in the tube were given irradiation with single dose from 0 to 100 Gy (1 Gy = 100 rad) and irradiated at the rate 375 rad/min at 70 cm distance from the source, using 60 Co (Thratron 780, AECL, Canada) gamma-ray.

Antigen preparation - *C. sinensis* MC were collected from *P. parva* and digested with artificial gastric juice, and orally administered to the experimental rabbits. Then adult worms were collected from rabbit liver after three month of infection. *C. sinensis* excretory-secretory antigen (ES Ag) was obtained by culturing living adult worm in RPMI

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1640 medium supplemented with antibiotics at 37°C and 5% CO₂. Culture medium was collected every two days and new medium was added to culturing living adult worm. Collected medium was centrifuged at 4°C, 10,000 rpm for 30 min, and the supernatant was used as ES Ag and stored at -70°C for use. Protein concentration of antigens was analyzed by the DC Protein Assay kit (Bio-Rad Lab, Hercules, CA, US) and stored at -70°C until used.

Study design - To determine the relationship between survival of worms and irradiation dose, rats were given irradiated MC of single dose that varied from 0 to 100 Gy. Then worm recovery and IgG antibody responses were measured. Thus, irradiation dose was selected with the highest titer of IgG and the lowest worm recovery. For the study of resistance to reinfection induced by selected irradiation dose and cytokine production, the experiment groups were designed as follows: uninfected control, rats infected with normal MC (non-irradiated MC), rats infected with irradiated MC, rats received challenge infection, previously infected with normal MC. Each group contained 10 rats.

Primary infection and challenge infection - In the experiments, rats were infected with 20 normal or 20 irradiated MC in the primary infection. After one month, challenge infections were given with 50 normal MC via orogastric tube under light ether anesthesia (Quan et al. 2000, 2002).

Worm recovery and evaluation of protection - Worms were collected in the liver duct of rats in 4th week in the primary infection for the determination of irradiation dose. Worm recovery for evaluation of resistance was performed in 4th week after challenge infection. Protection was evaluated by comparing the adult worms recovered from experimental groups and control group.

Sera collection - Blood was collected from each rat in week 4 after primary infection during the selection of irradiation dose. Blood was allowed to clot at room temperature and stored at 4°C. After removal of the clot, the sera were centrifuged at 3000 rpm for 10 min and stored at -20°C until needed. Sera were used to measure IgG levels.

Enzyme-linked immunosorbent assay (ELISA) - The solid phase ELISA test was performed in a microtiter plate as described elsewhere (McLaren et al. 1978) with some modifications. Plates for IgG was coated with 5 µg/ml *C. sinensis* ES Ag. Serum samples were diluted 1:100. Peroxidase-conjugated goat affinity purified antibody rat IgG (Cappel, Durham, NC, US) in 1:1000 dilutions were used in duplicate wells. The reactions were stopped using 5 N H₂SO₄ and the OD at 490 nm was recorded using an ELISA reader (Molecular Devices, Menlo Park, CA, US).

Lymphocyte proliferation assay - For detection of lymphocyte proliferation and cytokine production, lymphocytes in mesenteric lymph nodes (MLN) were collected in the 4th week after primary or secondary infection. MLN were excised from rats and cell suspensions were aseptically prepared by squeezing the MLN between two sterile glass slides. Lymphocytes were pooled from 5 rats and cultured at a final concentration of 5 × 10⁶ cells in 200 µl

RPMI 1640 supplemented with 10% fetal bovine serum, 100 units of penicillin/streptomycin, 2 mM glutamine, 25 mM HEPES, 1% non-essential amino acid solution, 0.1% 5 × 10⁻² M 2-mercaptoethanol and 1% sodium pyruvate (Invitrogen Co., Grand Island, NY, US). Cultures were performed in 96-well flat bottom culture plates for three days at 37°C in 93% air and 5% CO₂. Cells were stimulated with ES Ag at 25 µg/ml, or phytohemagglutinin (PHA) at 10 µg/ml final concentration. Each test was performed three times. Cells were pulsed for the last 18 h with 0.5 µCi/well [³H]thymidine (Amersham Co., Arlington Heights, IL, US) and then harvested on glass fibre filters with a semi-automatic cell harvester (Skatron, Norway). Incorporated radioactivity was measured in a liquid scintillation counter (LKB 1214 Racbeta) and expressed as geometric means after subtracting the background counts (ΔCPM).

