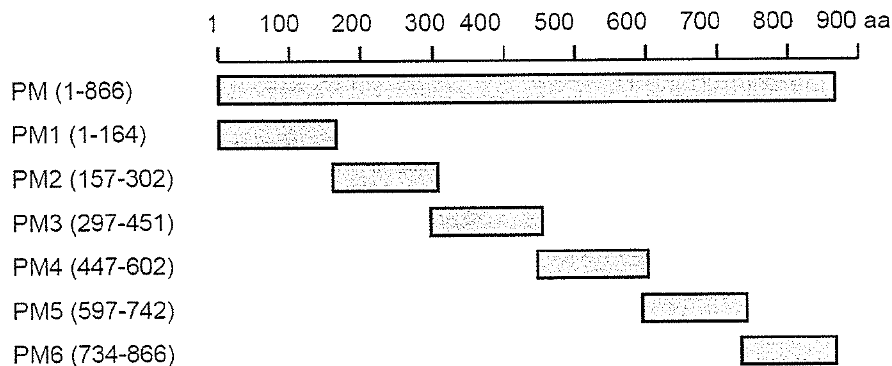

FIGURE 2. Schematic representation of recombinant *Schistosoma japonicum* paramyosin (PM) and its deletion derivatives used in this study. The scale and numbers indicate the amino acid (aa) positions. The full-length PM consists of 866 amino acids. The deletion mutants and their amino acid positions were as follows; PM1 (1–164 amino acids), PM2 (157–302 amino acids), PM3 (297–451 amino acids), PM4 (447–602 amino acids), PM5 (597–742 amino acids), and PM6 (734–866 amino acids).

letion mutants showed no significant correlations with ultrasonographic score and P-III-P levels, despite the positive correlation of anti-PM IgG3 levels with these markers (Table 3). Similarly, multiple regression analysis did not show significant correlations of IgG3 levels against any PM fragments with ultrasonographic score and P-III-P levels. It is likely that the multiple PM epitopes were associated with fibrosis and recognized differently by the patients.

The PM epitopes associated with fibrosis were recognized differently by IgA and IgG4. The IgA levels to PM2 and PM3 and IgG4 levels to PM1, PM4, and PM5 showed positive correlations with ultrasonographic score (Table 3). Multiple regression analysis showed correlations of ultrasonographic score with IgA levels against PM2 (R = 0.292, $P < 0.001$) and with IgG4 against PM1 and PM5 (R = 0.196, P = 0.028 and R = 0.258, P = 0.004, respectively).

Negative correlations between IgE titers and P-III-P levels were observed for PM3, PM4, PM5, and PM6, but the IgE responses against these deletion mutants were weak. Multiple regression analysis showed a correlation of P-III-P levels with anti-PM4 IgE levels (R = –0.309, $P < 0.001$). These results were consistent with the relationship between IgE levels and full-length PM, and suggested that PM4 recognized by IgE has a role in suppression of the progression of fibrosis.

DISCUSSION

Schistosome vaccines are expected to show both effects against infection and disease. In the present study, we addressed the relationships of antibody isotype responses to PM, not only with age-dependent resistance but also with fibrosis, because liver fibrosis is the most important lesion in schistosomiasis japonica.

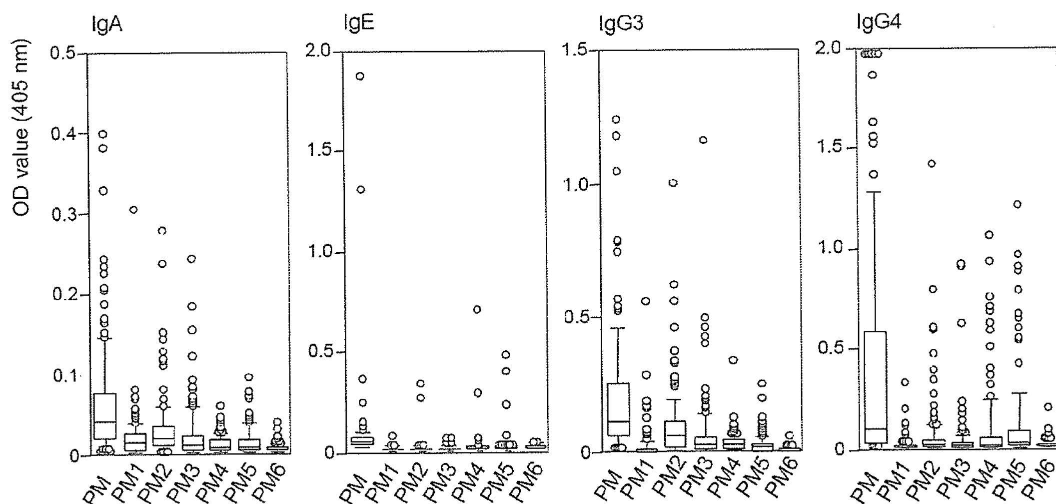

FIGURE 3. Box and whisker plots of IgA, IgE, IgG3, and IgG4 isotype responses to *Schistosoma japonicum* paramyosin (PM) and its deletion mutants. The full-length PM and a series of deletion mutants (PM1, PM2, PM3, PM4, PM5, and PM6) were used for an enzyme-linked immunosorbent assay. The box indicates the area ranging from the first to the third quartiles of each dataset and the median is indicated by the black centerline. The vertical bar represents 1.5 times the interquartile range (IQR) from the upper or lower quartile. Points at a greater distance from the IQR are plotted individually as circles. OD = optical density.

To determine the epidemiologic states of patients with schistosomiasis japonica in The Philippines, we first examined the relationships between age and fibrosis (Table 1). We observed a positive correlation between age and ultrasonographic score but not between age and any serologic markers of fibrosis. Correlations between age and ultrasonograpic score appear to reflect accumulation of fibrosis along with age rather than the current progression of fibrosis. For example, cases of schistosomiasis japonica in older persons in Japan, which showed advanced liver fibrosis by ultrasonographic scoring, did not excrete eggs.[26]

Positive correlations of ultrasonograpic score with P-III-P and type IV collagen levels are consistent with the previous findings for schistosomiasis japonica.[24,27] It is noteworthy that P-III-P level reflects mainly the progress of collagen synthesis, while the type IV collagen level reflects collagen degradation.[28] Thus, correlations between ultrasonographic score and these serologic markers may reflect the current pathologic progress.

The TBA level has been suggested to be a good indicator of hepatic fibrosis.[25] In the present study, however, the TBA level did not show any correlations with other indicators of fibrosis. This discrepancy may have been due to the difference in duration between the previous study design[25] and the present study design, in that the subjects in the present study had received mass treatment with praziquantel in the previous 10 years, which may have influenced the serum level of TBA.

The IgG3 levels to both AWA and PM showed a positive correlation with age (Table 2). A similar age-related trend with these IgG3 responses was reported previously in the human population in The Philippines.[7,29] In addition, multiple regression analysis showed a correlation of age with IgG3 levels against PM2 and PM3. These findings suggest that IgG3 responses to PM, especially anti-PM2 and anti-PM3 IgG3 levels, may be involved in protective immunity to *S. japonicum* infection.

Likewise, IgA responses to PM showed a positive correlation with age (Table 2). This was consistent with the previous report of a positive correlation between IgA levels against PM and age in Filipino patients.[9] Multiple regression analysis suggested that the anti-PM3 IgA level is likely to be associated with age-dependent resistance. In contrast, there was no correlation between any antibody responses to PM and age in China.[18] Thus, the correlation between levels of IgA to PM and age is likely to be distinctive in The Philippines, possibly because of differences in epidemiologic and immunologic features between China and The Philippines.

We did not find any correlations between IgE levels and age, whereas AWA- and PM-specific IgE were present in the sera of Filipino patients. Similarly, we found no significant correlations between IgE levels and frequency of treatment, which is an indication of intensity of reinfection. These observations were consistent with the report that levels of IgE against AWA did not show correlations with age.[18,29] In contrast, levels of IgE to AWA were higher in subjects who were unsusceptible to reinfection two years post-treatment in China.[8] Another group has also reported an association between IgE response to AWA and age in The Philippines.[7] Since there is no direct evidence that human IgE in combination with effector cells mediates killing of the parasites, further analyses are necessary to explain this discrepancy by verifying the precise role of IgE in age-dependent resistance.

In the present study, the relationships between antibody response levels to PM and the degree of liver fibrosis were investigated. We observed positive correlations of antibody isotypic responses to PM with the degree of liver fibrosis as follows: the IgA, IgG3, and IgG4 levels with ultrasonographic score and the IgG3 level with the P-III-P level (Table 3). Multiple regression analysis showed positive correlations of ultrasonographic score with IgA levels against PM2 and with IgG4 against PM1 and PM5, suggesting that anti-PM2 IgA and anti-PM1 and -PM5 IgG4 are likely involved in progressive fibrosis.

The anti-PM IgG3 level showed a positive correlation with ultrasonographic score and P-III-P level. It is important to note that the ultrasonographic score likely represents accumulation of fibrous tissues in the liver, while the serum P-III-P level indicates the currently active state of fibrosis.[28] Therefore, the IgG3 response to PM maybe involved in both progression and the subsequent accumulation of fibrosis, but the PM epitope(s) associated with the degree of fibrosis is unclear.

In contrast to the IgG3 response to PM, a negative correlation between IgE response and serum P-III-P level was found in the Filipino patients (Table 3). In addition, multiple regression analysis showed a negative correlation of P-III-P levels with anti-PM4 IgE levels. In the experimental rodent model, there are controversial observations concerning the role of IgE in schistosome infection: reduced granuloma formation in mice lacking IgE[19,20] and enhanced granulomatous inflammation in FcεRI-deficient mice.[30] The discrepancy in the mode of IgE in granulomatous development may suggest that the roles of IgE in fibrosis are dependent on parasite antigens; IgE to PM may interfere with the progression of fibrosis, and other combinations may enhance fibrosis in humans.

Recent studies have demonstrated the roles of surface PM as immunomodulators. Paramyosin is capable of binding *in vitro* to collagen and the complement components C1, C8, and C9, which results in inhibition of complement activation and formation of membrane attack complex.[31–33] Likewise, PM can bind to the Fc domain of immunoglobulin *in vitro*.[34] The modes of isotype responses to PM in granuloma formation are unclear. However, it is possible that the immune complex of immunoglobulins and PM released from the parasite surface binds to the endothelial or fibroblastic matrix surrounding the embolized eggs through interaction of PM with collagen, which leads to enhanced inflammation and granuloma formation.

