Tropical Biomedicine 38(2): 63-67 (2021)
https://doi.org/10.47665/tb.38.2.039

RESEARCH ARTICLE

Serine protease inhibitor, serpin, is a biomarker following *Schistosoma mansoni* re-infection in mice

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ARTICLE HISTORY

Received: 13 October 2020
Revised: 3 February 2021
Accepted: 6 February 2021
Published: 30 April 2021

ABSTRACT

Schistosomiasis is a chronic parasitic disease affecting mostly low income and resource-limited countries. Despite the distribution of the curative medicine, praziquantel (PZQ), the frequency of re-infection is commonly reported, thus, making a difficulty to discriminate treatment failure after re-infection. Therefore, assessing *Schistosoma mansoni* re-infection after praziquantel administration is crucial to prove the treatment efficacy and to break the transmission of infection in endemic areas. The evolution of highly sensitive and specific diagnostic markers, reliable to detect the re-infection and to evaluate the treatment efficacy, is required to control schistosomiasis. In this study, the potential role of serpin recombinant antigen of *S. mansoni* as a biomarker of re-infection and chemotherapeutic efficacy has been assessed. Therefore, 20 mice were experimentally challenged and re-challenged with 50 *S. mansoni* cercariae and divided into 4 equal groups; the first included infected mice (control positive), the second group was twice infected with *S. mansoni* and left untreated, the third included mice twice infected then treated with praziquantel following the last challenge, and the forth one remained uninfected and untreated (control negative). The current findings demonstrated that high levels of IgG and IgG1 bound to serpin were detected following the re-infection and rapidly declined post treatment. In summary, *S. mansoni* recombinant serpin could be used as a promising marker to discriminate *S. mansoni* re-infection and evaluated the efficacy of treatment. The translation of such a potential tool in endemic areas will provide a significant support for the elimination and control programs against schistosomiasis.

Keywords: *Schistosoma mansoni*, serpin, praziquantel, re-infection, antibody, ELISA.

INTRODUCTION

Schistosomiasis is a chronic helminthic disease caused by several species belonging to the genus *Schistosoma*. Humans and other mammalian hosts acquire the infection via freshwater snails, the potential intermediate host (Colley *et al.*, 2014). It is widely distributed in low income or limited-resources developing countries; however, it is prevalent in 76 countries (Steinmann *et al.*, 2006; Chadeka *et al.*, 2019). Despite of chemotherapeutic intervention and associated control measures, the disease is still prevalent with a potential risk of re-emerging (King, 2009). So far, praziquantel is the drug of choice, and extensively recommended by the mass drug administration (MDA) for the elimination of schistosomiasis (Cioli & Pica-Mattoccia, 2003). However, re-infections with the active transmission have been noted, together with a raised evidence of the potential development of the resistance to praziquantel (Wang *et al.*, 2012). In these circumstances, treatment failure/re-infection may be confused with the prior infection.

The life cycle of *schistosomes* comprises two hosts; mammals including humans, the definitive host, and freshwater snails, the intermediate host. In the later, the development of larval stages; miracidia, sporocysts, daughter sporocysts and furcocercus cercariae takes place. Cercariae penetrate human skin, forming schistosomulae that migrate through the blood circulation to pelvic/mesenteric venous plexuses where they grow into adult worms (Gurarie *et al.*, 2018).

Coprological examination and serological/molecular tools are routine methods to detect *S. mansoni* re-infection. Basically, conventional tools, like Kato-Katz assay have been used for the purpose of fecal egg count with the possibility of detecting various helminth eggs, however, they could not detect prepatent infections before egg release in stools, therefore, they are less sensitive in districts with low prevalences (Berhe *et al.*, 2004; McCarthy *et al.*, 2012).

Molecular assays like PCR have been evoked for the accurate detection of schistosomiasis. Those tools could detect DNA and parasite-free DNA in biological human

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samples (feces, urine, sera, plasma) as well asin snails (Pontes *et al.*, 2002; Sandoval *et al.*, 2006; Wichmann *et al.*, 2009; Fernández-Soto *et al.*, 2014; Aboelhadid *et al.*, 2016). Despite the increased and required degree of specificity and sensitivity, their use in endemic areas has various limitations like unaffordable cost, DNA preparation processes, applicability in field conditions and the requirement for well-trained personnel.

