2.5. Antibody ELISA

Recombinant As24-specific serum IgG, IgE and IgG-subclass antibodies (IgG1, IgG2a, IgG2b and IgG3) in vaccinated mice were measured by ELISA (Tsuji et al., 2003). IgG and IgG-subclass antibody levels were determined with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and IgG-subclass antibodies (Bethyl Laboratories Inc, Montgomery, TX). Affinity purified goat anti-mouse IgG, IgG1, IgG2a, IgG2b and IgG3 (Bethyl) were used as standards. For rAs24-specific IgE measurement, anti-mouse IgE was used as a capture antibody, and IgE was detected by using biotinylated anti-mouse IgE (Bethyl). Rat monoclonal anti-mouse IgE antibody diluted 1:10,000 was used as the standard (American Research Products, Belmont, MS).

ELISAs were performed in 96-well ELISA plates (MS-8496F; Sumitomo, Tokyo, Japan) in 50 μl reactions, with rAs24 antigen coated at 2 μg/ml of 0.1 M carbonate buffer, pH 9.6. The plates were incubated at 4 °C for 14 h and washed three times with PBS containing 0.05% Tween 20 (PBS-T). Wells were blocked with 100 μl of PBS-1% bovine serum albumin (Sigma) for 1 h at 37 °C. After the wells were washed five times with PBS-T, serial dilutions of the serum were added, followed by incubation at 37 °C for 1 h. After the incubation, the wells were washed five times with PBS-T, and 100 μl of HRP-conjugated anti-mouse IgG, IgE or IgG subclass antibodies were added to the wells. The plates were incubated at 37 °C for 1 h and washed five times with PBS-T. Mouse sera were diluted 1:1000. Conjugates were used at 1:10,000. Both test sera and conjugates were diluted in PBS (pH 7.5). Detection of antibody was performed at 37 °C with 100 μl of 2,2′-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) substrate solution (ABTS; Kirkegaard and Laboratories, Gaithersburg, MD), and the coloring reaction was terminated with 100 μl of 1% SDS. Plates were read at 405 nm in a microplate reader (Spectrafluor; Wako, Tokyo, Japan). Concentration of antibodies was calculated by reference to standard curve generated by using reference sera (Bethyl).

2.6. Spleen cell culture

Spleen cell suspensions were prepared as described previously (Tsuji et al., 2003). Briefly, spleens were cut into small pieces and minced through a sterile stainless steel mesh (Sigma) and suspended in cool Hank's balanced salt solution (HBSS) (GIBCO/BRL) supplemented with 5% (v/v) heat-inactivated FCS (Sigma) and antibiotics (penicillin and streptomycin, 100 μg/ml). Erythrocytes in cell suspensions were lysed by 0.2% sodium chloride. The suspended cells were washed three times in HBSS by centrifugation at $500 \times g$ at 4 °C for 5 min each. Finally, the spleen cells, mostly T lymphocytes were resuspended in complete RPMI 1640 (GIBCO/BRL), supplemented with heat-inactivated 10% (v/v) FCS, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, 1 mM non-essential amino acid, 1 mg/ml 5-fluorocytosine and 5×10^{-5} M 2-mercaptoethanol. The concentrations of splenic T cells obtained from individual mouse were adjusted to 4×10^6 cells/ml of complete RPMI 1640. For in vitro stimulation, splenic cells were seeded out on 96-well flat-bottomed tissue culture plates (MS-8096F; Sumitomo) in a volume of 100 μl per well. The cells were stimulated alone or with rAs24 antigens (10 μg/ml) for 72 h at 37 °C in a humidified atmosphere containing 5% CO_2, supernatants were removed and frozen at −80 °C until assayed for cytokine content.