It is commonly assumed that granulomatous pathology is $CD4^+$ T cell-mediated in mice. It is noteworthy that B cell- or FcRγ-deficient mice exposed to *S. mansoni* developed larger granulomas in the chronic stage of the infection and displayed unaltered T cell responses, which suggests a suppressive role of immunoglobulin receptor–mediated responses in granuloma formation.[35] In the present study, it is possible to speculate that PM or PM-immunoglobulin complex modulate positively immune responses at the site of lesions, which leads to accumulation of fibrous tissues, the mechanism of which remains unknown.

In the present study, it was difficult to identify the epitope(s) responsible for age-dependent resistance or granu-

loma formation. Epitope mapping of PM recognized by the isotypes and their correlation analyses indicated some trends, in that age tended to be associated with the responses to the N-terminal half of PM (Table 2). In contrast, no such tendency was observed in relationships between liver fibrosis and the epitopes. These results suggest that multiple epitopes are involved in both age-dependent resistance and liver fibrosis.

A positive correlation between IgG3 responses to PM and P-III-P levels was an undesirable finding in the context of schistosome vaccine development. In contrast, IgE response to PM is likely to play a suppressive role in the development of fibrosis, a desired feature for a schistosome vaccine. These contrasting results clearly indicate that PM has complex roles in modulating human immune responses.

Although PM can induce protective immunity against challenge parasite infection in experimental animal models, there are marked immunologic and pathologic differences between humans and animals. Our findings provide insights into the importance of combinations between PM and isotype responses for schistosome vaccine development, in which the desired immune responses should be provoked to avoid exacerbating the pathology. Further studies to characterize the precise mode of PM in antibody-dependent killing of the parasite and in granuloma formation in humans are required prior to clinical trials.

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Authors' addresses: Takeshi Nara, Kyoichi Iizumi, Soichi Tsubota, Akiko Tsubouchi, and Takashi Aoki, Department of Molecular and Cellular Parasitology, Juntendo University School of Medicine, Hongo 2-1-1, Bunkyo-ku, Tokyo 113-8421, Japan, Telephone: 81-3-5802-1043, Fax: 81-3-5800-0476, E-mail: tnara@med.juntendo.ac.jp. Hiroshi Ohmae, Laboratory of Imported Parasitic Diseases, Department of Parasitology, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan. Orlando S. Sy, Schistosomiasis Research Hospital, Palo, Leyte, The Philippines. Yutaka Inaba, Department of Epidemiology and Environmental Health, Juntendo University School of Medicine, Hongo 2-1-1, Bunkyo-ku, Tokyo 113-8421, Japan. Masanobu Tanabe, Department of Tropical Medicine and Parasitology, School of Medicine, Keio University, Tokyo 160-8582, Japan. Somei Kojima, Center for Medical Sciences, International University of Health and Welfare, Kitakanamaru 2600-1, Otawara, Tochigi 324-8501, Japan.

Reprint requests: Takeshi Nara, Department of Molecular and Cellular Parasitology, Juntendo University School of Medicine, Hongo 2-1-1, Bunkyo-ku, Tokyo 113-8421, Japan.

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2-Cys peroxiredoxins from *Schistosoma japonicum*: The expression profile and localization in the life cycle

Takashi Kumagai *, Yoshio Osada, Tamotsu Kanazawa

Department of Parasitology and Tropical Public Health, School of Medicine, University of Occupational and Environmental Health, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyusyu 807-8555, Japan

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Abstract

Peroxiredoxin (Prx) is known to be an antioxidant protein that protects the organisms against various oxidative stresses and functions as a signal transductor. Here, we determined the full-length cDNA sequences of three types of Prx from an Asian blood fluke, *Schistosoma japonicum*: Prx-1, Prx-2 and Prx-3. According to the deduced amino acid sequences, only Prx-3 had a mitochondria-targeting sequence. Using RT-PCR, it was shown that these Prx genes were constitutively expressed in the eggs, cercariae and adult worms of the schistosome. Western blot analysis using antisera specific for each Prx revealed that all the three Prx proteins existed in these developmental stages. By immunolocalization analysis, Prx-1 existed on the surface of a miracidium and in the space between a miracidium and an eggshell. Furthermore, Prx-1 was deposited in the host tissues around the eggs. In adult worms, Prx-1 was not only expressed in the tegument, but also contained in their excretory/secretory products. The surface of the 7 day-schistosomula was stained with anti-Prx-1 antiserum. On the other hand, Prx-2 only existed inside the miracidia in eggs. In addition, Prx-2 was mainly detected in the sub-tegumental tissues, parenchyma, vitelline gland and gut epithelium of the adult worms, but was not detected in the tegument of adults and schistosomula. Taken together with previous reports by other investigators, these data suggest that Prx-1 acts to protect the parasite against the ROS produced by host immune cells, and that Prx-2 plays important roles in intracellular redox signaling and/or in the reduction of ROS generated through the hemoglobinolytic process in the digestive tract.
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Keywords: *Schistosoma japonicum*; Peroxiredoxin; Thioredoxin peroxidase; Anti-oxidant protein; Excretion/secretion

1. Introduction

Schistosomiasis affects over 200 million people in tropical/sub-tropical areas and results in significant morbidity in over 20 million individuals [1]. *Schistosoma japonicum*, one species of Asian schistosome, lives a complex life cycle, surviving in the veins of the final host without elimination by immune attack [2]. The worms are also exposed to oxidative stresses due to reactive oxygen species (ROS), which are mainly produced in two situations. One is that the hemoglobinolytic process after erythrocyte ingestion by the worms produces ROS [3]. The other is that host effector cells (eosinophils and macrophages) adhere to the antibody-coated worms and release ROS [4]. ROS is known to be highly toxic to schistosomes; especially, hydrogen peroxide is responsible for the killing of schistosome larvae *in vitro* [5]. Thus, it has been proposed that the schistosome has antioxidant proteins to protect the worms from oxidative damage [6].

Peroxiredoxin (Prx) is a family of antioxidant proteins distributed in a variety of organisms, including parasitic species [7–10]. Prx proteins contain the conserved Cys residues, essential for the enzymatic activities of Prx: i.e. hydrogen peroxide reductase and alkylhydroperoxide reductase [8]. In the catalytic cycle, Prx requires reducing equivalents of thioredoxin (Trx) [11], and thioredoxin itself is maintained in the reduced state by thioredoxin reductase and nicotinamide adenine dinucleotide phosphatate (NADPH). Molecular cloning and the

Abbreviations: Cys, cysteine; EST, expression sequence tag; GPx, glutathione peroxidase; GST, glutathione-*S*-transferase; IPTG, isopropyl-β-D-thiogalactoside; NO, nitric oxide; ORF, open reading flame; Prx, peroxiredoxin; PVDF, polyvinyliden difluoride; RACE, rapid amplification of the cDNA end; ROS, reactive oxygen species; RT-PCR, reverse transcribed polymerase chain reaction; SOD, superoxide dismutase; Trx, thioredoxin

* Corresponding author. Tel.: +81 93 691 7431; fax: +81 93 602 4488.
E-mail address: tkuma@med.uoeh-u.ac.jp (T. Kumagai).

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characterization of Prxs from *Schistosoma mansoni* have been reported [12–14]. In addition, Trx and thioredoxin glutathione reductase of *S. mansoni* were also cloned [15,16]. In these reports, three types of Prx were identified from *S. mansoni* and their biochemical properties were also characterized. It was also shown that both Prx-2 and Prx-3, but not Prx-1, exhibited unusual biochemical flexibility, reacting with both Trx and a glutathione reducing equivalent system [14]. However, both the expression profiles and localization of Prxs in the various developmental stages of the schistosome have still not been investigated.

In this context, we identified the full-length cDNA sequences of Prxs from *S. japonicum* and examined expression of each Prx in the life cycle stages using RT-PCR. Furthermore, we determined the tissue distribution of both Prx-1 and Prx-2 by immunostaining.

2. Materials and methods

2.1. Preparation of the parasite at each stage

S. japonicum, isolated at Yamanashi, Japan, was maintained using standard laboratory procedures, using female ICR mice (SLC, Hamamatsu, Japan) and its snail host, *Oncomelania hupensis nosophora*. Cercariae were shed from the crushed snails, collected and used for infection and other experiments. At 8 weeks after percutaneous infection with 40 cercariae, the adult worms were recovered by hepatic portal perfusion of the infected mice [17]. Eggs were isolated from the livers of the infected mice using a previously described method [18]. Miracidia were collected after the eggs were hatched in fresh water. The animal experiments were performed under the control of the Ethics Committee of Animal Care and Experimentation in accordance with The Guiding of Principles for Animal Care Experimentation, University of Occupational and Environmental Health, Japan, and the Japanese Law for Animal Welfare and Care (no. 221).

2.2. Seven-day schistosomula

In order to prepare the schistosomula, anesthetized ICR mice were percutaneously infected with more than 500 cercariae through shaved abdominal skin and kept at room temperature for 1 h. Their skin regions containing schistosomula were peeled off after euthanasia. Then, they were floated on RPMI1640 medium and cultured at 37 °C in 5% CO_2 overnight. After the removal of the skin, the schistosomula released into the medium (skin-penetrated schistosomula) were incubated for an additional 6 days and designated as "7 day-schistosomula".

2.3. Preparation of total RNA and cDNA

Total RNA was isolated from each developmental stage of the parasite using TRIzol reagent (Invitrogen, Sandiego, USA). Complementary DNAs were synthesized with Ready-To-Go You-Prime First-Strand Beads (GE Healthcare Bio-Sciences Corp., Piscataway, USA).

2.4. Identification of full-length cDNA sequences of Prxs

To identify the Prxs of *S. japonicum*, open reading frame (ORF) regions from three Prx cDNAs were obtained from the *S. japonicum* expressed sequence tag (EST). Partial fragments of Prx-1, Prx-2, Prx-3 (GenBank^{TM} accession numbers BU718028, BU719716, BU717726, respectively) were identified as highly homologous sequences to Prxs from *S. mansoni* [14]. In order to determine the 5′ end regions of each cDNA from Prxs, we obtained PCR products containing the 5′ end region, using 5′-Full RACE Core Set (TAKARA BIO, Shiga, Japan) from the cDNA of adult worms. The 3′ regions of Prxs were obtained by the 3′ rapid amplification of the cDNA ends (RACE). Briefly, first strand cDNA was synthesized from the total RNA of adult worms with a 3′ Full RACE Core Set (TAKARA BIO), and the fragments of the 3′ end regions were obtained by PCR with an adapter primer. The sequence of the products was determined for both strands with a BigDye terminator cycle sequencing kit (Applied Biosystems, CA, USA) on an ABI310 automated sequencer (Applied Biosystems). The full-length cDNA sequences of Prxs were determined by direct sequencing of the PCR products amplified with specific primer sets from *S. japonicum* adult cDNA as templates. The PCR products were purified and cloned into pCR 2.1 plasmid vector (Invitrogen). The full-length cDNA sequences of Prx-1, Prx-2 and Prx-3 are available in GenBank^{TM} at accession numbers AB126036, AB215040 and AB215041, respectively. Prx amino acid sequences were deduced using the alignment software ClustalW, available the web (http://www.ddbj.nig.ac.jp/search/clustalw-e.html).

