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Immunization and challenge shown by hamsters infected with *Opisthorchis viverrini* following exposure to gamma-irradiated metacercariae of this carcinogenic liver fluke

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Abstract

Here we report findings to optimize and standardize conditions to attenuate metacercariae of *Opisthorchis viverrini* by ionizing radiation to elicit protective immune responses to challenge infection. Metacercariae were gamma-irradiated and the ability of irradiated metacercariae to prevent patent infection of challenge metacercariae in hamsters was determined, as well as their ability to induce a host antibody response. Metacercariae irradiated in a dose-dependent manner, with 3, 5, 10, 12, 20, 25 and 50 Gray, were used to infect Syrian golden hamsters by stomach gavage to ascertain the effect of irradiation on ability of the worms to establish infection. In addition, other hamsters were infected with metacercariae irradiated with 20–50 Gray, followed by challenge with intact/wild-type (non-irradiated) metacercariae to determine the protective effect as established by the numbers of adult flukes, eggs of *O. viverrini* in hamster faeces and anti-*O. viverrini* antibody titres. Significantly fewer worms were recovered from hamsters immunized with metacercariae irradiated at 20, 25 and 50 Gray than from control hamsters infected with intact metacercariae or 0 Gray, and the worms showed damaged reproductive organs. Faecal egg

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Conflict of interest

None.

Ethics statement

Male Syrian (golden) hamsters (*Mesocricetus auratus*) were reared at the animal facilities of the Faculty of Medicine, Khon Kaen University. Protocols for the experiments were approved by the Animal Ethics Committee of Khon Kaen University, approval number AEKKU25/2554, according to the Ethics of Animal Experimentation of the National Research Council of Thailand.

numbers were decreased significantly in hamsters immunized with 25 and 50 Gray metacercariae of *O. viverrini*. Moreover, hamsters administered metacercariae that were protected elicited a robust, specific anti-fluke immunoglobulin G response compared to control hamsters, suggesting a role for antibody in protection elicited by radiation-attenuated metacercariae.

Introduction

Helminth infections remain a persistent public health problem in resource-poor areas of the world (Hotez *et al.*, 2008b). Three helminth pathogens, the liver flukes *Opisthorchis viverrini* and *Clonorchis sinensis* and the blood fluke *Schistosoma haematobium*, are categorized as Group I carcinogens by the World Health Organization's International Agency for Research on Cancer (IARC) (Bouvard *et al.*, 2009; IARC, 2012).

Opisthorchiasis is endemic in the lower Mekong Basin countries of Thailand, Lao PDR, Cambodia and Vietnam (Sripa *et al.*, 2011). About 9 million people in Thailand alone are infected with *O. viverrini*, with many progressing to cholangiocarcinoma (Sripa *et al.*, 2012). The geographical distribution of opisthorchiasis reflects the distribution of its intermediate hosts, both *Bithynia* spp. snails and cyprinid fish, as well as the dietary habits of the people resident in these regions (Smout *et al.*, 2011; Sithithaworn *et al.*, 2012). Infection with *O. viverrini* is caused by consumption of undercooked fish, in which metacercariae (MC), the infective stage, of *O. viverrini* have encysted. Metacercariae excyst in the host duodenum and migrate into the intrahepatic biliary system, where they develop into adult flukes (Sripa *et al.*, 2011).