2.7. Cytokine ELISA

Cytokines produced by activated spleen cells were measured by standard sandwich ELISA using purified anti-IL-4, IL-10, IL-2 and γ-IFN as capture antibody and the corresponding biotinylated antibody as reporter antibody according to manufacturer's instructions (eBioscience, San Diego, CA). The concentrations of primary capture antibody and biotinylated reporter antibody were used according to protocol (eBioscience). Purified recombinant IL-4, IL-10, IL-2 and γ-IFN were used as standards (eBioscience). TMB microwell peroxidase was used as substrate and the reaction was terminated by 1 M H_3PO_4. Absorbance of wells was read at 450 nm in a microplate reader (Spectrafluor; Wako, Tokyo, Japan). Cytokine levels were calculated by reference to standard curves constructed from supernatants containing known amounts of each molecule.

2.8. In vitro moulting assay for A. suum lung-stage larvae

A. suum lung-stage L3 obtained from an infected rabbit were cultured in vitro in the presence of affinity purified anti-rAs24 mouse IgG to evaluate the possible in vivo functions of endogenous As24 proteins in the moulting processes of the parasites (Islam et al., 2003). Briefly, 50–100 *A. suum* lung-stage L3 were cultured in 1 ml RPMI 1640 (GIBCO/BRL), pH 6.8 supplemented with 10% (v/v) fetal bovine serum (Sigma) and antibiotics (penicillin and streptomycin, 100 μg/ml) in 24-well flat-bottomed tissue culture plates. The cultures were incubated at 37 °C, in humidified 5% CO_2 in the presence of increasing concentrations of anti-rAs24 mouse IgG and mouse preimmune IgG (as control) for 10 days. The number of L4 which had moulted from L3 in the culture well was determined as previously described (Islam et al., 2003).

2.9. Localization of endogenous As24 in A. suum lung-stage larvae by immunofluorescence

To localize the As24 endogenous proteins, *A. suum* lung-stage L3 obtained from an infected rabbit were washed in

cool PBS containing 0.1% Tween 20 (PBS-T) three times and fixed in cool acetone for 2 min. After washing in PBS-T, the fixed larvae were blocked in 10% goat serum for 1 h and incubated with primary anti-rAs24 antibody (1:20) for 2 h at room temperature. The larvae treated with mouse pre-immune sera (1:20) were served as control. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma) was used as secondary antibody at a 1:100 dilution. After labeling, larvae were washed in PBS-T and whole larvae were mounted on slides with 70% glycerol. The larvae were analyzed and photographed with an Axiophot fluorescence microscope (Carl Zeiss, Jena, Germany) using appropriate filter sets.

2.10. Statistics

The data are expressed as mean ± SD for the experimental groups. The statistical significance ($P<0.05$) was determined by Student's t test.

3. Results

3.1. Protective effect of recombinant As24

Protective efficacy of rAs24 molecule against *A. suum* lung-stage infection was investigated in BALB/c mice received one primary vaccination and two boosters followed by an oral challenge 1 week apart with 2500 *A. suum* infective embryonated eggs. Data showed that mice vaccinated with rAs24 in FCA had a significant ($P<0.001$) reduction in recovery of challenged *A. suum* lung-stage L3 (55.2 ± 20.23) compared with either non-vaccinated controls (132 ± 22.49) or mice vaccinated with FCA alone (112.8 ± 34.43) (Fig. 1). To test whether mice protective immunity could also arrest larval development, we measured 50 challenged *A. suum* lung-stage L3 from mice vaccinated with rAs24-FCA, FCA alone and from non-vaccinated controls for body length and body width. We recorded a stunted development in body length (897 ± 114.80 μm) and body width (51.60 ± 6.83 μm) of challenged *A. suum* larvae recovered from rAs24-FCA-vaccinated mice compared with body length and body width of those grown either in the non-vaccinated controls (1254.50 ± 103.96 μm × 59.65 ± 8.76 μm) or in mice vaccinated with FCA alone (1145.5 ± 133.70 μm × 56.65 ± 6.58 μm). Lungs removed from vaccinated mice showed significantly reduced verminous pneumonitis (pulmonary haemorrhages) following challenge infections compared with the non-vaccinated controls (data not shown). Moreover, histopathological sections of these lungs (not from non-vaccinated controls) revealed fewer red blood cells and no eosinophils; however, mononuclear cells (for example, lymphocytes, macrophages, etc.) were never seen following infections (data not shown).