2.5. RT-PCR

Ten micrograms of total RNA from each developmental stage of *S. japonicum* was reverse transcribed in 50-μl reaction mixture using random hexamer oligonucleotides as primers. As a template, 1-μl of cDNA solution was applied to PCR in 20 μl reaction mixture. The specific primer sets for each gene were as follows: Prx-1 (5′-TCCAGCTGATTTCACATTCG-3′, 5′-ATCGTCCAACCGGTTTATCA-3′), Prx-2 (5′-GTTTCGCGGGAGTTATGTGT-3′, 5′-AGACGAATCGCCTCATCAAC-3′), Prx-3 (5′-CGTGGTTATGCAGCTCATGT-3′, 5′-TCGCGTGTAATCTGCTTTTG-3′) and triose-phosphate isomerase (TPI) (5′-ATGGCAGTAGAGCCGACAAC-3′, 5′-AACGCTTAGACCTCCTGCAA-3′), which is expressed constitutively through all developmental stages [19]. The PCR consisted of 30 cycles, with each cycle consisting of 95 °C for 20 s, 60 °C for 30 s and 72 °C for 1 min. The PCR products were resolved with agarose gel electrophoresis, followed by ethidium bromide staining.

2.6. Expression and purification of Prx recombinant proteins

The complete *S. japonicum* Prx-1 ORF was amplified by PCR using a primer pair with the addition of *Xho*I and *Bam*HI restriction sites to the 5′ end and 3′ end, respec-

tively. The amplified ORF fragment was inserted into the pCR2.1 vector with a TOPO™-TA cloning kit according to the manufacturer's instructions (Invitrogen), and the sequence of the insert was confirmed to be identical to Prx-1 from *S. japonicum*. The insert was isolated from the purified constructs by digestion with *Xho*I and *Bam*HI, and then subcloned into pET15 vector (Novagen, Darmstadt, Germany). *Escherichia coli* strain BL21 (Novagen) was transformed with the recombinant plasmids. Expression of the recombinant protein fused with the hexamer histidine tag at the N-terminus was induced with isopropyl-β-D-thiogalactoside (IPTG) at 0.1 mM. The recombinant protein was then purified with the TALON™ His-Tag Purification Resins (Clontech, CA, USA). Using a similar procedure, the histidine-tagged fusion recombinant proteins of Prx-2 and Prx-3 were expressed in the *E. coli* expression system. *Nde*I and *Xho*I restriction sites were added to the 5′ end and 3′ end of the inserts, respectively. As for Prx-3, the 23-amino acid sequence containing the mitochondria-targeting motif was removed from the N-terminal region. The purity of each recombinant Prx protein was confirmed using SDS-PAGE. All recombinant Prx proteins showed a molecular mass of about 23 kDa on SDS-PAGE.

2.7. Polyclonal antibodies (antisera) specific for each Prx

Antiserum against each Prx was generated by the immunization of female Syrian hamsters (SLC) with recombinant proteins. Fifty micrograms of each protein was injected subcutaneously three times at 2-week intervals. At primary immunization, the proteins were emulsified with Freund's complete adjuvant (Difco Laboratories, MI, USA). Freund's incomplete adjuvant (Difco Laboratories) was used for the two subsequent booster immunizations. Antisera were obtained by bleeding of the animals at 1 week after the last booster, and were stored at −20 °C until use. All animals handling was performed under anesthesia with pentobarbital sodium.

2.8. Western blot analysis

Soluble antigens from eggs, cercariae and adult worms of *S. japonicum* were prepared according to a previously described method [18]. The supernatant was collected after a 12 h-culture of adult worms in RPMI1640 medium at 37 °C in 5% CO_2 and designated as excretory/secretory (ES) antigens. Their protein concentrations were determined with a Protein Assay Kit (Bio-Rad, CA, USA). One microgram recombinant protein of each Prx and 30 μg of the parasite antigens were fractionated on 15% SDS-polyacrylamide gels (Bio-Rad) and electrically transferred onto polyvinyliden difluoride (PVDF) membranes (Bio-Rad). The non-specific binding of antibodies to the membranes was blocked with 3% BSA in PBS (blocking buffer) for 3 h, and then the membranes were incubated with anti-Prx antibodies appropriately diluted with the blocking buffer for 2 h. After washing, the membranes were incubated with biotin-conjugated anti-hamster IgG (H+L) antibody (Vector Laboratories, CA, USA) for 1 h. Subsequently, avidin-conjugated alkaline phosphatase from VECTASTAIN ABC kit (Vector Laboratories) was added to the washed membranes. Finally, specific binding of the anti-Prx antibodies was detected using Alkaline Phosphatase Substrate Kit 1 (Vector Laboratories) as a substrate. All experimental steps were performed at room temperature.

2.9. Immunohistochemistry

The granulomatous livers and the adult worms were collected from infected mice and frozen sections were prepared as previously described [20]. The preparations were treated with anti-Prx-1 or anti-Prx-2 antiserum at 4 °C for 3 h. Thereafter, the washed sections were incubated with biotin-conjugated anti-hamster IgG (H + L) antibody (Vector Laboratories) for 1 h at room temperature. After washing with PBS, the sections were treated with avidin-conjugated alkaline phosphatase from Vectastain ABC kit (Vector Laboratories). After the final washing, the preparations were stained with Alkaline Phosphatase Substrate Kit 1 (Vector Laboratories) and counter-stained with haematoxylin.

2.10. Immunofluorescence

The 7 day-schistosomula of *S. japonicum* were fixed with cold acetone according to previously described methods [20]. Especially, whole schistosomula were fixed without reagents to increase permability of schistosomula to antisera. The acetone-fixed whole schistosomula were treated with anti-Prx antisera and biotin-conjugated anti-hamster IgG (H+L) antibody, as described in Section 2.9. Then, the preparations were treated with avidin-conjugated fluorescencein (Vector Laboratories) for 30 min at room temperature. Immunofluorescence was observed using epifluorescence microscopy.

3. Results

3.1. Cloning and sequencing of S. japonicum peroxiredoxins

By screening for the sequence information at the *S. japonicum* EST database, we identified three types of 2-cystein (2-Cys) Prx (Prx-1, Prx-2 and Prx-3) cDNA sequences. The information about them was confirmed by the direct sequencing of cDNA isolated from *S. japonicum* adult worms. The sequence information is shown in Fig. 1, and is also available at the accession numbers AB126036 (Prx-1), AB215040 (Prx-2) and AB215041 (Prx-3) of GenBank™. The deduced amino acid sequences of each Prx are ∼90% identical to each orthologue of *S. mansoni* described previously [14]. They are about 60% homologous to each other (Prx-1 versus Prx-2: 67%, Prx-1 versus Prx-3: 60%, Prx-2 versus Prx-3: 55%). Two conserved cystein residues and the consensus FVCP sequence places them in the 2-Cys Prx family [11]. Prx-3 has a mitochondria-targeting sequence homologous to the Prx-3 of *S. mansoni* [14].

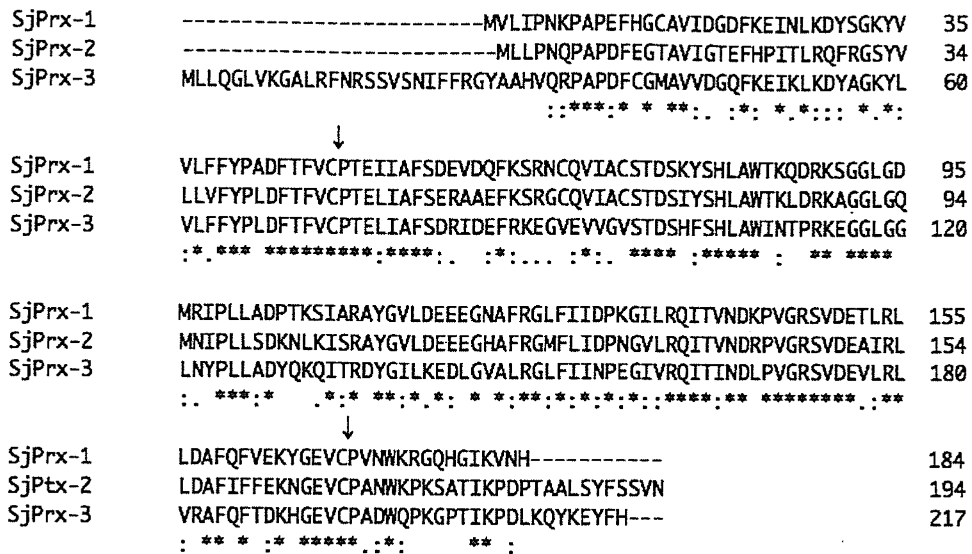

Fig. 1. Alignment of amino acid sequences of Prxs from *S. japonicum*. (A) The alignment was obtained using ClustalW (version 1.8). Identical residues are marked with asterisks, highly conserved residues are marked with colons, and moderately conserved residues are marked with dots. Arrows indicate the reactive cysteines. (B) Chart outlining the percent identity between Prxs of *S. japonicum*.

3.2. The expression levels of Prxs at each developmental stage determined by RT-PCR

A RT-PCR technique was employed to determine the relative levels of each Prx transcript at the different developmental stages. TPI, constitutively expressed in schistosomes, was used as an internal control in the assay to standardize the results [21]. All Prx transcripts were expressed throughout all the developmental stages we examined (Fig. 2). The expression levels of Prxs were almost comparable between the developmental stages, except that the Prx-3 expression level in adult female worms was slightly low.