Residents of endemic regions are often infected for a lifetime. Opisthorchiasis induces inflammation of the epithelia lining the intrahepatic bile ducts, with subsequent periductal fibrosis. Chronic opisthorchiasis can in turn progress to severe hepatobiliary disease, including cholangitis, obstructive jaundice, hepatomegaly, cholecystitis, cholelithiasis and cholangiocarcinoma (CCA), bile duct cancer (Mairiang *et al.*, 2012). The mechanisms by which the parasite causes CCA are likely multi-factorial and include chronic immunopathology, mechanical damage from the worms grazing the epithelium lining the bile ducts, elevated dietary nitrosamines, and the secretion by the parasite of growth factors and other proteins that stimulate cell proliferation and interfere with homeostatic apoptosis (Smout *et al.*, 2011). Despite the humoral and cell-mediated immune response elicited in both humans and rodents, it is unclear whether a protective immune response is acquired to *O. viverrini* (Wongratanaheewin *et al.*, 2003). Due to the chronic nature of opisthorchiasis and its carcinogenicity, control measures to combat this infection are a public health priority, especially in Thailand and Lao PDR (Prichard *et al.*, 2012). Praziquantel and, recently, tribendimidine are effective anthelmintics to treat opisthorchiasis (Soukhathammavong *et al.*, 2011; Keiser *et al.*, 2013). However, successful clearance of the infection does not lead to protection against reinfection, and there is concern of increased risk of CCA from repeated rounds of praziquantel for reinfection (Sripa *et al.*, 2012).

Despite the pernicious impact of opisthorchiasis and the inability of drug treatment to induce lasting protection, there are few reports on attempts to develop a vaccine against this neglected tropical disease. Syrian golden hamsters (*Mesocricetus auratus*) can be reliably

infected with MC of *O. viverrini*, leading to patent infection (Flavell & Lucas, 1983; Thamavit *et al.*, 1987). Notwithstanding the clinical relevance of this model, it has been under-studied as a model for the development of vaccines. This is despite the outcome of passive transfer to naïve hamsters of spleen cells and/or sera from infected hamsters that results in reductions in fecundity compared to control hamsters, implying that both humoral and cellular factors contribute to this protective outcome (Flavell *et al.*, 1980). In addition, immunization with parasite extracts has also induced modest reductions in fluke burdens (Sirisinha & Wongratanacheewin, 1986). However, a key step in vaccine development is proof of concept that protective immunity can indeed be induced. Many successful viral and bacterial vaccines are attenuated versions of the microbe. Whereas diverse forms of attenuation have shown utility, e.g. serial passage, attenuation by irradiation of the infective stages of helminth parasites has been used widely. Examples include irradiation of the third-stage larvae of *Ancylostoma caninum*, which provided the platform for industrial production of a vaccine for hookworm disease of dogs (Fujiwara *et al.*, 2006) and the irradiated cercariae model of schistosomes (Yole *et al.*, 1996). This report presents the initial steps towards proof of concept that attenuation of *O. viverrini* by gamma irradiation of metacercariae can induce a protective immune response as a basis for vaccine development. We anticipate that this model will facilitate screening of vaccine candidates and formulations for consideration in human clinical trials against this carcinogenic liver fluke.

Materials and methods

Irradiation and viability of metacercariae

Metacercariae of *O. viverrini* (*Ov*-MC) were obtained from naturally infected cyprinid fish (Sithithaworn *et al.*, 1997). These larvae were dispensed into a glass block (4 × 4 × 1 cm) containing 0.85% NaCl (1 ml) and exposed to gamma (γ) radiation at increasing doses. The γ -rays were discharged from a ^{60}Co linear accelerator (Theratron 780C, Theratronics, Ottawa, Ontario, Canada) at a rate of 112 rad/min, with target *Ov*-MC located 80 cm from the ^{60}Co source, in a radiation field of 400 cm² (20 × 20 cm).

To monitor the effect of γ -irradiation on viability of *Ov*-MC *in vitro*, 20 *Ov*-MC were irradiated with 0, 5, 10, 12, 20, 25 or 50 Gray (Gy). Thereafter, the irradiated *Ov*-MCs were induced to excyst *in vitro* for 20 min in 1 ml 0.25% trypsin in phosphate-buffered saline (PBS) containing 0.2% EDTA (excystation buffer). Newly excysted juveniles were washed several times in saline (0.85% NaCl), then examined and quantified for activity/viability by light microscopy.

Experimental infection and vaccination

One day before infection with irradiated *Ov*-MC, blood samples were collected from the retro-orbital vein of hamsters. To determine the effect of radiation on infectivity of irradiated *Ov*-MC, groups of five hamsters were infected with 50 *Ov*-MC that had been irradiated with increasing doses of γ -radiation – 3, 5, 10, 12, 20, 25 and 50 Gy. Infection of hamsters with *Ov*-MC was accomplished by orogastric gavage (Bhamarapavati *et al.*, 1978; Sripa & Kaewkes, 2002). Hamsters infected with non-irradiated (intact/wild-type) *Ov*-MC were

included as controls. Six weeks after infection, hamsters were euthanized, after which livers, blood and faeces were collected.