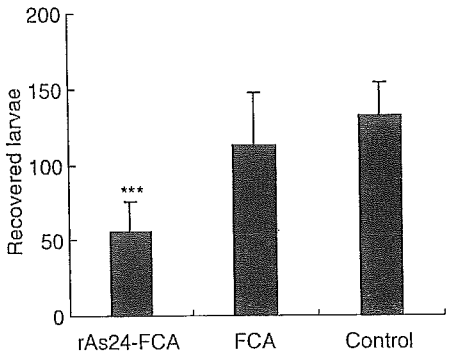

Fig. 1. Number of *Ascaris suum* lung-stage larvae recovered from mice vaccinated with recombinant As24 in Freund's Complete Adjuvant. Mice were vaccinated and then challenged with 2500 *A. suum* infective embryonated eggs 1 week after the final vaccination. Mice were sacrificed 1 week after challenge and the lung-stage larvae were recovered. The results are expressed as mean ± SD in each group of five mice. Asterisks indicate that the mean value was significantly lower than that of non-vaccinated controls and group vaccinated with FCA alone. ***$P<0.001$.

3.2. Serum antibody responses to recombinant As24

To assess the potential of rAs24 molecule in eliciting host-protective antibody, we measured rAs24-specific total IgG, IgE and IgG-subclass antibodies in protected mice sera. Results showed that mice vaccinated with rAs24-FCA displayed a significantly ($P<0.0001$) high level of rAs24-specific IgG compared with either non-vaccinated controls or mice vaccinated with FCA alone (Fig. 2). Antigen-specific IgE responses were not seen in these mice sera (data not shown), indicating that rAs24 may be a non-allergenic molecule for the host. To further assess the efficacy of rAs24 in induction either of an in vivo Th1 or Th2 response, IgG-subclass antibody levels were measured. Our results demonstrate that rAs24-FCA vaccination induced a mixed pattern of IgG1 ($P<0.0001$), IgG2b ($P<0.005$), IgG3 ($P<0.04$) and IgG2a ($P<0.006$) responses (Fig. 2).

3.3. Cytokine responses to recombinant As24

We examined the cytokine levels in rAs24-stimulated splenic cell culture supernatants to determine the T cell response to rAs24 molecule in vaccinated mice. The in vitro-stimulated splenic T cells from rAs24-FCA-vaccinated mice exhibited a significantly high level of Th2-type cytokine IL-10 ($P<0.001$); however, only a modest increase of IL-4 ($P<0.09$) level was observed compared with stimulated T cells from both FCA-vaccinated and non-vaccinated control groups (Fig. 3). Interestingly, stimulated T cells from rAs24-vaccinated mice also showed a significantly increased level of γ-IFN ($P<0.005$) but not IL-2 ($P<0.11$) (Th1-type cytokines) compared with those from non-vaccinated controls and FCA-vaccinated animals (Fig. 3). These data suggest that rAs24 molecule induces a Th1/Th2-mixed type immune response in mice.