Moreover, the urine-based point of care assay (POC-CCA) was used to detect circulating antigen of living worms in urine samples providing valuable findings in terms of diagnosis and treatment. Such assay is convenient and efficient compared to the KK test due to easy and rapid performance (Greter *et al.*, 2016). However, more studies are required to assess the field sensitivity of this technique, particularly in less endemic areas (Stothard *et al.*, 2006; Lodh *et al.*, 2013; Adriko *et al.*, 2014). On the other hand, the serological assays using *S. mansoni* crude antigen derived from *Schistosoma* adult worms/eggs have been used to overcome obstacles revealed by conventional parasitological tests. Although this sensitivity, they have a drawback of cross-reactivity with other helminths. In addition, these immunodiagnostic tests could not be used to evaluate the efficacy of treatment since specific antibodies might be detected in blood several months post treatment (Zhu *et al.*, 2005).

As a result of the risk of species cross-reactivity as well as failure of the antibody detection using the crude antigens in distinguishing the persisting infection from past one, as the occurrence of antibodies even after cure is considerably responsible for the false positive infections (Doenhoff *et al.*, 2004; Hinz *et al.*, 2017), the use of *S. mansoni* recombinant antigen might be greatly useful to detect parasite-specific antibodies and, thus, to determine the actual prevalence of schistosomiasis in low prevalence-endemic areas (Hamilton *et al.*, 1998). Furthermore, these limitations could be minimized by the proper selection of specific recombinant antigens to induce species-specific and short-lived antibodies in response to the parasite infection, thus, antibodies should be strongly sensitive to chemotherapy or consecutive exposure (Mohammed *et al.*, 2020).

Serine protease inhibitor (serpin) recombinant antigen of *S. mansoni* has been reported a promising species-specific diagnostic antigen for diagnosis of schistosomiasis (Tanigawa *et al.*, 2015). It had a strong reactivity to sera of *S. mansoni*-infected patients, and with sera from experimentally infected rats as well (Mohammed *et al.*, 2020). Since anti-bodies to recombinant serpin are species-specific and they are elevated at the early stage of experimental *S. mansoni* infection, the present study aimed to determine the potential use of such antigen to monitor the re-infection with *Schistosoma* spp. and evaluate the treatment efficacy of praziquantel in mice experimentally infected with schistosomes.

MATERIALS AND METHODS

Parasite culture

S. mansoni Puerto Rican strain has been used. In the animal facility building at Nagasaki University, Japan, the life cycle of the parasite was done by the successive passage of the parasite in *Biomphalaria glabrata* snails and ICR mice. Each mouse was percutaneously infected with 250 cercariae and sacrificed 7 weeks after infection. Following cardiac perfusion with phosphate buffer saline, adult worms are extracted from the portal vein and eggs are isolated from the livers of infected mice (Mohammed *et al.*, 2020).

Animals and experimental design

Female C57BL/6 mice aged 28 weeks were purchased from Japan SLC Inc. (Shizuoka, Japan) and they are kept under hygienic and strict pathogen-free conditions. Animal housing, handling and control were carried out in accordance with the Nagasaki University agreement. The experimental design was approved by the Nagasaki University Ethical Committee (approval number 1505181226) and was carried out in compliance with the animal facilities guidelines in Nagasaki University and Japanese law for Humane Treatment and Management of Animals.

Experimentally, mice were divided into 4 equal groups: Mice in the first group (G1) were infected with *S. mansoni* cercariae and left untreated (control positive), mice of the second group (G2) were re-infected and left untreated, and animals of the third group (G3) were re-infected and treated with PZQ (re-infected treated group). For the re-infection, mice of both G2 and G3 were re-challenged with 50 *S. mansoni* cercariae in the inguinal area 9 weeks following the first infection. In G3, mice were first treated at 8 weeks post infection with PZQ (Sigma, St. Louis, MO) 500 mg/kg body weight twice for two consecutive days, then treated again 5 weeks post re-infection (14 weeks post the primary infection). PZQ was dissolved in distilled water and 230 µl of the solution was administered orally by gavage. To estimate the worm burden, one mouse was sacrificed one week post treatment (9 weeks post infection), and adult worms were checked in the portal vein via perfusion. All mice were sacrificed at the end of the experiment (18 weeks post infection) and the effectiveness of the second treatment was assessed. Uninfected mice of the fourth group (G4) were kept untreated and used as a negative control.