A putatively protective dose of irradiated *Ov*-MC for vaccination was predicted to be that which resulted in impaired parasite development, as ascertained by reduced adult fluke numbers in the biliary tree, but which also stimulated a strong antibody response to *O. viverrini* fluke antigens. Immunization was carried out using *Ov*-MC subjected to increasing doses of radiation. Six treatment groups ($n = 5$ per group) were included: group 1, control hamsters infected with 50 intact/wild-type (non-irradiated) *Ov*-MC (0 Gy); groups 2, 3 and 4 immunized with a single dose of 50 *Ov*-MC that had received 20, 25 and 50 Gy γ -radiation, respectively. Hamsters were challenged with 50 intact *Ov*-MC 4 weeks after exposure to irradiated *Ov*-MC. Faeces of the hamsters were collected 4, 5 and 6 weeks later to ascertain numbers of fluke eggs per gram of faeces. Figure 1 presents a schematic of the experimental design and timetable for immunization with irradiated *Ov*-MC, challenge infection, euthanasia and sample collection.

Faecal eggs, hamster sera, recovery of adult flukes

Faeces from each group of hamsters were pooled and the eggs of *O. viverrini* counted as eggs per gram (EPG) of faeces using a modified formalin–ethyl acetate technique (Elkins *et al.*, 1986). Food was not provided to the hamsters for 12 h prior to euthanasia. After euthanasia, blood was recovered from the heart and allowed to clot at 4°C for 30 min, centrifuged at 2100 *g* for 10 min at 4°C, and serum separated from blood cells and stored at –20°C. Adult worms of *O. viverrini* were recovered by dissecting the liver, with the number of worms determined by examination under light microscopy (Flavell *et al.*, 1983).

Preparation of *O. viverrini* antigen

Worms of *O. viverrini* were recovered from bile ducts of hamsters at necropsy. Worms were washed in phosphate-buffered saline, pH 7.2 (PBS) and resuspended in PBS containing a 1 $\times$ protease cocktail (GE Healthcare Life Sciences, Pittsburg, Philadelphia, USA). Worms were homogenized in a hand-held tissue grinder on ice and subsequently the lysate was subjected to ultrasonic sonication with amplitude 20% for 2-s bursts with 5-s intervals for 20 cycles (Vibra-Cell, Sonics & Materials Inc, Newtown, Connecticut, USA). The lysate was clarified by centrifugation at 18,000 *g* for 30 min at 4°C, the supernatant collected, and the protein concentration determined using the bicinchoninic acid assay (Amresco, Solon, Ohio, USA). The soluble lysate of adult liver flukes, for use as antigen in serological assays (below), was divided into aliquots and stored at –20°C.

Serology

To detect *O. viverrini*-specific immunoglobulin (Ig) G, flat-bottom 96-well microtitre plates (Maxi-Sorp Immunoplates, Nunc, Denmark) were coated with 1 μ g/ml of the soluble lysate of adult worms at 100 μ l per well (Saichua *et al.*, 2013) in 15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6, and incubated at 4°C overnight. Wells were washed with 300 μ l of 154 mM NaCl, 0.05% Tween 20 (wash solution) five times before being blocked with 200 μ l of 5% skimmed milk powder buffer. Plates were incubated at 37°C for 2 h and then washed three times with wash solution (above). Sera were diluted 1:1000 in incubation buffer (2%