Cytokine analysis - For detection of cytokine production, triplicate cultures of 5 × 10⁶ cells as described above were incubated with antigens in 96-well culture plates at 37°C for three days, in 5% CO₂. The supernatants of three wells were pooled following centrifugation and stored at -70°C. The concentrations of interferon (IFN-γ) and interleukins (IL-2, IL-4, IL-10) were determined in the culture supernatants by rat Cytoscreen™ Immunoassay Kits (Biosource, California, US).

Statistical analysis - Statistical significant differences from all groups were carried out using ANOVA of SAS system. A value of *P* < 0.05 was considered significant. Cytokine productions were carried out using pooled cells from five rats and as such were not subject to statistical analysis.

RESULTS

Relationship between survival of worms and irradiation dose - Some different number of adult worms was recovered from rats infected with 20 irradiated or non-irradiated MC of *C. sinensis* after four weeks of infection. As shown in Fig. 1, worm burdens were reduced following increasing dose of radiation. Worms were recovered when MC were irradiated at 1, 3, 5, 7, 10, and 12 Gy irradiation dose, including non-irradiated control (0 Gy) (Fig. 1), whereas we could not observe survival worms at 15, 20, 40, 60, and 100 Gy irradiation dose.

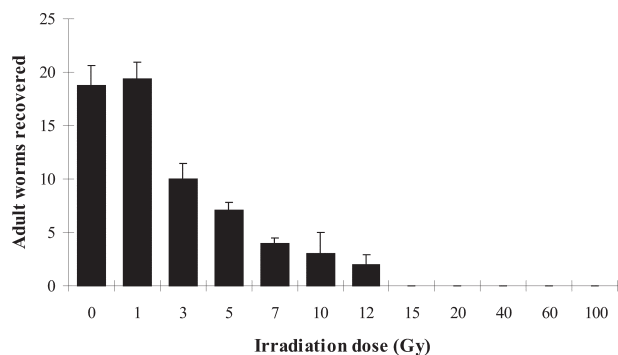


Fig. 1: adult worms were recovered in rats on week 4 after infection with the *Clonorchis sinensis* metacercariae irradiated with different dose.

Antibody responses in rats infected with irradiated MC - All rats (n = 9/each dose) had been infected with MC irradiated with a range of dose (1-20 Gy) showed significant increases in IgG antibody responses from 1 to 12 Gy range compared with the uninfected control (Fig. 2, $P < 0.01$). But, IgG antibody responses are low level in rats infected with 15 and 20 Gy irradiated MC. These data are consistent with the above that adult worms were recovered from the range of 1 to 12 Gy. The high antibody responses were detected in rats infected with 1 and 12 Gy irradiated MC, including non-irradiated (0 Gy) MC.

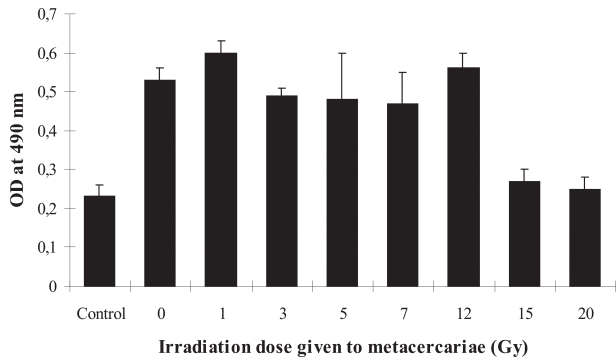


Fig. 2: IgG antibody responses from sera samples of rats infected with irradiated metacercariae (MC) of *Clonorchis sinensis* (n = 9/ each dose). Each rat was given 20 MC irradiated with different dose.