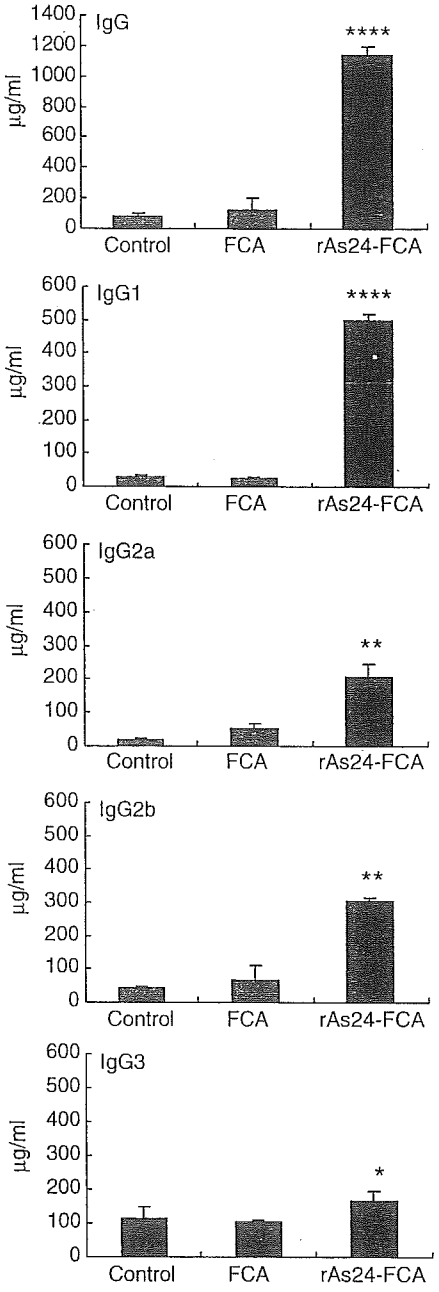

Fig. 2. Serum IgG and IgG-subclass responses in mice vaccinated with recombinant As24 in Freund's Complete Adjuvant. Mice were vaccinated and sacrificed 2 weeks after the final vaccination. The values are expressed as mean ± SD for each group of five mice. $^{*}P<0.05$, $^{**}P<0.01$, $^{****}P<0.0001$.

3.4. In vitro moulting inhibition of A. suum lung-stage larvae by anti-rAs24 IgG

We hypothesized that if the endogenous As24 protein plays role in the moulting processes of *Ascaris* parasites, then its neutralization by specific antibody would inhibit larval moulting process. The experimental findings showed that affinity purified anti-mouse IgG inhibited moulting of *A. suum* lung-stage L3–L4 in vitro by 26% at a maximum concentration of 20 µg/ml culture medium (Fig. 4), larval

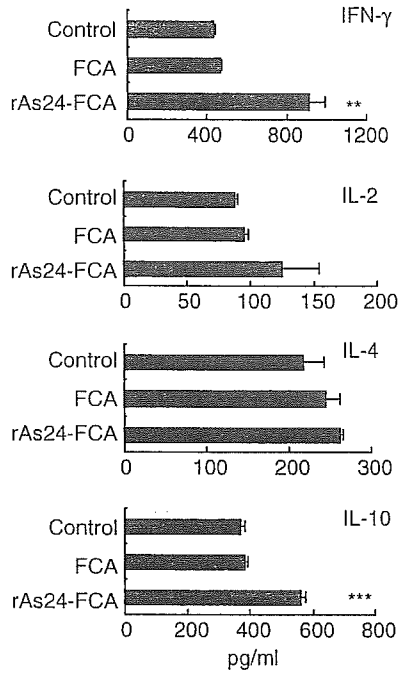

Fig. 3. Th1- and Th2-type immune responses in rAs24-vaccinated mice. Splenic T cells were isolated from mice 2 weeks after the final vaccination. Th1-type (i.e. γ-IFN and IL-2) and Th2-type (i.e. IL-4 and IL-10) cytokines were measured by cytokine-specific ELISA of cell culture supernatants from rAs24-FCA vaccinated, FCA alone and non-vaccinated control at 72 h in vitro stimulation with 10 µg/ml rAs24. The results are expressed as mean ± SD for each group of five mice. Each sample was examined in triplicates. $^{**}P<0.01$, $^{***}P<0.001$.

viability was, however, remained unaffected (data not shown). These results suggest a partial role for endogenous As24 proteins in the moulting processes of *Ascaris* larvae. The mean moulting percentage in control culture (in the presence of mouse pre-immune IgG) was recorded as 54.85 ± 6.28.

