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Potential of recombinant 2-Cys peroxiredoxin protein as a vaccine for *Fasciola gigantica* infection

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ABSTRACT

Helminth 2-cys peroxiredoxin (Prx) is a major antioxidant enzyme that protects parasites against hydrogen peroxide-generating oxidative stress from the hosts' immune responses. This enzyme has been found in all stages of the tropical liver fluke, *Fasciola gigantica*. To investigate the potential of the recombinant *F. gigantica* Prx-2 (rFgPrx-2) as a vaccine candidate, vaccine trials in mice were carried out. In this study, the ICR mice were immunized with rFgPrx-2 combined with Freund's adjuvant and infected with *F. gigantica* metacercariae. The vaccine efficacy was estimated by quantitate fluke recovery, antibody levels and liver function. The protection by rFgPrx-2 against *F. gigantica* infection was achieved at 43–46% compared with adjuvant-infected and non-immunized-infected control groups, respectively. The vaccine elicited both Th1 and Th2 humoral immune responses with predominance of Th2 as indicated by the higher level of IgG1 in sera of immunized mice. However, the levels of liver damage markers, serum glutamate oxalic transaminase (SGOT) and serum glutamic pyruvate transaminase (SGPT) in rFgPrx-2 immunized group did not show significant difference in comparison with the controls. This study suggested that rFgPrx-2 may have a potential as a vaccine against tropical fasciolosis.

1. Introduction

Tropical fasciolosis is a food-borne zoonosis disease affecting a range of livestock, which was caused by infection with the liver flukes, *Fasciola gigantica*, through of water or water plants contaminated with metacercariae. Humans can also be infected and are considered accidental hosts (Pockros and Capozza, 2004). Pathological sequelae of fasciolosis comprise two stages, prehepatic and hepatic stages, based on the sites where the flukes are migrating in the definitive hosts (Behm and Sangster, 1999). Pre-hepatic stage begins when newly excysted juveniles (NEJ) pass through the intestinal wall, and reach the abdominal cavity (Behm and Sangster, 1999). Hepatic stage is when the migrating juvenile flukes reach and cause damage to the liver parenchyma. Ultimately, the flukes reach the bile ducts where they become mature and commence egg production. Acute fasciolosis is associated with pre-hepatic stage, which may result in severe abdominal pain and ascites with heavy infection. Chronic fasciolosis is indicated by the presence of eggs in feces, eosinophilia, bile duct fibrosis and liver cirrhosis (Behm and Sangster, 1999). The disease causes loss of meat and milk production, anemia, and mortality in economic livestock (Torgerson and Yilma, 1999). Controls of fasciolosis are mainly achieved by anthelminthic drug administration, especially triclabendazole. However, the development of resistance after long-term anthelminthic drug use has been reported (Brennan et al., 2007). Thus, the vaccine against fasciolosis is thought to be an alternative strategy that provides a more sustainable protection for the animals that is safe for the consumers (Dalton and Mulcahy, 2001). Several recombinant proteins encoded by functionally important genes of *F. gigantica* have been tested for their vaccine potentials in animal models, including recombinant glutathione-S-transferase (Preyavichyapugdee et al., 2008), leucine aminopeptidase (Changklungmoa et al., 2013), saposin-like proteinases-2 (SAP-2) (Kueakhai et al., 2013), cathepsin B (Chantree et al., 2013), and fatty acid binding protein (Nambi et al., 2005) with different protective efficacies.

During helminth infection, T helper 2 (Th2) immune responses are upregulated and generated reactive oxygen species (ROS) by the

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activated eosinophils and macrophages that attack the parasite surfaces (Moreau and Chauvin, 2010). Peroxiredoxins (Prxs) are the central antioxidant enzyme produced in many metazoan parasites to counteract against host-generated hydrogen peroxides, allowing their survival within the hosts (Gretes et al., 2012). This enzyme is classified, based on the location and numbers of cysteine residues, into typical 2-Cys, atypical 2-Cys, and 1-Cys (Wood et al., 2003). In *F. gigantica*, there are two genes encoding 2-Cys Prxs, namely FgPrx-1 and FgPrx-2, which have been characterized (Chaithirayanon and Sobhon, 2010). Between the two genes, FgPrx-2 is more highly expressed and identical to *F. hepatica* Prx. The expression of FgPrx could be detected in all developmental stages and in mice sera after 3 and 4 weeks of infection (Sangpairoj et al., 2014). Vaccination using recombinant Prx protein with Quil A adjuvant has been tested against *F. hepatica* infection in goat that showed 33.1% protection with significant reduction of liver damage (Mendes et al., 2010). Notably, *Schistosoma mansoni* Prx had been trialed and showed 40% protection against infection in mice with elicited Th1 immune response (El Ridi and Tallima, 2009). Similarly, vaccination with Prx antigen promoted a long-term protection against *Leishmania major* infection in mice and monkey (Campos-Neto et al., 2001). Substantial protection by *L. major*, Prx antigen against visceral Leishmaniasis caused by *L. infantum* was also reported in mice with 99.6% parasite reduction (Coler et al., 2007). Effective protection of *Brugia malayi* Prx against its infection had also been reported (Anand et al., 2008; Madhumathi et al., 2011). So far, vaccination using recombinant Prx against *F. gigantica* (rFgPrx-2) has not been tested. In this study, we demonstrated the efficacy of rFgPrx-2 as a vaccine candidate against *F. gigantica* infection in mice model.