Resistance to reinfection - For detection of resistance to reinfection, rats were primarily infected with 20 irradiated MC ranging from 7 to 15 Gy. Then challenge infections were performed in rats using 50 non-irradiated MC after four weeks of primary infection. Fig. 3 showed that the number of *C. sinensis* adult worms recovered from rats was gradually decreased from range of 7 to 12 Gy, in which the highest resistance to reinfection was found in 12 Gy irradiation dose. Whereas high worm burdens were recovered in rats when challenge infection was carried out after primary infection with 15 Gy irradiated MC (Fig. 3). This result is the same as when rats only received challenge infection. And because these data are consistent with high IgG antibody responses in rats infected with 12 Gy irradiated MC, we assumed that MC died, and therefore lost ability to infect rats when MC were irradiated with dose over 15 Gy.

Lymph node cell proliferation and cytokine production - *C. sinensis* adult ES Ag specific stimulated proliferative responses of MLN cells were determined in relation to resistance. Twelve Gy irradiation dose was chosen to evaluate proliferative activity in primary and secondary infection. ES Ag specific stimulated proliferative responses were similar level with primary and secondary infection at 12 Gy irradiation dose (Fig. 4). Cytokine productions by *C. sinensis* ES Ag specific stimulated MLN cells were also measured in selected 12 Gy irradiation group. IFN- γ and IL-2 were mainly increased in secondary infections to compare with primary infection, whereas IL-4 and IL-10 were almost same level with primary and secondary infections at 12 Gy irradiation dose (Fig. 5).

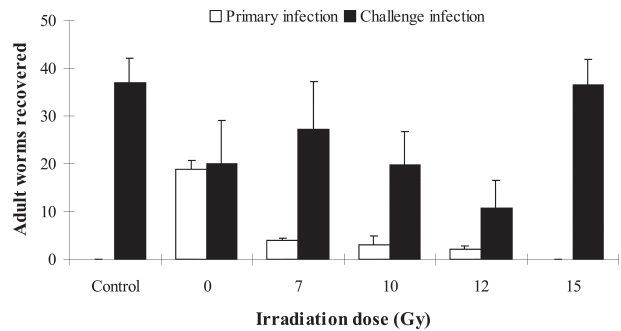


Fig. 3: worms were recovered from rats infected with primary infection or secondary infection. Primary infections were performed 20 irradiated metacercariae (MC), secondary infections were given 50 non-irradiated MC. Irradiation varied on dose 0, 7, 10, 12, and 15 Gy. Fifty MC were just infected to rats without primary infection in control group.

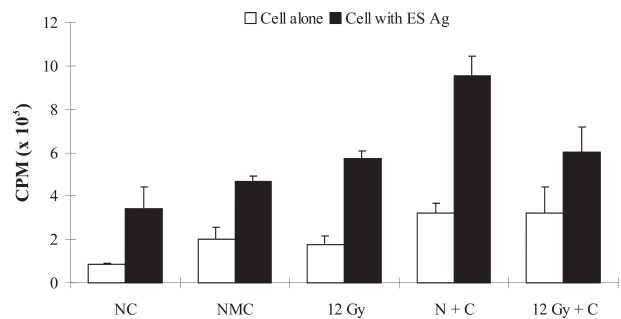


Fig. 4: in vitro proliferation of lymphocytes from rat mesenteric lymph nodes (MLN). Cellular proliferation was evaluated by [³H]thymidine incorporation on three days cultures with medium (cell alone) and in the presence of 10 μ g/ml *Clonorchis sinensis* ES Ag. NC: normal control; NMC: non-irradiated metacercariae (MC) control; 12 Gy: MC irradiated with 12 Gy; N + C: challenge infection after primary infection with non-irradiated MC; 12 Gy + C: challenge infection after primary infection with 12 Gy-irradiated MC.

DISCUSSION

Attempts to induce protection induced by γ -irradiated infective stages were performed in some parasites, *Fasciola hepatica*, *S. mansoni*, *S. japonicum*, *Loa loa*, *Brugia malayi*, *Achantocheilonema viteae*, *B. pahangi* (Creaney et al. 1995, Zhang et al. 1999, Ungeheuer et al. 2000). In these studies, that the infective larvae when optimally attenuated cannot develop into adults will die in the host, which induce high level of protection was proved. Differences in irradiation dose, number of vaccination and host showed different effects (Zhang et al. 1999).