3.5. Localization of endogenous As24 in A. suum lung-stage larvae by immunofluorescence

To localize the As24 endogenous proteins, *A. suum* lung-stage L3 were reacted with anti-rAs24 primary antibody,

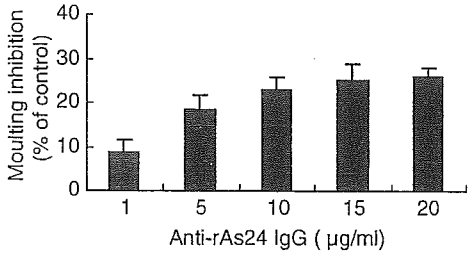

Fig. 4. Moulting inhibition of *Ascaris suum* lung-stage L3–L4 in vitro by anti-mouse rAs24 IgG. *Ascaris suum* lung-stage L3 obtained from an infected rabbit were cultured in serum supplemented RPMI 1640 in the presence of affinity purified anti-mouse rAs24 IgG as described in Materials and methods. Moulting of L3–L4 was evaluated at day 10 post-culture. Moulting percentages are relative to the control in the presence of mouse preimmune IgG (100%). Moulting percentage of control culture was 54.85 ± 6.28. The results are expressed as mean ± SD of triplicates.

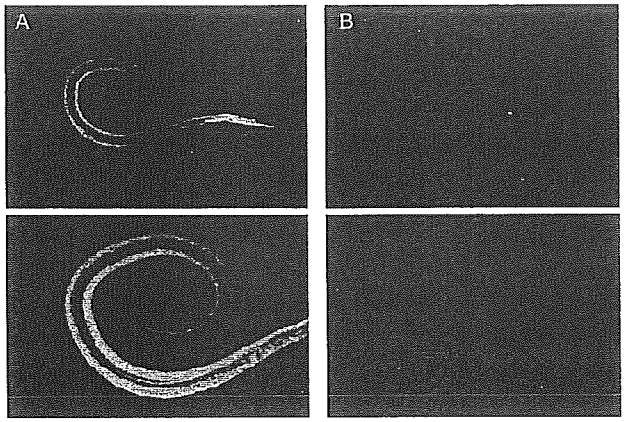

Fig. 5. Localization of endogenous As24 in *Ascaris suum* lung-stage L3 by immunofluorescence. Immunofluorescent staining of whole mounted lung-stage larvae was performed. (A) Lung-stage larvae were washed in cool PBS-Tween 20 (0.1%), fixed in acetone and incubated with anti-rAs24 primary antibody (1:20). FITC-labeled goat anti-mouse IgG was used as a secondary antibody. (B) Lung-stage larvae incubated with mouse preimmune sera (1:20) were regarded as control. Mouse anti-rAs24 antibody bound to the endogenous As24 proteins localized in the hypodermis along the whole body length and in the gut epithelial tissues of *A. suum* lung-stage larvae were observed under an Axiophot fluorescent microscope using appropriate filter sets and photographed (upper panel magnification×80, lower panel magnification×160).

and then incubated with FITC-labeled goat anti-mouse IgG as secondary antibody. Immunofluorescence microscopy revealed the bound antibodies in the hypodermis along the whole body length and in the gut epithelial tissues of anti-rAs24 antibody-treated *A. suum* lung-stage L3 (Fig. 5A). In contrast, no reactivity was seen in the larvae treated with mouse pre-immune sera (Fig. 5B).