2. Materials and methods

2.1. Production of recombinant FgPrx-2 protein

The cDNA encoding FgPrx-2 was cloned from adult *F. gigantica* cDNA library. A recombinant protein encoded for FgPrx-2 gene (GenBank Accession no: ABY85785) was produced by competent *Escherichia coli* BL21 (DE3) and purified using nickel-nitrilotriacetic acid (Ni-NTA) affinity column under the native condition as previously described (Sangpairoj et al., 2014).

2.2. Animal experimentation and parasite preparation

Eight-week-old outbred female Imprinting Control Region (ICR) mice weighing 20–25 g were randomly divided into three groups for immunization and infection experiments: unimmunized and infected, immunized with Freud's adjuvant (Sigma-Aldrich, St Louis, MO, USA) and infected (n = 10 for each group); and immunized with 50 μg of recombinant FgPrx-2 protein (rFgPrx-2) with Freud's adjuvant and infected. All mice were kept in steel cages in an air-conditioned room at 22–25 °C, lighting with a light-dark cycle of 12 h light/12 h dark, 50–60% relative humidity, and provided food and water *ad libitum*. Blood samples were taken by tail clipping 6 times at 2-week intervals prior to experiment on each time point: at day 0 (pre-vaccination), 2nd and 4th weeks (first and second boosters), 6th week (infection), 8th and 10th weeks (termination). The animal experimentation was approved by the Animal Care and Use Committee (SCMU-ACUC), Faculty of Science, Mahidol University.

F. gigantica metacercariae were prepared as follows: eggs collected from gallbladder of naturally infected cattle at slaughterhouses in Pathumthani Province, Thailand. They are then incubated in fresh water about 2 weeks until miracidia hatched. *Lymnaea ollula* snails were each infected with a single miracidium. The cercariae were shed from snails after 8 weeks of infection and settled on cellophane membrane, then transformed into metacercariae. The metacercariae were collected from the membrane and washed several times with 0.85% NaCl solution before use.

2.3. Vaccination

Vaccination was performed following a previously described protocol (Changklungmoa et al., 2013). The first immunization of each mouse was performed by injection of 50 μg rFgPrx-2 mixed with Freud's complete adjuvant (50 μl) subcutaneously on day 0. Two other boosts (first and second boosters) were administered with 50 μg rFgPrx-2 mixed with 50 μl of Freud's incomplete adjuvant at 2nd and 4th week after priming, respectively. Two weeks after the last immunization, each mouse was infected with 30 metacercariae orally. At the termination (4 weeks after infection), mice were anesthetized by CO_2 inhalation, and their peritoneal cavities were opened and washed thoroughly with 0.85% NaCl solution. The livers were immersed and teased in 0.85% NaCl.

2.4. Fluke recovery

After termination, the livers of infected mice were teased in a plate containing 0.85% NaCl and flukes were collected from the biliary ducts and counted. The persons who performed the operation and recovered the flukes did not know the treatments that were given to each group of mice. Protection of rFgPrx-2 protein was determined by calculating percent of fluke reduction in rFgPrx-2 immunized mice group compared with control unimmunized and infected group, or immunized with Freud's adjuvant and infected group, using the following equation: % protection = (A-B)/A*100, where "A" represents the mean fluke recovery from challenged control mice, and "B" represents the mean fluke recovery from challenged immunized mice.

2.5. Antibody level determination

Levels of IgG1 and IgG2a during immunization and infection were determined from serum samples by indirect enzyme-linked immunosorbent assays (ELISA). A 96-well plate was coated with 1 μg/ml rFgPrx-2 in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) and incubated for overnight at 4 °C. The coated plate was washed with phosphate buffer saline diluted with 0.05% Tween 20 (PBST), and then non-specific binding was blocked with 1% bovine serum albumin for 1 h at room temperature. After washing with PBST, the diluted mouse sera (diluted 1:50,000 in PBS) were added and incubated for 2 h at room temperature. The plate was washed and incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG1 and IgG2a (Southern Biotech, Birmingham, USA) diluted at 1:5000 for 1 h at room temperature. After washing with PBST, 3, 3′, 5, 5′-tetramethylbenzidine (TMB) (KPL, Gaithersburg, USA) was added to each well. The plate was incubated at room temperature for 10 min and the reaction was stopped by adding 100 μl/well 1 N HCl. The optical density at 450 nm was measured by automatic spectrophotometer (Flow Laboratories, VA, USA).

2.6. Liver enzyme assay

The degrees of liver parenchymal damage in mice was estimated by the levels of serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT). The levels of these enzymes were measured in pooled mice sera collected at pre-vaccination (0 weeks), infection (6 weeks) and termination (10 weeks), and done in triplicates using Automatic Chemistry Analyzer (Cobas Mira, Roche, Switzerland).

2.7. Assessment of pathological lesions of the infected liver

Gross pathological lesions of the livers after termination were evaluated by a pathologist who was not aware of the conditions of treatment for each group of mice. The damage, based on severity and lesion intensity, was scored from 0 to 5 according to the criteria