skimmed milk powder in 137 mM NaCl, 9 mM Na₂HPO₄·2H₂O, 0.8 mM NaH₂PO₄·2H₂O, 0.05% Tween-20), added to plates at 100 µl per well, with the plates incubated for 2 h at 37°C. The washing process was repeated as above, after which 100 µl goat anti-hamster IgG–horseradish peroxidase (HRP) (Invitrogen, Grand Island, New York, USA) diluted 1:2500 in PBS was added (100 µl per well) and incubated for 1 h at 37°C. Plates were washed four times with wash solution (above), followed by a final wash with 1 × PBS. *O*-Phenylenediamine (Life Technologies/Invitrogen) was added (100 µl/well) and the plates incubated at 37°C for 30 min. Finally, substrate development was halted by addition to wells of 100 µl 0.5 M H₂SO₄. Optical densities (OD) at 490 nm were determined (Spectramax, Molecular Devices, Sunnydale, California, USA), with data collected and stored using SoftMax Pro (Molecular Devices). Duplicate wells without control or experimental sera, but with the above reagents, were included to determine the extent of non-specific binding of the antibodies.

SDS-PAGE, Western blot analysis

Fifty microgram of PBS-soluble adult worm antigen extract were separated by 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using the mini-PROTEIN electrophoresis system (Biorad, Hercules, California, USA) and transferred to nitrocellulose using a rapid semi-dry blotting system, Fastblot B43/B44 (Biometra, Göttingen, Germany). The nitrocellulose membrane was cut into 14 strips (~3.5 µg soluble adult worm antigen extract per strip) and blocked with 5% skimmed milk powder in PBS-T (0.1% Tween-20 in PBS) for 2 h at room temperature. Membranes were briefly rinsed twice with PBS-T, before incubating in pooled hamster sera diluted 1:100 in 2% skimmed milk powder in PBS-T for 2 h at room temperature. After washing three times for 10 min each with PBS-T, membranes were incubated with goat anti-hamster IgG-HRP (Invitrogen) diluted in 2% skimmed milk powder in PBS-T for 1 h. Membranes were washed twice for 10 min each with PBS-T, followed by a final 10-min rinse with PBS. The bands were visualized using a chemiluminescence detection system (ECL Plus^{TM}, GE Healthcare).

Data analysis

Protection levels were calculated by comparing worm recovery of control and vaccinated groups. Data were analysed by one-way analysis of variance using Prism 5 (GraphPad, La Jolla California, USA); *P* values of 0.05 were considered to be statistically significant.

Results

Gamma-radiation attenuated metacercariae of *O. viverrini*

Metacercariae of *O. viverrini* exposed to 5, 10, 12, 20, 25 and 50 Gy of γ -radiation from ⁶⁰Co exhibited 85–100% active excystation *in vitro*. Excystation rates of MC that had been γ -irradiated did not differ from that of controls, i.e. non-irradiated *Ov*-MC (not shown). To address the *in vivo* effect of γ -radiation on the establishment of irradiated *Ov*-MC in hamsters, as well as to determine the optimal dose to attenuate irradiated the *Ov*-MC to elicit a protective immune response, hamsters were infected with *Ov*-MC that had been exposed to increasing doses of radiation. Radiation doses greater than 12 Gy resulted in progressively negative impacts on the ability of the *Ov*-MC to develop into adult worms. Whereas similar

numbers of adult worms were recovered from hamsters infected with *Ov*-MC irradiated at 10 Gy and intact MC, significantly fewer adult worms were recovered after infection with 12 Gy *Ov*-MC. Less than 10% survived at 25 Gy, and none survived to the adult stage when *Ov*-MC were exposed to 50 Gy. Overall, the number of adult worms recovered from the hamster biliary tract ranged from 32 to 0 (fig. 2A). The numbers of eggs in faeces of infected hamsters reflected the numbers of adult worms (fig. 2B), where egg output was significantly decreased in groups of hamsters infected with *Ov*-MC irradiated with 12, 20, 25 and 50 Gy, compared to hamsters infected with control, intact (non-irradiated) MC ($P = 0.001$) which shed faeces containing ~10,000 EPG.