4. Discussion

Previous studies from our laboratory have shown that mice immunized with *A. suum* L3 are efficiently protected (99%) against challenged *A. suum* migrating larvae (Tsuji et al., 2001). Protection was typically measured by recovering migrating larvae that reach to the lungs after challenge infections. Other studies from the same laboratory have revealed that intranasal immunization of mice with *E. coli*-expressed recombinant 14-kDa and 16-kDa antigens from *A. suum* can induce a significant level of protection (58–64%) against parasite challenge (Tsuji et al., 2001, 2003). In the present study, we evaluated whether a nematode-specific recombinant As24 protein from *A. suum* could be used as a potential vaccine candidate for the control of *Ascaris* roundworm infections in *A. suum*-mouse model. Encouragingly, our data showed that immunization of BALB/c mice (one primary immunization and two boosters) with rAs24 coupled with FCA results in protection against challenged *A. suum* lung-stage L3. Protection was manifested by a significant reduction (58%) in the recovery and stunting of *A. suum* lung-stage L3 at day 7 post-challenge compared with either non-immunized controls or mice immunized with FCA alone (Fig. 1). Recombinant As24-FCA immunization also results in a significant decrease in verminous pneumonitis (data not shown). It was shown that when *A. suum* L3 were administered orally to mice, the larvae penetrate the gastrointestinal tract after approximately 24 h, and the administered larvae reach the lungs, where they cause verminous pneumonitis after 72 h (Slotved et al., 1998). Mice vaccinated orally with *A. suum* L3 were found to be protected against verminous pneumonitis after challenge infections (Guerrero and Silverman, 1972; Tsuji et al., 2001). In a recent report, Girod et al. (2003) showed that mice vaccinated with γ-irradiated larvae of human hookworm *Necator americanus* had reduced pulmonary larval burdens and haemorrhages compared with the non-vaccinated controls following challenge infections. Our results indicated that subcutaneously administered rAs24 in FCA efficiently induced protective immunity to *A. suum* lung-stage larval infection.

Sera obtained from protectively immune mice had high levels of antigen-specific IgG antibody. Further, analysis of IgG-subclass profiles revealed that anti-rAs24 IgG1 showed the greatest increase followed by IgG2b, IgG2a and IgG3, suggesting that rAs24-FCA immunization resulted in a Th1/Th2-mixed type protective immunity. Interestingly, IgE responses were not detected in these mice, suggesting that immune responses to rAs24 are not mediated by hypersensitivity reactions. Although several studies have shown that parasite-specific IgG and IgE responses are associated with protective immunity to human ascariasis (McSharry et al., 1999; Cooper et al., 2000; Geiger et al., 2002), the protective function of these antibodies are yet to be defined. Our data show that protection against challenge by *A. suum* larvae is associated with high levels of all IgG-subclasses examined. However, we did not perform passive immunization with pooled sera from rAs24-FCA immunized mice to examine the potential of antibody in blocking *A. suum* larval migration. Recently, Frontera et al. (2003) reported that acquired resistance against *A. suum* larvae is correlated with the induction of IgG1 and IgM, and not with IgG2; rAs24-specific IgM, however, was not examined in the present study. Our results are in agreement with the report of Ghosh and Hotez (1999) who demonstrated that dog hookworm *Ancylostoma caninum* burden reduction from alum-precipitated recombinant *A. caninum* secreted protein 1 immunization is associated with elevations in all IgG-subclasses but the greatest rise observed in host IgG1 and IgG2b. These authors also showed that *A. caninum* burden reduction is antibody-dependent.

The effector mechanisms that induce stunted development and prevent migration of *A. suum* L3 being reached to the lungs after exit through host's gut wall remain poorly understood. It is reasonable to assume that anti-rAs24 IgG may penetrate the cuticle of *A. suum* larvae during the course

of their liver-lung migration, and mediate neutralization of As24 endogenous protein which has shown to be highly expressed in hypodermis and gut epithelium of *A. suum* lung-stage L3 (Fig. 5), and thus may interfere with larval migration and/or growth and development. Data obtained on moulting inhibition by anti-rAs24 antibody (Fig. 4) support such an assumption. Studies have shown that the nematode cuticle is a dynamic structure with important absorptive, secretory and enzymatic functions (Maizels et al., 1993) and not merely an inert covering as was once believed. The absence of eosinophils and mononuclear cells (e.g. macrophages) in the lung tissues of rAs24-immunized mice (data not shown) where migration by L3/L4 occurs for 2–3 days indicates that damage and/or killing of target *A. suum* larvae may not be induced by macrophages, further supporting our hypothesis of antibody-mediated protection described above. The immune mechanisms that operate against adult stage worms, however, were not examined in the present study. It has been reported that immune responses against tissue-dwelling helminths are different from those against gastro-intestinal parasites (Garside et al., 2000; Hoffmann et al., 2000).