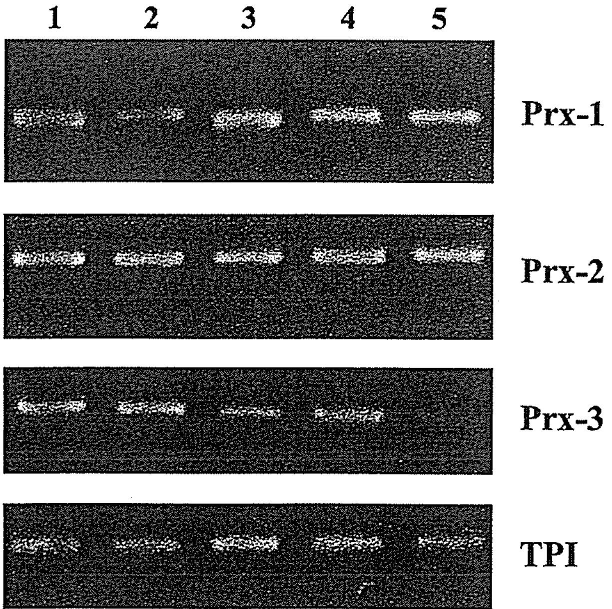

Fig. 2. Transcription of Prx-1, Prx-2 and Prx-3 from *S. japonicum* at various developmental stages. cDNA templates were prepared from the different stages in the life cycle and PCR amplification was carried out. The PCR product was detected by ethidium bromide staining after separation by agarose gel electrophoresis. Lane 1, eggs; lane 2, miracidia; lane 3, cercariae; lane 4, male adult worms; lane 5, female adult worms. Triose-phosphate isomerase (TPI) gene, which is constitutively expressed throughout the life cycle, was used as an internal control.

3.3. Western blotting

To examine the expression levels of Prx proteins, Western blotting using anti-Prx antisera was performed. At first, it was confirmed that each Prx-antiserum did not react with the two other types of recombinant Prx protein (Fig. 3A). Western blotting using these antisera demonstrated that all native Prxs were expressed in the eggs, cercariae and adult worms (Fig. 3B). This result is consistent with the results of RT-PCR (Fig. 2). Prx-1 is more highly expressed in the eggs than in the adult worms, whereas Prx-2 is most highly expressed in the adult worms among all the stages examined. In contrast, Prx-3 was only weakly detected in crude schistosome antigens with our anti-Prx-3 antiserum (Fig. 3B). We did not find any considerable differences between the expression of Prx-1 and that of Prx-2, except the finding that only Prx-1 was detected in the culture supernatant containing the excretory/secretory products from the adult worms (Fig. 3C).

3.4. Immunostaining

To investigate the tissue distribution of Prx-1 and Prx-2, we performed an immunolocalization assay with frozen sections prepared from the eggs and the adult worms. As shown in Fig. 4C, Prx-1 was localized on the surface of the miracidium, and in the space between the miracidium and the eggshell. In contrast, Prx-2 existed inside the miracidium, but did not exist on the surface of it (Fig. 4E). Prx-1, but not Prx-2, also existed in the host liver tissue around the eggs (Fig. 4C). When the adult worms were treated with anti-Prx-1 antiserum, the antibody bound strongly to the tegument of the worms (Fig. 4D). Especially, the male worms expressed Prx-1 much stronger than

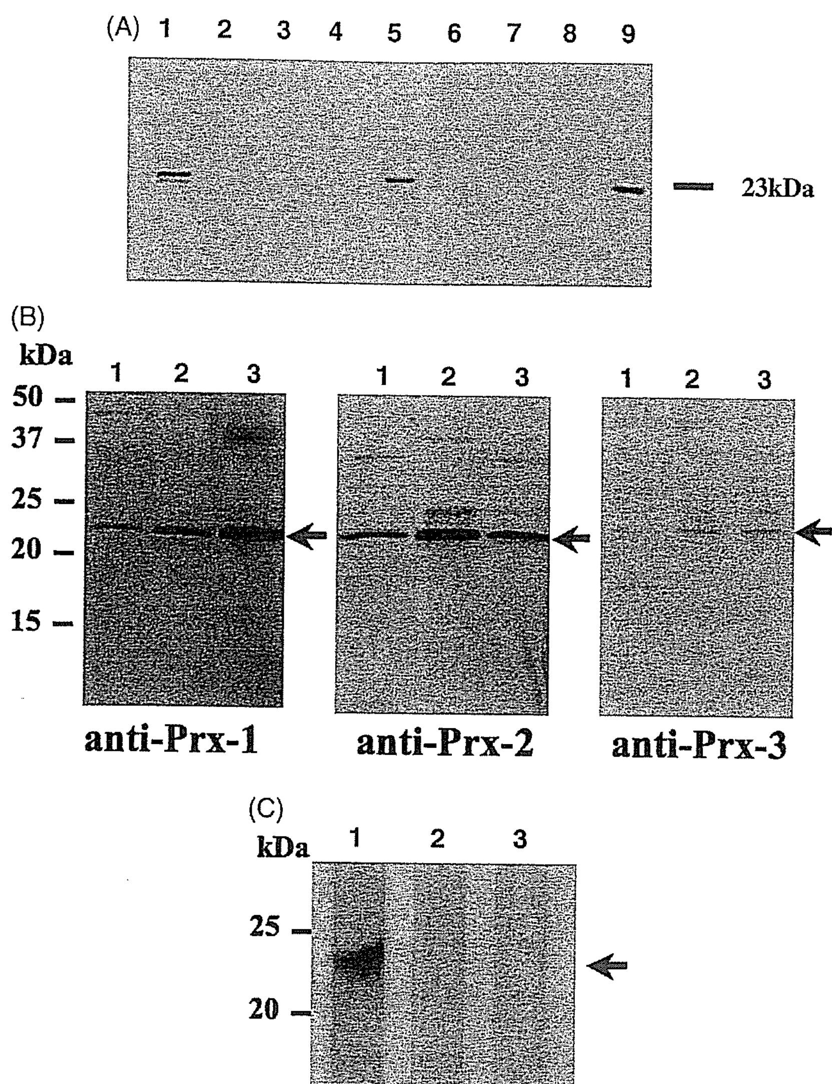

Fig. 3. Western blot analysis of Prx-1, Prx-2 and Prx-3 in soluble antigen preparations of *S. japonicum* at various developmental stages. (A) Each recombinant was transferred onto a PVDF membrane after SDS-PAGE and was reacted with anti-Prx antisera from hamsters immunized with each Prx. Lanes 1–3, lanes 4–6 and lanes 7–9 were subjected with 1 μg recombinant protein of Prx-1, Prx-2 and Prx-3, respectively. Lanes 1, 4, 7 were treated with anti-Prx-1 antiserum (1:1000); lanes 2, 5, 8 were treated with anti-Prx-2 antiserum (1:2000); lanes 3, 6, 9 were treated with anti-Prx-3 antiserum (1:180). (B) Thirty micrograms of soluble proteins extracted from the parasites of three different stages were separated by 15% SDS-PAGE under reduced conditions and were transferred to PVDF membranes. These membranes were treated with anti-Prx-1 antiserum (1:100), anti-Prx-2 antiserum (1:200), anti-Prx-3 (1:100), respectively. Lane 1, soluble cercaria antigens; lane 2, soluble adult worm antigens; lane 3, soluble egg antigens. (C) The excretory/secretory antigens (30 μg) from adult worms cultured for 12 h were separated by electrophoresis and were analyzed by Western blotting using anti-Prx antisera. Lane 1, anti-Prx-1 antiserum (1:100); lane 2, anti-Prx-2 antiserum (1:200); lane 3, anti-Prx-3 antiserum (1:100). Molecular weights in kDa are indicated on the left.

the female worms. Conversely, Prx-2 was localized in the subtegumental area, parenchyma, vitelline gland and gut epithelial tissues of the adult worms, but was not found in their tegument (Fig. 4F). In additon to the experiments, in order to know whether Prx-1 is localized on the surface of the 7-day schistosomula, As a result, it was revealed that Prx-1, but not Prx-2, was highly expressed on the surface of schistosomula (Fig. 5). Normal hamster serum as a negative control did not bind to all the preparations we used (Figs. 4 and 5).

4. Discussion

Reactive oxygen species (ROS) potentially play an important role as effective molecules against protozoan and helminthic parasites. They are generated through normal cellular metabolism, and also by certain anti-parasite drugs [22]. Schistosomes are also exposed to ROS throughout their life cycle. In particular, they have to remove the ROS produced both by host immune cells and by the metabolized erythrocytes taken in by the adult worms [6]. Until now, several anti-oxidant proteins have been shown to exist in the schistosome. Superoxide dismutase (SOD) catalyses the dismutation of the superoxide anion to hydrogen peroxide and oxygen [23,24]. Catalase, working to decrease hydrogen peroxide, does not exist in schistosomes [23], and glutatione peroxidases (GPxs), which are in the phospholipid hydroperoxide GPx class, may not effectively regulate hydrogen peroxide [25]. Recently, three types of peroxiredoxins (Prxs), classified as novel antioxidant proteins, were cloned