Immunoglobulin responses to soluble lysate of adult *O. viverrini*

Immunoglobulin G (IgG) responses against the soluble lysate of adult *O. viverrini* worms were detected in infected, but not uninfected, hamsters after the primary exposure to MC (fig. 3). Titres of IgG greater than $OD_{490} = 0.6$ against soluble lysate of adult *O. viverrini* were elicited in hamsters exposed to *Ov*-MC exposed to 0, 3, 5, 10 and 12 Gy. Exposure to MC exposed to greater doses of radiation (*Ov*-MC at 20, 25 and 50 Gy) also elicited strong IgG responses against soluble lysate of adult *O. viverrini*, although not as markedly elevated as evident in sera from rodents receiving MC exposed to less radiation. As noted, an appropriate immunizing dose of γ -radiation would require the impairment of parasite development upon reaching the small intestine/biliary system as well as eliciting a strong anti-adult *O. viverrini*-IgG response. A radiation dose of 20 Gy induced a robust antibody response against adult soluble lysate, along with significant reductions in adult worms and faecal eggs (fig. 2). Accordingly, a dose of 20 Gy, as well as the higher 25 Gy and 50 Gy doses of radiation were utilized in the immunization study (below).

Anti-worm and anti-fecundity effects against challenge infection

Metacercariae of *O. viverrini* irradiated with 20, 25 and 50 Gy were used to immunize hamsters before challenge infection with intact MC (fig. 1). Immunized groups were compared with non-immunized control groups, which were challenged with 50 intact *Ov*-MC. Significantly fewer worms were recovered from hamsters immunized with *Ov*-MC irradiated at 20, 25 and 50 Gy than from the control hamsters infected with intact *Ov*-MC or 0 Gy (fig. 4). Similarly, faecal egg numbers were decreased significantly in hamsters immunized with 25 Gy and 50 Gy *Ov*-MC, although not with the 20 Gy *Ov*-MC immunization dose. Reductions in faecal egg counts were marked at weeks 4 and 5. Although the reductions waned by week 6, significantly fewer eggs were still apparent in comparison to the control hamsters challenged with wild-type metacercariae (fig. 5).

IgG levels following challenge infection

The serum IgG antibody profiles in hamsters immunized with irradiated *Ov*-MC were determined by enzyme-linked immunosorbent assay (ELISA) (fig. 6). IgG responses against *O. viverrini* were induced in all hamsters infected with irradiated *Ov*-MC, with significantly increased IgG responses against soluble lysate of adult *O. viverrini* in groups that received 0 and 20 Gy irradiation compared to non-vaccinated but challenged rodents (fig. 6). Immunoblot analysis showed that serum IgG from the group of hamsters vaccinated with non-irradiated MC (0 Gy) reacted to the soluble lysate of *O. viverrini* worms with bands of

molecular masses of ~89, 34, 25 and 20 kDa. In hamsters that received irradiated *Ov*-MC at 25 Gy and 50 Gy, proteins of 89, 34 and 20 kDa were recognized; the 25 kDa protein was not recognized (fig. 7). The most prominent band recognized by vaccinated animals was at 89 kDa.

Discussion

The energy from ionizing radiation, such as γ -rays, X-rays and ultraviolet (UV) light, damages cellular nucleic acids, proteins and lipids, leading to physiological changes or cell death (Spitz *et al.*, 2004). These types of radiation have been used to attenuate diverse species of helminths for subsequent administration, as proof of concept that vaccines can be developed against these pathogens (Bain, 1999; Bickle, 2009). Several efficacious vaccines currently in use, including some against helminths, are based on immunization with irradiated microbes. The use of ionizing radiation to attenuate helminth parasites forms the basis of a number of commercial vaccines for companion animals and livestock (Bain, 1999; Bickle, 2009). In a pioneering report from half a century ago, it was demonstrated that irradiated larvae of the cattle lungworm *Dictyocaulus viviparus* fail to develop to adulthood, yet still stimulate a protective response, an outcome that propelled commercial development of a vaccine for this infection (Jarrett *et al.*, 1958).