Collection of sera

Blood samples were collected from mice of all groups via the tail vein biweekly until 18 weeks post infection. They were allowed to coagulate at room temperature for 2 hours at 1000 x g for 10 min at 4°C before centrifugation. Serum samples were collected and processed in Eppendorf tubes at -30°C before their use (Kalenda *et al.*, 2015).

Preparation of recombinant protein

Serine protease inhibitor (serpin) was prepared as previously described by Tanigawa *et al*. (2015) through a collaborative work. The protein was expressed using bacterial expression system as a His-tagged protein at N-terminal.

Detection of antibodies using ELISA

In order to detect specific antibodies against serpin antigens, enzyme-linked immunosorbent assay (ELISA) was performed on mice sera. Nunc MaxiSorp 96 wells (Nalgen Nuncint, Roskilde, Denmark) microtiter plates were coated with 50 µl per well of antigen solution (5 µg/ml) (PBS) of recombinant antigen overnight. Plates were washed 3 times at room temperature with PBS containing 0.05% Tween 20 (PBST), then blocked for 2 hours at room temperature with 1% bovine serum albumin (BSA) (Sigma) in PBS. Plates were washed with (PBST) and filled with mice sera that were diluted 50 times in PBST containing 0.1% BSA and incubated at room temperature for 2 hours. After washing as mentioned above, the plate was incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (R&D Systems, Minneapolis, MN) diluted in PBST 1:1000 containing 0.1% BSA for 1 hour. For anti-mouse IgG1-HRP (Invitrogen) antibodies were used in PBST containing 0.1% BSA at 1:1000 dilution. The plates were cleaned, accompanied by the addition of tetramethylbenzidine (TMB) colorimetric substrate (BD

pharmingen, Allschwil, Switzerland). Using the Multiskan FC microplate reader (Thermo Scientific), absorbance was determined at 450nm. Duplicate serum samples were tested. Control negative sera on each ELISA plate were also included. This method was applied according to (Kalenda *et al.*, 2015; Mohammed *et al.*, 2020).

Statistical analysis

For statistical analysis and graph creation, GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA) and Excel software are used. The ELISA cut-off values for infected mice were determined as the mean plus three normal optical density (OD) deviations (mean + 3SD) of the control group serum. OD readings equal to or less than the cut-off value were considered negative, while those were considered negative, whereas such readings were considered positive above the cut-off value. For typically distributed samples, ANOVA and a t test were used and the Mann-Whitney test was used for the other non-normally distributed samples. $P< 0.05$ was deemed statistically significant when comparing variations between groups (Kalenda *et al.*, 2015).

RESULTS AND DISCUSSION

Dynamics of IgG and IgG1 subtype reacting with serpin during *Schistosoma mansoni* re-infection

Currently, levels of both anti-serpin IgG and IgG1 significantly ($P\leq 0.01$) increased one week post re-infection (from 10 weeks) in sera of mice of the re-infected group compared to those detected in the infected group. This increase was maintained during the re-infection (Figure 1 a, c) suggesting that the level of specific IgG1 antibodies bound to serpin could distinguish re-infected mice from firstly infected ones. Such observation was consistent with Demeure *et al*. (1993) who found that a higher production of IgG4 (in human sera) or IgG1 (in mice) is correlated with the re-infection. Previous immunological studies of *S. mansoni* infection have been shown a higher level of IgG4 correlating with *S. mansoni*