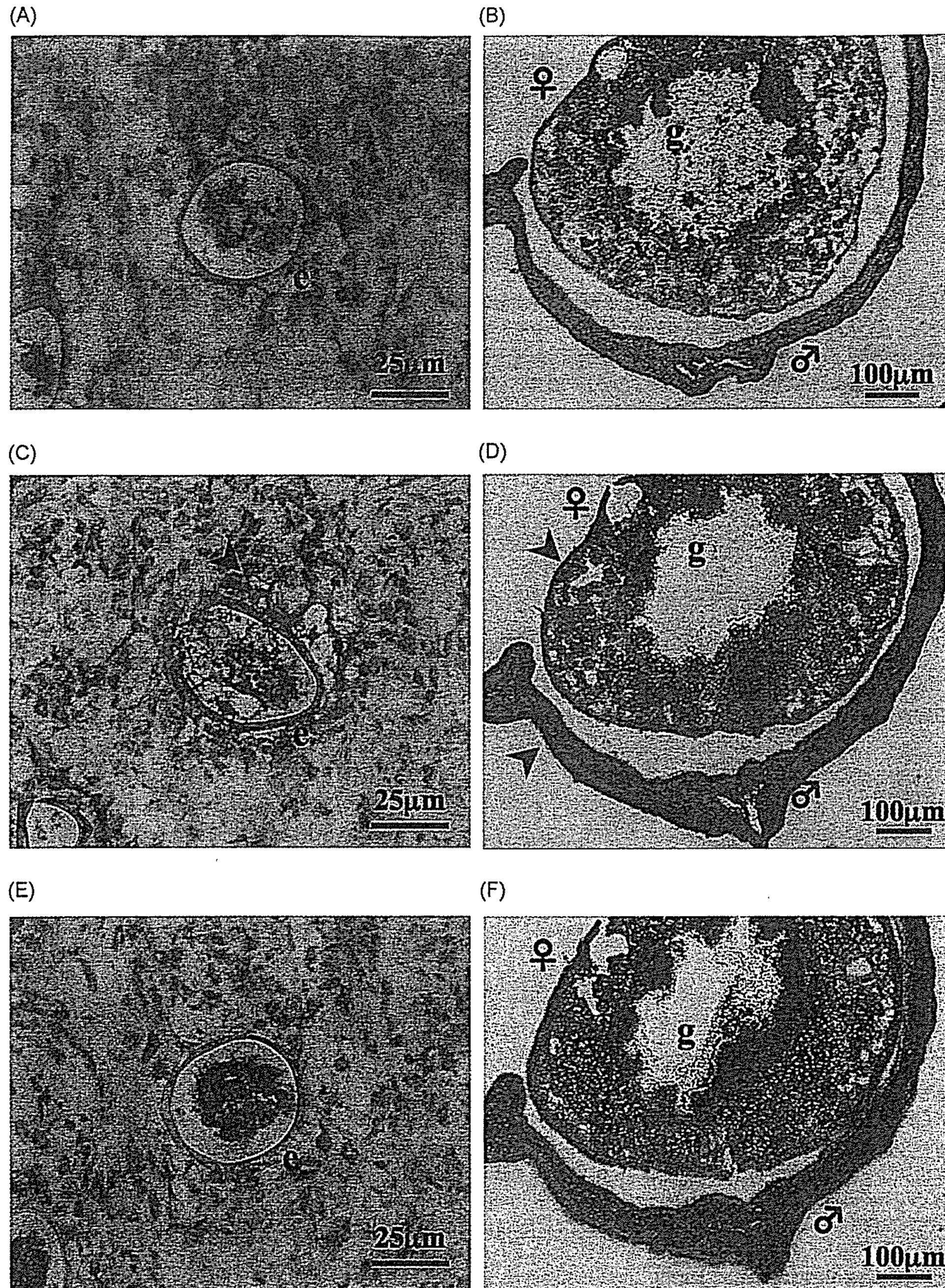

Fig. 4. Immunolocalization of Prx-1 and Prx-2 in eggs and adult worms of *S. japonicum*. (A, C, E) The liver sections from the *S. japonicum*-infected mouse were reacted with normal hamster serum (1:200) (A), anti-Prx-1 antiserum (1:100) (C), or anti-Prx-2 antiserum (1:200) (E), respectively. Eggs (e) in the liver were surrounded by granulomatous inflammatory cells. The arrowhead shows the existence of Prx-1 secreted to the host tissue. (B, D, F) Adult worm sections were observed by immunohistochemistry. The adult worms were treated with normal hamster serum (1:200) (B), anti-Prx-1 antiserum (1:100) (D) and anti-Prx-2 antiserum (1:200) (F), respectively. The tegument was stained with anti-Prx-1 antiserum. Sub-tegmental, parenchyma (asterisk), vitelline gland (v) and gut (g) epihelial tissues (arrowhead), but not tegument, were stained with anti-Prx-2 antiserum.

and sequenced from *S. mansoni* [12–14]. Several Prxs were shown to act as the terminal peroxidase that reduced hydrogen peroxide and organic hydroperoxides [14]. It is likely that Prxs predominantly work to remove hydrogen peroxide in the schistosomes.

Here, we cloned three Prxs (Prx-1, Prx-2 and Prx-3) from *S. japonicum*, and determined their full-length cDNA sequences. Then, they were compared with the corresponding orthologue of *S. mansoni*. The deduced amino acid sequences of Prx-1, Prx-2 and Prx-3 from *S. japonicum* were 83%, 89% and 88%, identical to those of the corresponding *S. mansoni* Prx proteins, respectively. These results mean that Prx sequences are highly conserved between *S. japonicum* and *S. mansoni*. These Prxs of *S. japonicum*, as well as another general organisms, contained two conserved cystein residues (Fig. 1). Only Prx-3 has the mitochondria-targeting sequence at the N-terminal region, suggesting that this protein works as a ROS scavenger in mitochondria [26].

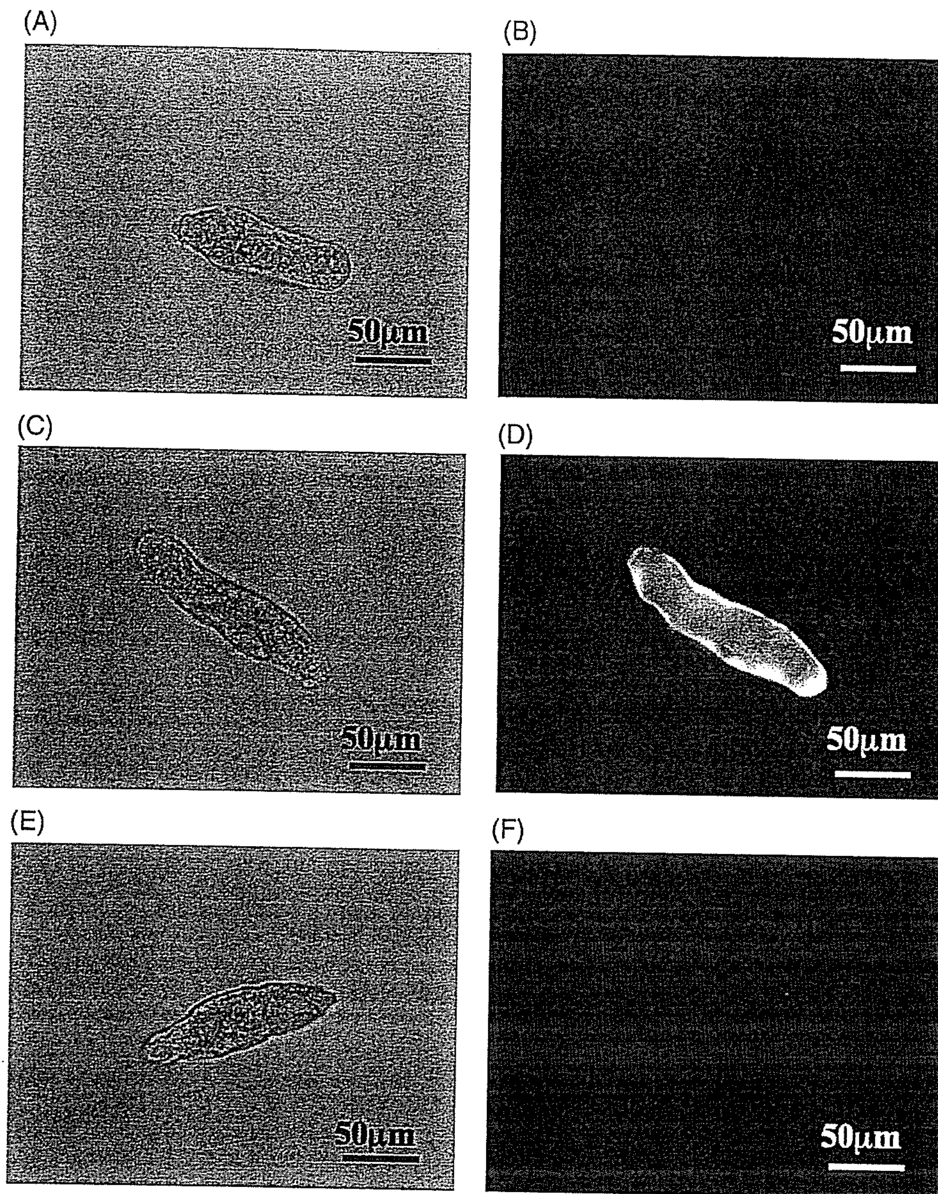

Fig. 5. Localization of Prx-1 and Prx-2 in 7-day schistosomula by immunofluorescent staining. (A, C, E) The schistosomula were shown using light microscopy. The schistosomula were treated with normal hamster serum (B), anti-Prx-1 antiserum (1:100) (D) and anti-Prx-2 antiserum (1:200) (F), respectively. Prx-1, but not Prx-2, existed on the surface of schistosomula.

Previous studies clarified some biochemical characteristics of Prxs: e.g. Prx-2 and Prx-3, but not Prx-1, could use not only thioredoxin, but also glutathione as an electron donor [14]. However, the expression profiles of Prxs through the developmental stages and the localization of Prxs in the schistosome are still unclear. Thus, in this paper, we have firstly examined the expression of each Prx through the developmental stages of *S. japonicum*. Using RT-PCR, the transcripts of all Prxs were detected at all stages we tested (Fig. 2), indicating that the expression of Prxs did not vary in the life cycle of *S. japonicum*. By Western blotting using polyclonal antibodies against each Prx, these native Prx proteins were expressed during the egg, cercariae and adult worm stages, consistent with the result of RT-PCR (Fig. 3B). As only Prx-1 was detected in the excretory/secretory products from the adult worms (Fig. 3C), Prx-1 may be secreted from the parasites to protect themselves against ROS generated by their hosts. To our regret, Prx-3 in soluble adult worm antigens was weakly detected, as shown by Western blot analysis (Fig. 3B), because the reactivity of our antiserum against recombinant Prx-3 may be very low (Fig. 3A).

Therefore, we performed an immunolocalization assay using anti-Prx-1 and anti-Prx-2 antisera. When the granulomatous liver sections from the infected mouse were examined, Prx-1 was localized on the surface of a miracidium, in the space between the miracidium and the eggshell, and in the host tissues surrounding the eggs (Fig. 4C). This fact implies that Prx-1 was secreted from the eggs across the eggshell, coinciding with two previous reports on *S. mansoni*, which showed that both Prx-1 and thioredoxin were secreted from the eggs [13,15]. In contrast with Prx-1, Prx-2 existed only inside of the miracidium and

seemed to not be secreted from the eggs to the surrounding tissues (Fig. 4E). Next, we examined the tissue distribution of Prxs in adult worms. Prx-1 was detected in the tegumental layer of the adult worms (Fig. 4D), and Prx-2 was predominantly localized in the sub-tegumental layer, parenchyma, vitelline gland and gut epithelial tissues (Fig. 4F). These observations are the first demonstration of the differential tissue distribution between Prx-1 and Prx-2 of the schistosome. These results, especially the localization of Prx-1 in the tegument, were consistent with the result of immunofluorescent staining on the surface of *S. japonicum* schistosomula cultured for 7 days (Fig. 5).