In *Schistosoma mansoni*, radiation-attenuated cercariae induce protective immunity in mammals, including non-human primates (Bickle, 2009). With vaccines for schistosomes, the protection obtained with cercariae that have been attenuated with ionizing radiation is considered to be the 'gold' standard against which other vaccine candidates are compared for clinical development (McManus & Dalton, 2006; Hotez *et al.*, 2008a; McManus & Loukas, 2008; Loukas *et al.*, 2011). Elevated levels of protection against *Schistosoma japonicum* can be induced by exposure to X-irradiated cercariae in macaques (Hsu *et al.*, 1969) and cattle (Hsu *et al.*, 1983), by exposure to γ -irradiated cercariae in bovines (Hsu *et al.*, 1984), and UV-attenuated cercariae in pigs (Tian *et al.*, 2010). Vaccine efficacies are variable, however, as exemplified by immunization of mice with γ -irradiated cercariae of *S. japonicum*, which elicits only modest resistance to reinfection (Zhang *et al.*, 1999). Whereas it would be impractical to use γ -radiation of fish to block transmission of *O. viverrini*, we examined radiation-attenuated MCs here with the goal of obtaining a baseline level of protection (either strong or limited protection against challenge) against which to compare future vaccine preparations.

At the outset, we determined that exposure to γ -radiation reduced the infectivity of metacercariae of *O. viverrini*; i.e. doses >10 Gy reduced infectivity, with, for example, >50% reduction caused by 12 Gy. At 50 Gy, flukes failed to develop from irradiated larvae. Similar findings have been described with radiation sensitivity of MC of the minute intestinal fluke *Metagonimus yokagawi* (Chai *et al.*, 1995). Metacercariae of the closely related liver fluke *C. sinensis* may be even more sensitive to damage from γ -radiation than *O. viverrini*, with only ~25% of worms exposed to 5 Gy developing to the adult stage in rats (Park & Yong, 2003; Quan *et al.*, 2005). We also observed that immunization of hamsters with γ -irradiated MC of *O. viverrini* resulted in decreased infection and fecundity upon challenge infection with intact/wild-type MC. Passive transfer of immune spleen cells and serum to protect

against *O. viverrini* has been examined, but failed to induce resistance to reinfection in hamsters (Flavell *et al.*, 1980). The immunization of hamsters with a soluble lysate of adult worms before challenge infection afforded modest protection, a 30% reduction of worms (Sirisinha & Wongratanaheewin, 1986). Vaccination with radiation-attenuated MC of related liver flukes has also been reported, e.g. a single dose of γ -irradiated (12 Gy) MC induced resistance in rats to challenge infection with *C. sinensis* (Quan *et al.*, 2005).

When helminth parasites are exposed to ionizing radiation, they can suffer growth retardation, vacuolization of the interstitial tissues, elevation of the tegument, malformation of reproductive organs, including vitellaria, the uterus and numbers of eggs *in utero*, failure of reproduction and attenuation of pathogenicity (Bickle *et al.*, 1979; Chai *et al.*, 1995). Irradiation of MC of *O. viverrini* led to malformation of reproductive organs and impairment of, and/or failure to, produce eggs. Moreover, an abnormal morphology, particularly of the reproductive organs, was apparent in worms exposed to 25 Gy. A dose of 50 Gy was lethal, with no adult worms surviving. The number of eggs released in the faeces of hamsters that were immunized with 25 Gy was significantly reduced in the fourth and fifth weeks of infection compared to hamsters infected with wild-type parasites. We selected this dose of γ -radiation for vaccine development because 25 Gy *Ov*-MC induced robust IgG responses yet attenuated adult flukes sufficiently to arrest their development and ensure failure of normal reproduction. Metacercariae of *O. viverrini* exposed to 25 Gy were capable of inducing a robust IgG response to soluble lysate of adult *O. viverrini*, which we conjecture is the vaccine-induced protective response that negatively influenced fecundity of the challenge cohort of wild-type flukes.