This study is about the resistance induced by irradiated MC of *C. sinensis*. Previous studies on parasite *C. sinensis* showed that 50 Gy irradiation doses on isolated MC or 150 Gy on fish is an effective control measure that can be used in preventing MC (Lee et al. 1989, Song et al. 1992). Present study used different doses that varied from 1 to 100 Gy to select optimal dose, in which worms remained the least in rats to reduce damage of liver tissue (Fig. 1). Thus, 12 Gy irradiated MC was selected in which

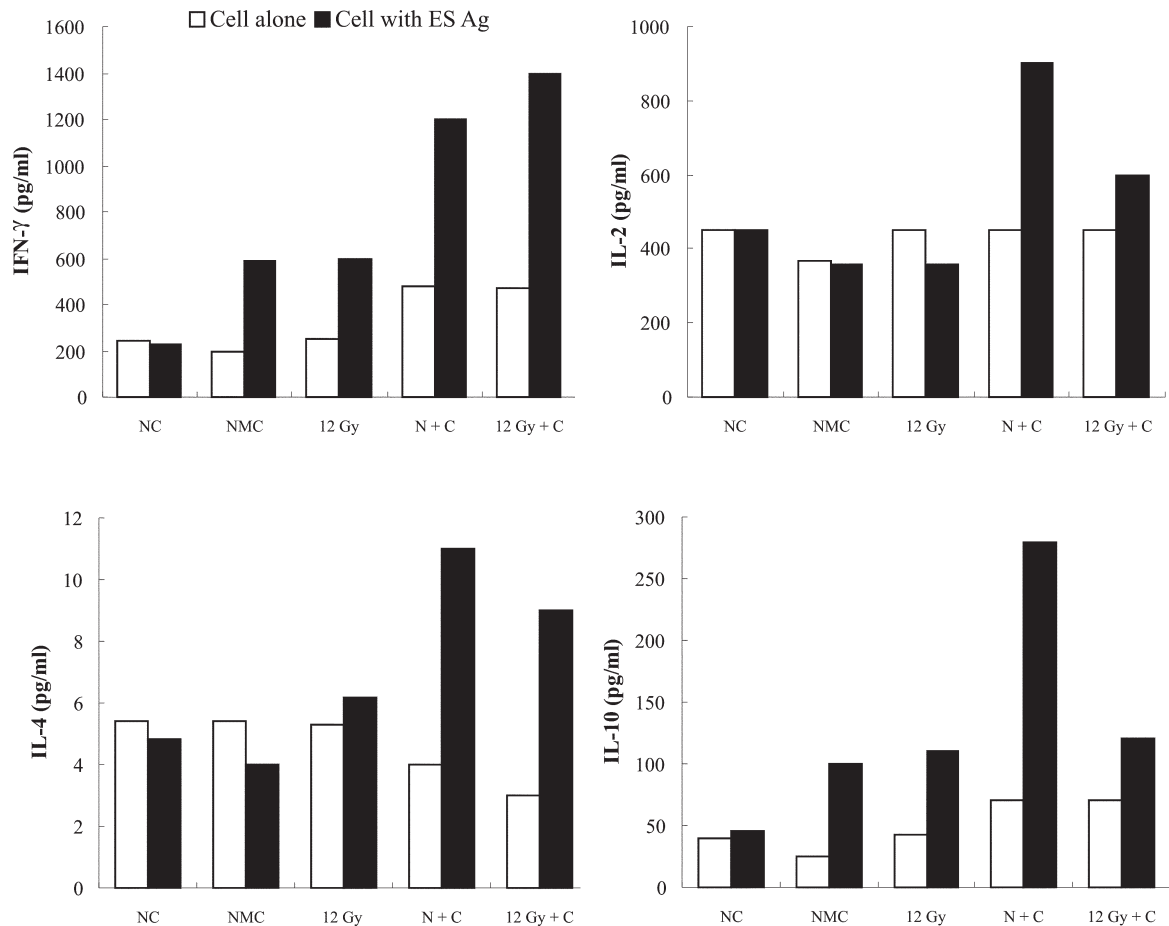


Fig. 5: in vitro production of cytokines, interferon (IFN- γ) and interleukins (IL-2, IL-4, IL-10), from rat mesenteric lymph nodes (MLN). NC: uninfected control; NMC: non-irradiated metacercariae control; 12 Gy: metacercariae irradiated with 12 Gy; N + C: challenge infection after primary infection with non-irradiated metacercariae; 12 Gy + C: challenge infection after primary infection with 12 Gy-irradiated metacercariae. In this experiment, it used lymphocytes that were pooled from five rats.

rats showed high level of IgG antibody responses to the same level as those infected with non-irradiated MC (Fig. 2). Rats showed no antibody responses and no worms were recovered from 15 to 100 Gy irradiation dose compared to uninfected control and non-irradiated rat control. It may be that no worms survived on these irradiation doses. So an attempt to induce resistance was carried out on 12 Gy irradiation doses, in which worm burdens were significantly decreased after challenge infection (Fig. 3).

Little is known about the mechanisms by which protection against parasite infection is induced by irradiation-attenuated vaccines, but several studies have been performed including disruption of protein synthesis, changes in carbohydrate expression (Wales & Kusel 1992), alteration of parasite antigens, alteration of expression of cathepsin-B protease and WGA- and Con A-specific sugars which may be detrimental to parasite invasion and contribute to the protective immune responses generated in the host (Creaney et al. 1996). Maybe 12 Gy irradiation dose can induce maximum changes described above correlated with the capacity of the irradiated larvae (highest level of antibody responses) to protect rat against

subsequent challenge with non-irradiated MC. Different levels of antibody responses from different doses need further study.