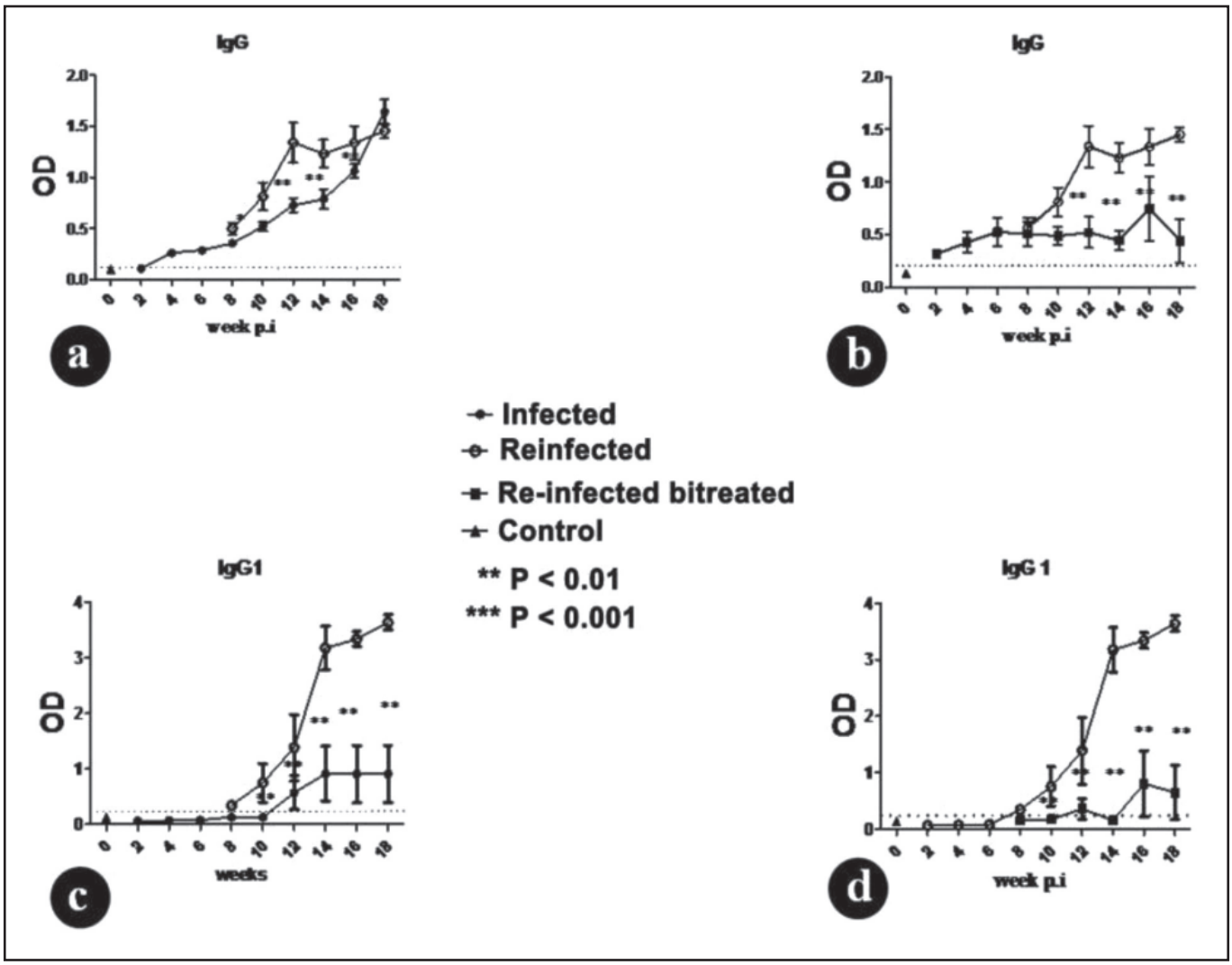

Figure 1. Total serum IgG and IgG1 responses to recombinant *S. mansoni* antigens serpin during *S. mansoni* infection, re-infection and treatment. Sera from five mice were used at each time point. Data are presented as graphs, and means are shown with the standard errors. The dashed line represents the cut-off value (mean + 3SD of the OD of the uninfected controlgroup). a, c The total IgG and IgG1 levels at each time point in the period 2-18 weeks post-infection for infected group untreated (black circle) compared to that in the re-infected untreated group (white circle). b, d The total IgG and IgG1 levels at each time point in the period 2-18 weeks post-infection forre-infected untreated group (white circle) compared to that in the re-infected treated group (black square). Black triangle represented mice of the uninfected control group. $^{**}P< 0.01$ and $^{***}P< 0.001$.