In summary, vaccination of hamsters with irradiated *Ov*-MC induced significant levels of protection, as evidenced by reduced worm numbers and reduced fecundity of the parasites. The methods and findings provided proof of concept for assessment of performance of vaccines for human opisthorchiasis in the context of informative vaccine endpoints – titre of IgG antibody to antigens of *O. viverrini*, the numbers of flukes resident in the biliary system after immunization, and the number of fluke eggs in hamster faeces, i.e. fecundity effects. Hamsters provide an informative model host for development of a vaccine against infection with *O. viverrini* because not only is this rodent a permissive, definitive host, but also because the hamster will develop *O. viverrini*-induced CCA (Bhamarapravati *et al.*, 1978; Smout *et al.*, 2011; Lvova *et al.*, 2012; Sripa *et al.*, 2012). Efforts toward developing a vaccine for opisthorchiasis might now focus on refining this model involving infection with radiation-attenuated metacercariae. The iterative process might include multiple immunizations, size of the inoculum of irradiated *Ov*-MC, duration between immunization and challenge, and analysis of cellular and cytokine responses. With advances in immunoproteomics, and using mass spectrometry-based approaches, sera from hamsters vaccinated with irradiated MC could be used to identify the protective antigens. It would also be informative to investigate the role of the immunization on biliary tree and intestinal microbiota, which are modified by opisthorchiasis (Plieskatt *et al.*, 2013). Progress toward a vaccine that would not only protect against opisthorchiasis but also against opisthorchiasis-induced bile duct cancer would be welcomed, given that these are among the most problematic neglected tropical diseases and infection-induced cancers of East Asia.

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Fig. 1.

Experimental design and the timelines of immunization with irradiated *O. viverrini* metacercariae, challenge infection and necropsy. Upper panel: infection of hamsters with radiation-attenuated metacercariae. Lower panel: vaccine study involving immunization with radiation-attenuated metacercariae, followed by challenge with intact parasites. Ov-MC, *Opisthorchis viverrini* metacercariae; Gy, Gray; EPG, worm eggs per gram of hamster faeces; W, weeks.

Fig. 2.

The number of adult worms of *O. viverrini* in the bile ducts, and eggs recovered in pooled faeces, from hamsters infected with *O. viverrini* metacercariae subjected to γ -radiation. (A) The number of worms recovered 6 weeks after infection with 50 irradiated *Ov*-MC subjected to different doses of γ -radiation. (B) Faecal egg counts of hamsters infected with irradiated and wild-type metacercariae (0 Gy). *** Denotes significant differences between worms treated compared with control groups infected with wild-type parasites ($P = 0.001$).

Fig. 3. IgG responses to a lysate of adult *O. viverrini* worms in the sera of hamsters 6 weeks after infection with irradiated metacercariae. C: uninfected hamsters.

Fig. 4.

The number of adult worms of *Opisthorchis viverrini* recovered from hamsters after immunization with irradiated metacercariae and subsequent challenge with 50 wild-type metacercariae. Control: hamsters infected with 50 intact *Opv*-MC. Levels of significant differences: ** $P = 0.01$ and *** $P = 0.001$.

Fig. 5.

The number of eggs per gram of faeces (EPG) in hamsters exposed to irradiated metacercariae at 4, 5 and 6 weeks after infection and challenged with 50 wild-type metacercariae. Levels of significant differences: * P 0.05, ** P 0.01 and *** P 0.001.

Fig. 6.

IgG responses against soluble lysate of adult *O. viverrini* as determined by ELISA in sera of hamsters 6 weeks after challenge infection with wild-type *O. viverrini* metacercariae following immunization with radiation-attenuated metacercariae at 20, 25 and 50 Gy. The term ‘– ve control’ (C–) refers to uninfected hamsters whereas ‘control’ (C+) refers to non-vaccinated but infected hamsters. Levels of significant differences: ** P 0.01 and *** P 0.001.

Fig. 7.

Immunoblot analysis of IgG in hamster sera against a soluble lysate of adult *O. viverrini* worms. The terms 20, 25 and 50 Gy refer to hamsters immunized with radiation-attenuated *O. viverrini* metacercariae that had been exposed to 20, 25 and 50 Gy. The term 0 Gy refers to hamsters vaccinated with non-irradiated metacercariae and infected with wild-type metacercariae; C- refers to a pool of pre-immunized serum. C+ refers to non-vaccinated hamsters infected with 50 wild-type metacercariae.