Our data suggest that Prx-1 is a constitutive antioxidant protein throughout the developmental stages against hydrogen peroxide, because other antioxidants including SOD, GPx and GST have been reported to express more in the adult stages than the egg and larval stages [27]. Taken together with a previous report, which described that Prx-1 in the eggs of *S. mansoni* was localized to the von Lichtenberg's envelope and was secreted from eggs [13], Prx-1 expressed during the egg stage may work to protect the eggs from hydrogen peroxide generated by granulocytes, including eosinophils [28]. Furthermore, Prx-1 expressed in adult male worms and schistosomula may eliminate the hydrogen peroxide produced as part of the protective immune responses of the hosts. The low level expression of Prx-1 in female worms implies that the female worms may not need protection against hydrogen peroxide by themselves because they lie in the gynecophoral canal of the male.

Several *in vitro* studies have been reported that *S. mansoni* schistosomula were highly susceptible to ROS, in which resistance seems to develop rapidly after the parasite arrives at the liver [23,29,30]. This phenomenon seems to be contradictory to the localization of Prx-1 at the surface of schistosomula from *S. japonicum*, but we suppose that the expression level of Prx-1 is enough to protect the larvae from hydrogen peroxide, because the concentration of hydrogen peroxide released from leukocytes is very low *in vivo* (<1 nM/min) [5], and the killing assay *in vitro* with high concentration of hydrogen peroxide (>100 μM) would not always reflect the *in vivo* situation. Considering other protective immunity to schistosomes, a host macrophage is known to be an effector cell against schistosomula [31]. The larvae seem to be attacked by nitric oxide (NO) released from macrophages activated by type 1 cytokines, including IFN-γ [32]. However, resistance of lung stage schistosomula to NO has been also reported [33,34]. Collectively, these reports suggest that lung schistosomula have molecules to protect themselves from the NO produced by macrophages. We propose that Prx-1 can reduce NO generated from host macrophages on the surface of the parasite. In fact, the bacterial Prxs was shown to have peroxynitrite reductase activity [35].

Prx-2 from *S. japonicum* also has the YF motif in the C-terminal helix, and the Prx-2 of adult worms and eggs from *S. japonicum* was localized in the sub-tegumental layer and the parenchymal tissues, but not in the tegument (Fig. 4). Recently, Sayed and Williams [14] suggested that Prx-2 from *S. mansoni* might be involved in intracellular redox signaling as this protein had the YF motif in the C-terminal region, which was related to signal transduction [36]. Other groups showed that Prx-2 from the mammalian organisms might function as a signal transducer [37,38]. This Prx-2 expression inside the parasite supports the hypothesis of Sayed and co-workers, that Prx-2 of *S. mansoni* might be involved in cellular redox signaling. Here, we have found that Prx-2 was also expressed in the gut epithelial tissue (Fig. 4F), suggesting that Prx-2 may not only be a signal transducer, but also a scavenger against the hydrogen peroxide generated through the hemoglobinolytic process.

In the present study, the full-length cDNA sequences of Prx-1, Prx-2 and Prx-3 from *S. japonicum* were determined. The transcripts and the proteins of all Prxs were detected in the egg, the cercaria and adult worm stages of *S. japonicum*. By immunolocalization analysis, the distinct tissue distributions of both Prx-1 and Prx-2 were obsereved. These results suggest that Prx-1 acts to protect worms from host protective immunity, and Prx-2 functions as a signal transducer and scavenger against the ROS generated from the metabolized hemoglobin. We expect that the essential roles of Prx in the schistosome will be clarified by further investigation. Based on these findings, novel anti-parasite drugs and vaccines against the parasite will be developed.

Acknowledgements

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Evaluation of the anthelmintic effects of artesunate against experimental *Schistosoma mansoni* infection in mice using different treatment protocols

Lu Shaohong [a,b], Takashi Kumagai [b], Ai Qinghua [b], Yan Xiaolan [a,b], Hiroshi Ohmae [c], Yoshisada Yabu [b], Li Siwen [a], Wen Liyong [a], Haruhiko Maruyama [b], Nobuo Ohta [b,*]

[a] *Institute of Parasitic Diseases, Zhejiang Academy of Medical Sciences, Hangzhou 310013, China*
[b] *Department of Molecular Parasitology, Nagoya City University Graduate School of Medical Sciences, Nagoya 467-8601, Japan*
[c] *Institute of Basic Medical Sciences, University of Tsukuba, Ibaraki 305-8575, Japan*

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Abstract

The therapeutic effects of artesunate against experimental *Schistosoma mansoni* infection in mice were analyzed. Previous studies showed that artesunate is highly effective against *S. japonicum* infection, but the action of this drug against *S. mansoni* remained uncovered. The present study examines the optical conditions for artesunate against *S. mansoni* and evaluates the effects of inhibiting the sexual maturation of adult worms. Mice infected with *S. mansoni* were orally administered with artesunate according to different schedules. Four consecutive administrations of 300 mg/kg of artesunate at 2-week intervals conferred almost total protection without the development of pathological lesions in the liver. The significant reduction in the number of eggs produced by surviving worms and the status of egg maturation suggested that artesunate inhibits sexual maturation. Electron microscopy revealed that artesunate caused morphological damage, especially on the worm tegument. Artesunate was also very effective in iron-deficient mice. Furthermore, the efficacy of artesunate was equal to or better than that of artemether against *S. japonicum* infection. Considering that artemether is more toxic, artesunate is currently one of the most efficient drugs against immature *S. mansoni*.
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Keywords: *Schistosoma mansoni*; Artesunate; Prophylaxis; Iron-deficiency; Fecundity

1. Introduction

Schistosomiasis remains an important parasitic disease in terms of large endemic area and the number of infected individuals [1]. Among the five major species of human schistosomes, *Schistosoma mansoni* is the most prevalent, being endemic in 55 countries mainly of sub-Saharan Africa as well as in some parts of South America [2].

Metrifonate, oxamniquine and praziquantel have been used to treat schistosomiasis over the past few decades [3]. Among these, praziquantel is presently the only drug that is highly effective against the adult stage of all human schistosome species with no or minimal side effects [4–6]. However, a series of recent laboratory studies and clinical trials has indicated that schistosomes are developing resistance to praziquantel [7–10]. Thus, the present widespread use of praziquantel might eventually negate the benefits of this drug. Considering that the current state of vaccine development is still far from practical application [11], effective drugs for the prophylaxis and therapy of schistosomiasis are urgently required.

Artesunate (dihydroartemisinin-10-α-succinate) is a derivative of artemisinin that has improved solubility and chemical stability, as well as enhanced anti-malarial activity [2,12,13]. It was originally synthesized and used as an anti-malarial drug in China in 1987 [14]. It has low toxicity and no mutagenicity [15]. Li et al. discovered that artesunate could kill schistosomula and that it had prophylactic properties against *S. japonicum* [16]. Malic dehydrogenase, 6-phosphate mannosidase and acid phosphatase are inhibited in *S. japonicum* and tegument damage arises in worms after exposure to artesunate

Abbreviations: CMC-Na, sodium carbonyl methylcellulose; FWRR, female worm reduction rate; PBS, phosphate buffered saline; PI, post infection; SD, Standard deviation; WRR, Worm reduction rate.

* Corresponding author. Tel.: +81 52 853 8184; fax: +81 52 842 0149.
E-mail address: nohta@med.nagoya-cu.ac.jp (N. Ohta).

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[17–20]. The worm reduction rates (WRR) were 77.5–99.5% when *S. japonicum*-infected mice (300 mg/kg), rabbits (20–40 mg/kg) and dogs (30 mg/kg) were orally administrated with artesunate once each week for 4 consecutive weeks [21]. Patients infected with *S. japonicum* in endemic areas of China were treated with praziquantel, and then divided into one group that was inoculated with 6 mg/kg of artesunate and another that received a placebo [22]. The protective rates of artesunate were 83.9–100% and 68.2% in areas of light to moderate, and heavy endemic infection, respectively. Recently, artesunate has also been reported to be effective against *S. mekongi* infection [23].

The efficacy of artesunate against infection with *S. mansoni* as opposed to *S. japonicum* remains controversial. An early study by Araujo et al. showed that artesunate obviously modified the reproductive organs of *S. mansoni* [24]. The results of our preliminary study showed that artesunate was therapeutically effective against *S. mansoni* infection in mice. We investigated details of the prophylactic and therapeutic aspects of artesunate against *S. mansoni* infection with a view of expanding the use of this drug. Since recent report has suggested that artesunate is less effective against *S. mansoni* than artemether, we attempted to reproduce these findings [25]. Chronic *S. mansoni* infection is associated with a latent iron-deficiency and iron is thought to be important for the activities of artemisinin-derivatives [26–28]. We therefore studied the effects of artesunate against *S. mansoni* infection in iron-deficient and in normal mice.

2. Materials and methods

2.1. Parasites and infection of mice

Cercariae of Puerto Rican strain of *S. mansoni* were released from *Biomphalaria glabrata* snails after exposure to artificial light for 3–5 h. BALB/c female mice weighting about 20 g (SRL, Hamamatsu, Japan) were infected with 200 cercariae by the tail dipping method. Developmental stages of the parasites were divided into the following: pre-lung stage at <7 days after infection, lung stage at 7–8 days after infection, post-lung stage at 2–3 weeks after infection, young adult stage at 4–5 weeks after infection, and adult stage at 6 or more weeks after infection. The Ethical Committee for Animal Experiments, Nagoya City University Graduate School of Medical Sciences, approved our experimental protocol.

2.2. Treatment protocol

Artesunate provided by the Guilin Pharmaceutical Corp (lot #021205) (Guilin, China) was suspended in 1% sodium carbonyl methylcellulose (CMC-Na) for treatment of *S. mansoni*-infected mice. Mice were administrated orally with the aid of stainless stomach tube attached to a syringe. In each experiment, there was a control group(s) being administrated with 1% CMC-Na alone. In all experiments described below, one group contained 6 or 7 mice.

The first experiment was to determine which developmental stage of the parasite was most susceptible to artesunate. Five groups, each of which contained 7 mice, were orally administrated with dose of 300 mg/kg for 2 consecutive days on the day 7–8, 14–15, 21–22, 28–29, or 35–36 post infection (PI). This covered different developmental stages (7–36 days) of the parasite. Adult worms were collected by perfusion method 56 days PI. The dosage of 300 mg/kg was referenced from our previous results from testing *S. japonicum* and *S. mansoni* [19,24].

In the second experiment, 6 groups, each of which again contained 6 or 7 mice, were treated orally in different time schedule at the dose of 300 mg/kg. Comparisons were made for three points: start of treatment (14 or 21 days PI), frequency of treatment (3 or 4 times), and interval of treatment (every 2 weeks or 3 weeks). Adult worms were collected by perfusion method 70 days PI.