Immunoglobulin isotype-specific responses seen in rAs24-FCA-immunized mice are consistent with cytokine profiles that in vitro stimulation of splenic cells from immunized mice exhibited significant levels of Th1-type cytokine γ-IFN but not IL-2, together with Th2-type cytokine IL-10 levels. However, the level of IL-4, a Th2-type cytokine was only modest. These data suggest that As24 in FCA triggers a Th1/Th2-mixed type of immune response. These findings support the observations with a recombinant 16-kDa antigen from *A. suum* (Tsuji et al. 2003). It is proposed that a high level of γ-IFN production may suppress IL-4 production by splenic $CD4^+$T cells in mice subcutaneously immunized with rAs24-FCA, since γ-IFN and IL-4 affect each other as antagonists (Pene et al., 1988). On the other hand, the reason for a poor release of IL-2 is unclear. It is reasonable to think that a reduced synthesis of IL-4 in rAs24-immunized mice may account for little or no IgE response and tissue eosinophilia (see Finkelman et al., 1991).

To determine the functional significance of anti-rAs24 IgG in the moulting processes of *Ascaris* parasites, a previously developed bio-assay that allows moulting and development of *A. suum* lung-stage L3–L4 in vitro to be observed was employed (Islam et al., 2003). Our data show that neutralization by anti-rAs24 IgG of As24 endogenous proteins results in partial inhibition of moulting (26%) of *A. suum* lung-stage L3–L4 in vitro (Fig. 4). The anatomic location of As24 endogenous proteins in the hypodermis along the whole body length of *A. suum* lung-stage L3 (Fig. 5) supports such a role. Since the hypodermis of parasitic nematodes allows synthesis of components of the new cuticle and thus plays an important role in the moulting processes (Lee, 2002). Recently we observed that *A. suum* inorganic pyrophosphatase is highly expressed in the hypodermis of both adult and larval worms and is associated with molting and development processes of *A. suum* lung-stage L3–L4 (unpublished data). Furthermore, an intense expression of As24 endogenous proteins in the gut epithelium of *A. suum* larvae (Fig. 5) indicates that the protein may have functional role in the metabolic processes of *Ascaris* parasites.

In summary, the results with this vaccine formulation provides insights into rAs24-induced mechanisms that trigger a Th1/Th2-mixed type of response which may be important in host-protective immunity against *A. suum* larvae in the lung. This may assist in the development of a safe vaccine against ascariasis.

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INVESTIGATION ON THE EPIDEMIOLOGICAL FACTORS OF *CLONORCHIS SINENSIS* INFECTION IN AN AREA OF SOUTH CHINA