Our findings in this study indicated that ES Ag specific stimulated proliferative responses and cytokines productions were elicited. When the cytokines production compared to infection with 12 Gy irradiated MC and challenge infection with non-irradiated MC at ES Ag group, IFN- γ and IL-2 were significantly enhanced. This result indicates that irradiated MC like attenuated vaccine is able to induce Th1-type cytokines (IFN- γ and IL-2) and these cytokine effect to protection against *C. sinensis* infection. Resistance to different parasitic diseases has been associated with the both of Th1- or Th2-type immune responses. Different T-helper subsets appear when different parasite is presented in a host, or if the duration of infection is altered. *S. mansoni* infection can drive Th2 responses in rats or in mice and it favors protective immunity against reinfection (Cetre et al. 1999). *Trichinella spiralis* infection shows mixed Th1/Th2 response in rats (Stewart et al. 1999), *Trichuris muris* shows Th2-type response in mice involved in protective immunity (Koyama

et al. 1999). Chronic infection with *Taenia crassiceps* is characterized by high levels of production of both Th1 and Th2 cytokines in mice (Spolski et al. 2000). Mice showed low parasite numbers when receiving recombinant cytokines IFN- γ and IL-2, whereas significant increases in parasite loads were found when mice receiving IL-10 (Terrazas et al. 1999). It is well known that Th1-type response helps to eliminate intracellular microorganisms, whereas a Th2-type response specializes in the control of extra-cellular pathogens (Cox & Liew 1992, Stevenson & Tam 1993, Reiner & Locksley 1993). Development of an inappropriate immune response can be ineffective and even pathogenic to the host (Romagnani 1997). In *Leishmania major* infection, IL-10 administration leads to a higher parasite persistence in a mouse model and influenced the outcome of the disease by modifying the inflammation and local cell recruitment at the site of parasite penetration (Viana da Costa et al. 2002).

In conclusion, the single dose of 12 Gy could be adopted for induction of the highest resistance to challenge infection of *C. sinensis*. After primary infection with 12 Gy irradiated *C. sinensis*, reinfection with non-irradiated *C. sinensis* is induced high levels of production of IFN- γ and IL-2. These Th1-cytokines were involved in protecting against *C. sinensis* reinfection.

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