For electron microscopic observation and for in vitro oviposition study, we prepared mice with sub-optimal artesunate treatment, because full-dose treatment of artesunate eliminated almost all worms. For this purpose, mice were treated orally with 100 mg/kg of artesunate on 14 days PI, followed by two consecutive treatments on day 28 and 42. Adult worms were collected by perfusion method 56 days PI.

2.3. Iron-deficient mice

To test the efficacy of artesunate in the iron-deficient host animals, 2 groups had been fed with normal or iron-deficient feeding for 7 weeks, and we tested the iron concentration in serum every week by Fe-IC diagnostic kit (Wako, Osaka, Japan). After Fe level in the serum decreased significantly, groups of mice were infected with *S. mansoni*, and then given the drug on day 14 PI at dose of 300 mg/kg once a week for 4 consecutive weeks. WRR were compared between the two groups as described below.

2.4. Assessment of the therapeutic effects

Mice were sacrificed and worms were recovered by portal perfusion with phosphate-buffered saline (PBS). The reduction rates of total and female worms were calculated by comparing the mean worm number with that of control group. The whole small intestine and almost all of the liver, from which a small part was removed for histological examination, were digested in 4% KOH overnight at 37 °C in separate tubes. The number of eggs were counted after centrifugation and washing. The fecundity of female worm was assessed by the parameter of eggs per female worm calculated as follows: total number of eggs recovered from mice was divided by the number of female worms. Paraffin-embedded sections of the liver were examined histologically to assess the hepatic lesions due to *S. mansoni* infection. Wet weight of the liver from each mouse was measured.

2.5. Scanning electron microscopic analysis

Adult worms obtained from the group treated with artesunate and from the corresponding control group were

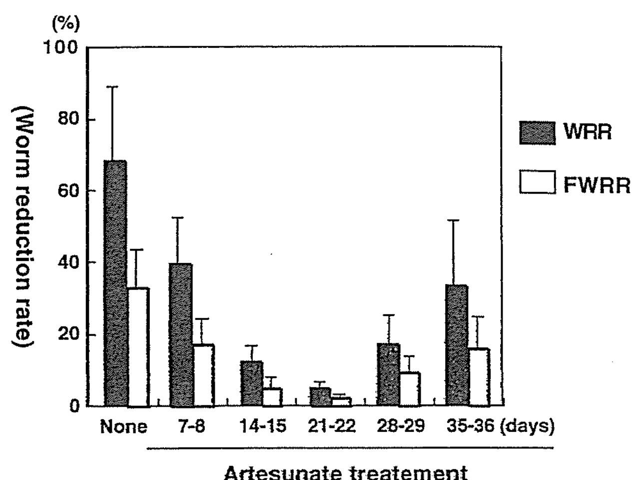

Fig. 1. Optimal time for artesunate prophylaxis against *S. mansoni* infection. Artesunate was orally administrated at the dose of 300 mg/kg to infected mice at different times from day 7/8 to day 35/36 after infection. Parasites were collected by perfusion 8 weeks after infection. WRR was highest at 21 days after infection.

tested for electron microscopic observation. The worms were collected by perfusion method 56 days PI, and processed for scanning electron microscopic analysis according to standard methods [18]. Briefly, samples were fixed with 1–2% glutaraldehyde, washed, and post fixed in 0.1 M osmium tetroxide, dehydrated using ethanol, coated with gold-palladium and then examined with a scanning electron microscope (S-4800, Hitachi, Japan).

2.6. In vitro evaluation of artesunate on oviposition by adult worms

Adult *S. mansoni* pairs of 8 weeks PI were obtained from control or artesunate-treated mice, and were cultured in vitro to compare their egg production in vitro. Two worm pairs were dispensed to each well of 24 well plates (Nunc, Roskilde, Denmark) in RPMI 1640 (Sigma, St Louis, USA) supplemented with 10% fetal bovine serum (FBS; GIBCO BRL, Rockville, USA) and 100 μg/ml streptomycin, 100 U/ml penicillin and 20 mM L-glutamine (GIBCO). After incubation for 3 days with 50 μg/ml of hemin in CO_2 incubator at 37 °C, produced eggs were collected and compared microscopically between the worms

Table 2
Effect of artesunate (300 mg/kg) with different regimens on oviposition and hepatomegaly in *S. mansoni* infection

Group	*N*	Administration (days)	Eggs/female[a] ± SD	Mean liver weight (g) ± SD
1	6	–	4449±641	2.00±0.40
2	5	14–28–42	448±91*	1.14±0.02*
3	4	14–35–56	155±26*	1.15±0.01*
4	4	14–28–42–56	270±33*	1.28±0.11*
5	5	21–35–49	702±226*	1.17±0.04*
6	6	21–42–63	1339±320*	1.28±0.13*
7	6	21–35–49–63	684±150*	1.23±0.16*
Cont[b]	4	–	–	1.10±0.01*

[a] Number of eggs deposited in the intestine and the liver divided by number of female worms recovered.
[b] Cont: Age-matched no infection control.
* $p<0.01$ (vs Group 1).

from artesunate-treated and non-treated mice. Results were shown as mean egg number per one worm pair.

2.7. Statistical analysis

Student's *t*-test was employed to assess the statistical difference between treatment group and control group. Differences were considered when p values were <0.05.

3. Results

3.1. Susceptible stages of S. mansoni

Fig. 1 shows the susceptibility of *S. mansoni* at various developmental stages to artesunate in mice. The anthelmintic effect was significant at a WRR of 41% in the group administered with artesunate at the lung stage (days 7–8 PI), and reached a maximum of 93% WRR in the group medicated at the post-lung stage (days 14–15 and 21–22 PI). The efficacy decreased when mice were treated after day 21 PI, but the effect against the young adult stage remained significant (days 35–36 PI) at a WRR of 46% ($p<0.01$).

3.2. Optimal protocol for artesunate treatment

To identify a suitable treatment schedule, groups of mice were initially given artesunate on either of day 14 or 21 PI, and

Table 1
Effect of artesunate (300 mg/kg) with different regimens against *S. mansoni* infection

Group	*N*	Administration (days)	Total worms ± SD	WRR (%)	Female worm ± SD	FWRR (%)
1	7	–	25.0±11.2		6.43±2.63	
2	6	14–28–42	3.17±3.58*,#	87	1.67±1.77*	73
3	6	14–35–56	4.16±2.61*,#	83	2.33±1.89*	63
4	6	14–28–42–56	0.67±0.90*	97	0.33±0.51*	95
5	6	21–35–49	4.83±2.90*,$	81	2.17±1.48*	66
6	6	21–42–63	8.50±4.17*,$	66	3.16±1.91*	50
7	6	21–35–49–63	1.00±1.63*	96	0.50±0.80*	92

All mice were sacrificed 70 days PI.
*$p<0.01$ (vs Group 1), #$p<0.05$ (vs Group 4), and $ $p<0.05$ (vs Group 7).
WRR: worm reduction rate, percent reduction in worm burden compared with Group 1.
FWRR: female worm reduction rate, percent reduction in female worm burden compared with Group 1.

repeatedly medicated at different frequencies and intervals. The WRR and FWRR were highest in mice given 4 doses of artesunate at 2-week intervals (Groups 4 and 7), regardless of whether drug administration was started 14 or 21 days PI (Table 1). The WRR values for the groups given 4 consecutive doses were significantly elevated compared with given 3 consecutive doses ($p<0.05$). Artesunate was more effective when administered at 2-week than at 3-week intervals, although the difference was not statistically significant. The reduction in oviposition of all groups given artesunate was statistically significant compared with the control group (Table 2) ($p<0.01$). The diminished egg production indicated a pathological improvement, because morbidity is mainly due to eggs being deposited in the liver and other organs. Mice that did not receive artesunate developed apparent hepatomegaly, whereas no changes were evident in infected mice given artesunate compared with uninfected mice (Table 2). Histological observations revealed that liver sections from mice treated with artesunate contained neither eggs nor circumoval granuloma (data not shown).

3.3. Efficacy of artesunate in iron-deficient mice

After 50 days of feeding with an iron-deficient diet, the mean serum iron concentration in mice decreased to 63 ± 12 μg/dl, compared with 272 ± 19.9 μg/dl in mice fed with a normal diet. Table 3 shows that worms were not recovered from either normal or iron-deficient groups treated with artesunate, while the worm burden was high in non-treated mice. The worm burdens in mice fed with iron-deficient and normal control diets did not differ. These results indicated that a serum iron-deficiency at the level tested here did not influence the efficacy of artesunate against *S. mansoni* infection.

3.4. Electron microscopic observation

Scanning electron microscopy revealed that artesunate induced damage mainly on the worm tegument. Fig. 2a and b shows normal tubercles with small sharp spines on the tegument of control 8-week-old adult worms and alterations on the tubercles of 8-week-old adult worms treated with artesunate, respectively. The tubercles on the surface of male worms were retracted and small, and the sharp spines were shortened or absent, although the sensory structures were unaffected. The suckers of both male and female worms were damaged and collapsed (Fig. 2c) and the tegument ridges were focally swollen and fused (Fig. 2d). Host leukocytes adhered to the damaged tegument, although cellular characterization of those leukocytes was not clarified (Fig. 2e).