re-infection, rendering IgG4 antibody subtype a useful biomarker of parasitic re-infections, particularly schistosomiasis (Sulbarán *et al.*, 2010; Tanigawa *et al.*, 2015; Mohammed *et al*., 2020). It has been found that serpin could be used as a diagnostic tool for the early detection of *S. mansoni* infection. Additionally, serpin of *S. mansoni* had a significantly higher reactivity to sera from *S. mansoni*-infected rats and patients suggesting that the immunodiagnostic potential of serpin arises from the important physiological role of host-parasite interactions that induce host immunity on releasing the host tissue (Smithers & Terry, 1965; Molehin *et al.*, 2014; Mohammed *et al.*, 2020).

Experimentally, it has been that IgG1 in mice is equivalent to IgG4 in humans, therefore, evaluating the level of mice IgG1 would reflect comparable information as for human IgG4 (Lilienthal *et al.*, 2018). Sera of infected and re-infected mice were probed against serpin recombinant antigen derived from *Schistosoma mansoni* to detect IgG and IgG1.

Dynamics of IgG and IgG1 subtype reacting with serpin during *Schistosoma mansoni* re-infection and treatment with PZQ

So far, few numbers of efficient markers to predict cure or re-infection have been reported. To evaluate the efficacy of treatment against the re-infection on kinetics of both IgG and IgG1 bound to serpin recombinant antigen, sera from re-infected untreated and re-infected bitreated mice group were assayed by ELISA. Levels of both IgG and IgG1 reacting to serpin were significantly declined ($P \leq 0.01$) one week after treatment with re-infection compared to the re-infection without treatment (Figure 1b, d). The decreased level of specific antibodies to serpin following treatment suggests that IgG and IgG1 against serpin are highly susceptible to treatment and correlate with parasite clearance in the treated group.

It is worthy to mention that the specific IgG and IgG1 rapidly declined post treatment with the re-infection. This finding might suggest that *S. mansoni* serpin induced a specific and short-lived antibody response which often concomitant with the persistence of the corresponding pathogenic antigen *in vivo* (Wang *et al.*, 2013). Previous literature proposed that evaluating short-lived antigen-dependent antibodies could be useful for the detection of the parasite as well as to properly evaluate the drug efficacy (Alderete *et al.*, 1991; Zang *et al.*, 2000).

The selection of target antigens is crucial and dependant on the diagnosis of the stage of the disease. For the diagnostic purpose, it could be helpful to select for an antigen that is expressed in all stages of the parasite life cycle inside the definitive host (schistosomulum, adult worm and egg) whereas the selection of antigens should emphasize on the adult worm expressed protein, excluding proteins that are abundantly expressed in the egg stage.

Indeed, following a successful treatment with PZQ, adult schistosomes as well as their antigens are eliminated; however, eggs remain trapped in the liver and intestinal tissues for seven months after a complete cure. The egg-released antigens are able to maintain a persistent antibody production (Traggiai *et al.*, 2003). Concomitantly, further works are mandatory to diagnostically use serpin as a biomarker in endemic areas where a cohort of subjects will be evaluated before PZQ and periodically afterwards to assess the treatment efficacy as well as re-infection. Additionally, samples from infected persons who visited endemic areas might be helpful as a single infection. The translation of such a potential tool in endemic areas will provide a significant support for the elimination and control programs against schistosomiasis.

CONCLUSION

This study has shown that the recombinant antigen (serpin) is likely be potential for guiding re-infection and efficacy of chemotherapeutic treatment against schistosomiasis.

ACKNOWLEDGMENT

We thank to Department of Eco-Epidemiology and Department of Parasitology, Institute of Tropical Medicine, Nagasaki University, Japan for providing recombinant antigen and mice beside to their help and support to conduct this study. The experimental design was approved by the ethical committee of Nagasaki University, Japan.

Conflicts of interest

None.

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