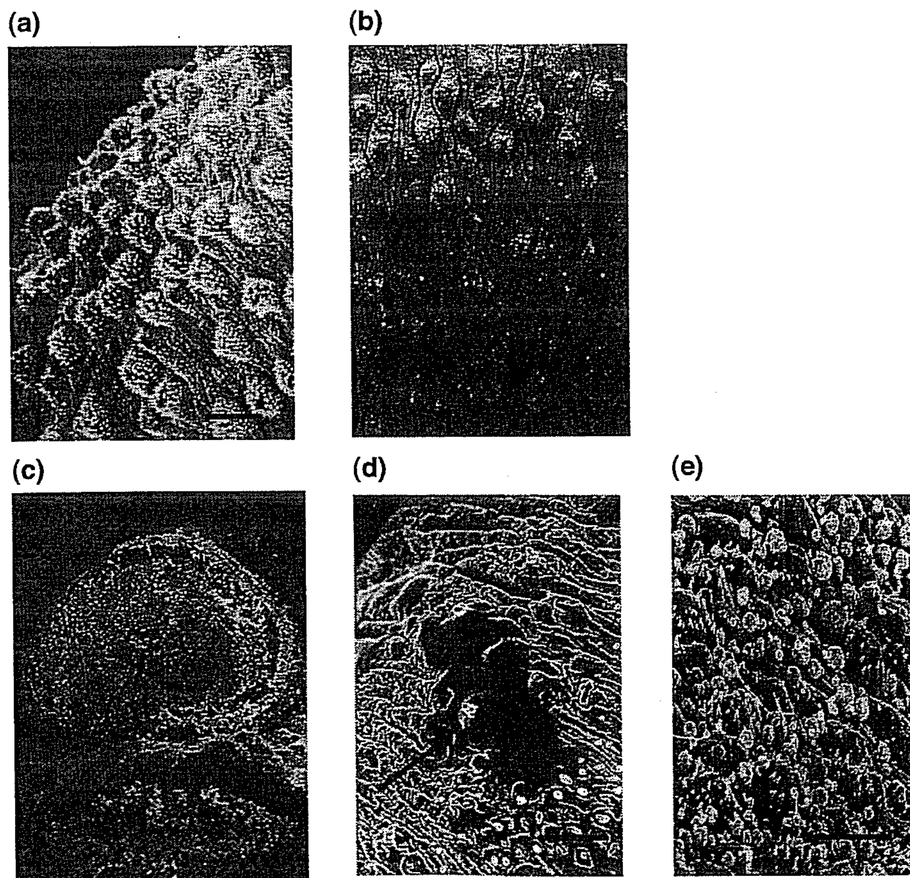

Fig. 2. Morphological damage observed by scanning electron microscopy. Worms were collected 8 weeks after infection from mice treated with artesunate at sub-optimal dose (100 mg/kg) on 14 days PI. (a) Normal tubercles on tegument of male worms from control group. (b) Alterations on tubercles of male worms from group given artesunate. (c) Damage to the oral sucker of treated group. (d) Focal swelling and fusion of tegumental ridges of treatment group (arrow). (e) Host leukocytes adhered to surface of parasites treated with artesunate, although cells were not characterized. Bars: 100 μm.

Table 3
Effect of artesunate on *S. mansoni* infection in iron-deficient or normal mice

Group	N	Fe-deficient	Artesunate[a]	Total worms recovered	WRR (%)	No. of female worms	FWRR (%)
F-1	6	+	–	24.8±3.9		13.3±2.3	
F-2	6	+	+	0*	100	0	100
N-1	6	–	–	28.8±9.1		14.0±4.0	
N-2	6	–	+	0*	100	0	100

All mice were sacrificed 70 days PI.
[a] Artesunate was treated at the dose of 300 mg/kg on day 14–21–28–35 PI.
* $p<0.01$ vs artesunate non-treated groups.

3.5. Inhibitory effects of artesunate on egg production in vitro

The number of eggs and degree of egg maturation were compared between adult pairs of *S. mansoni* recovered from mice with or without artesunate treatment. Worm pairs from control mice produced significantly more eggs in vitro than those from mice given a sub-optimal dose of artesunate (45.0±3.6 vs 12.3±1.8 eggs/pair, $p<0.01$). Over 80% of eggs produced by worms from control mice were mature and/or normally developed, whereas worms from mice treated with artesunate did not produce any mature eggs in vitro.

4. Discussion

The present study found that artesunate prominently affected the immature stages of both *S. mansoni* and *S. japonicum* but at different levels of efficacy, since *S. mansoni* was the most susceptible to artesunate 1 or 2 weeks later than *S. japonicum* in a previous study in mice [21]. The lung stage schistosomula of *S. japonicum* were the most susceptible to artesunate [21], whereas immature adult *S. mansoni* worms at the post-lung stage were most affected by the drug. This coincides with their growth profiles: *S. mansoni* takes approximately one week longer than *S. japonicum* to develop from the schistosomula to the adult stage. A common metabolic profile, which is sensitive to artesunate, might be expressed in the highest level at a particular developmental stage of schistosome parasites, but there might be a time lag of one week between the two parasite species. Our results regarding the effectiveness of artesunate are equal to the efficacy of artemether reported by Xiao et al. [29]. The difference in developmental time course could explain the different time schedule for effective drug prescription between *S. mansoni* and *S. japonicum*. To combat *S. japonicum* infection, the first medication should be given 7 days after infection and 3 consecutive administrations once a week are recommended [21], although early diagnosis method should be developed when we implement the protocol. For *S. mansoni*, drug administration starting 14 or 21 days after infection followed by 3 repeated doses at 2-week intervals provided optimal protection. Considering the lengthy exposure period in heavy endemic areas, a treatment regimen with long intervals might be practical for field application. Other morphological observations have shown that damage caused by a single administration of artemether recovers within one week [24], indicating a need for repeated administration.

In addition to immature worms, artesunate also seems to kill mature adults, because the WRR was enhanced when additional doses were administered at 8 or 9 weeks after infection (Group 4 vs Group 2, or Group 7 vs Group 5: Table 1). The mechanism of the effects of artesunate has not been defined. Electron microscopy showed that artesunate caused morphological changes in *S. mansoni*, especially tegument damage similar to that caused by artemether [18,20,30]. The adhesion of host leukocytes to the surface of the parasites suggests that parasite antigen is released and these cells could cause immune-mediated damage, although no direct evidence is available.

Artesunate not only diminished the number of eggs produced, but also retarded their maturation. Although the reduced worm burden and inhibition of sexual maturation might be responsible for preventing the development of hepatic lesion, one study has shown that artesunate protects against liver injury induced by acetaminophen and carbon tetrachloride by stimulating hepatic-metabolizing enzymes [31]. Artesunate also inhibits the growth of hepatocellular carcinoma by inducing cancer cells to undergo apoptosis and by increasing topoisomerase activities [32,33]. We speculate that the schistosomicidal effects suppressed hepatic lesions, but an additional mechanism of hepatic cell protection conferred by artesunate could not be ruled out.

Iron-dependent free radicals generated by interaction between artemisinin and a high concentration of heme generated by extensive hemoglobin digestion might be toxic for the parasite [26]. Iron is an important factor for artesunate action. Mansour et al. suggested that chronic *S. mansoni* infection is associated with a high incidence of latent iron-deficiency [34]. Artesunate exerted striking schistosomicidal effects against *S. mansoni* in iron-deficient mice at levels comparable to those in control mice with normal iron levels. We thus conclude that even a severe iron-deficiency would not influence the efficacy of the artesunate.

Among several artemisinin derivatives, artesunate and artemether are usually used as anti-malarial drugs. Artesunate is less toxic than artemether [35], but a recent study has reported that artemether is more effective than artesunate against *S. mansoni* [25], although the experimental design in these studies and artesunate lots in the present study differed. Further studies are required to conclude which should be recommended for use as an anti-schistosome drug in humans.

In conclusion, artesunate is a promising prophylactic and therapeutic agent with which to combat *S. mansoni* infection,

and human trials should be implemented in the near future. In view of the short half-life of artesunate, parasite will probably not develop tolerance or resistance. Nevertheless, artesunate should be extensively applied in areas where malaria and schistosomiasis are co-endemic to minimize any latent risk of resistance.

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BEHAVIORS ASSOCIATED WITH WATER CONTACT AND *SCHISTOSOMA JAPONICUM* INFECTION IN A RURAL VILLAGE, THE DONGTING LAKE REGION, CHINA

Shouhei Takeuchi[1,2], Yuesheng Li[3], Yongkang He[3], Huan Zhou[1],
Kazuhiko Moji[2], Ryutaro Ohtsuka[4], Chiho Watanabe[1]
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Abstract: Although identification of water contact patterns is one of the most important factors for the prevention of *Schistosoma japonicum* infection, it is still insufficient for clarifying specific high-risk behaviors and their implications. Parasitological studies and behavioral observations were carried out in a rural village, the Dongting Lake region, China. A time-allocation study conducted by a time-saving spot-check method was implemented to quantify the behavioral risks. Of the 122 participants, 18 (14.8%; 95% confidence interval: 8.5, 21.0) were positive for *S. japonicum*. Among those diagnosed, the median (25−75% quartile) eggs per gram was 8 (8−16). A significant positive correlation with worm intensity was found among people who repair ships on the marshland (p<0.001), and this potential risk was consistent with previous suggestions. Although the parasitological techniques and study design require further improvements, our observational methods may be of use to explicitly identify behaviors at the local level that could be relevant to prevention.
Key words: *Schistosoma japonicum;* Schistosomiasis; Water microbiology; Environmental exposure; Behavior; China

1. INTRODUCTION

Schistosomiasis japonica, which is caused by the helminth *Schistosoma japonicum,* is a parasitic zoonosis with more than 40 species acting as definitive hosts [1]. The World Health Organization (WHO) estimates the global number of cases of schistosomiasis due to *Schistosoma* spp. at 200 million, among which 120 million are symptomatic. Moreover, the report states that about 600 million individuals may be at risk worldwide [2]. Since *Oncomelania* snails are still present in numerous areas, and because cattle and buffaloes frequently harbor the infection, schistosomiasis control is a serious challenge even in the 21st century [3].

In mainland China, schistosomiasis japonica remains a major public health problem in eight provinces. Although schistosomiasis gradually decreased after the 1950s and four of 12 provinces succeeded in eliminating the disease, *S. japonicum* was still endemic in 240 counties, with 44 million individuals estimated to be at risk as of 1989 [4]. According to a more recent survey in 2001, 0.8 million humans and 31,500 buffaloes were still infected in China, where the snail habitat area covers 3,436 km^2 [5].

As the disease is mainly transmitted through contact with infected water, it is of practical importance to identify the most risky behaviors and adopt specific preventive measures. In particular, identification of specific water contact at the local level will contribute to reducing the potential risks. Previous studies explored behaviors associated with water contact by means of questionnaires [6,7] and activity diaries [8-10]. But these do not sufficiently clarify behavioral characteristics, and more detailed quantifications, based on direct observation, are required. In this study, we conducted a cross-sectional behavioral study in a rural village, the Dongting Lake region, and analyzed the behavioral risk factors for *S. japonicum* infection that would be relevant for the design of local lifestyle-oriented prevention programs.

2. MATERIALS AND METHODS

2. 1. Study area

Parasitological studies and behavioral observations were carried out in a rural village located in the area called the Dongting Lake region, Hunan Province. Located about

[1]Department of Human Ecology, School of International Health, Graduate School of Medicine, the University of Tokyo, Japan
[2]Research Center for Tropical Infectious Diseases, Nagasaki University Institute of Tropical Medicine, Japan
[3]Hunan Institute of Parasitic Diseases, Hunan, China
[4]National Institute for Environmental Studies, Tsukuba, Japan