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Abstract. To detect the epidemiological factors of *Clonorchis sinensis* infection in Hengxian County, one of counties in Guangxi Zhuang Autonomous Region where *Schistosoma japonicum* was endemic but eliminated in the late 1980s, a questionnaire was designed with 37 questions covering socioeconomic conditions and human behavior, contamination of the environment and fish ponds, inadequate farming/fishery practices; and the formalin-ether sedimentation technique was used to examine the feces of cats, dogs and pigs for the eggs of *C. sinensis*. Fresh fish was sliced and digested to detect the metacercariae of *C. sinensis* under a stereomicroscope. Of 1,521 people interviewed, 64% of the interviewees did not know about fluke disease or its transmission route, 46% of those who knew about the fluke believed that the infection caused no harm or only slight harm to their health. More than half of the interviewees (51%) ate raw fish at least 1-2 times per month, more among the middle-aged males. Eight percent of them used the same utensils for both raw fish and cooked food. When advice was given not to eat raw fish, 73% of the interviewees thought it was not feasible. In relation to pisciculture, 25% and 9% of the owners of fish ponds fed their fish with feces of domestic animals and human feces, respectively. The prevalences of *C. sinensis* infection in cats, dogs and pigs were 70, 50 and 27%, respectively, and the infection rate in fish was 40%. These results indicate the poor knowledge, and beliefs, and unhealthy behaviors in the human population. They also show poor environmental hygiene and inappropriate farming/fishery practices are important in the increase of *C. sinensis* prevalence in humans. Combined interventions, including health education, environmental modification, reform of traditional farming/fishery practices, mass screening and chemotherapy for humans, and the management of domestic animals are needed in developing control strategies to decrease *C. sinensis* infection in the human population.

INTRODUCTION

The Guangxi Zhuang Autonomous Region is one of the *Schistosoma japonicum* endemic provinces in China, which has been successful in eliminating the disease in both the human and cattle populations since 1989 (Sleigh *et al*, 1998). However, other helminthes are highly prevalent in the Region due to relatively poor socioeconomic development, particularly in the areas of ethnic populations. Hengxian County is one of ethnic counties in Guangxi Province where schistosomiasis was prevalent before 1989. In surveys of human parasites carried out in 1989 and 2002, the average prevalences of ascaris, hookworm and trichuris infection decreased from 71, 34 and 55% to 15.9, 7.5 and 12.8%, respectively while those of *Clonorchis sinensis* infection increased from 18% to 31.5%(Yu *et al* ,2003;Lin *et al*, 2004). The prevalence of intestinal helminthes decreased due to a chemotherapy campaign, especially in schoolchildren, and an improvement in living standards, environmental hygiene, and health literacy among local population, which also helped to reduce the transmission of soil-transmitted helminthes. However, the *C. sinensis* infection has been increasing in the past decade.

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In order to understand the factors relative to the transmission of *C. sinensis* so as to provide a basis for developing control intervention strategies for preventing transmission, an epidemiological study in Hengxian County was proposed. This paper reports the findings of that survey regarding the epidemiological factors for *C. sinensis* infection.

MATERIALS AND METHODS

Areas and subjects

Hengxian County was selected for the investigation, where the earlier survey was conducted in 2002. Three spots (villages) were identified for the study, the entire population in each place, about 500 people, were interviewed. Meanwhile, fecal materials from cats, dogs and pigs were collected to examine for the eggs of *C. sinensis*. Fishes were caught from the ponds /canals for metacercariae detection.

Methods

A questionnaire was designed with 27 questions covering demographic information, knowledge regarding liver flukes, fish-eating habits, source of fish and processing, and history and attitudes regarding the disease. Nine questions were added for fish pond owners and one more question for restaurant owners and cooks. The answers to the questionnaire were confidential. All the interviewers were briefly trained before the interviews.

The formalin-ether sedimentation method was used to examine the feces of cats, dogs and pigs for the eggs of *C. sinensis*. The fish specimens were sliced and digested with artificial gastric juice in a 37°C incubator for 12 hours to examine for the metacercariae of *C. sinensis* under a stereomicroscope. SPSS software and the χ^2 test were used for statistical analysis.

RESULTS

Questionnaire

The numbers of residents who received questionnaire were 520, 497 and 504 in Shitang, Xiaoyi and Lingzhu, respectively. Only 36% of the 1,521 interviewees showed a knowledge of clonorchiasis; 32% knew the mode of transmission. Of those who were aware of the fluke, 54% (296/551) believed the infection could be hazardous to people's health, while 46% believed the fluke caused no or only slight harm. Fifty-one percent of those interviewed (773/1521) ate raw fish at least 1-2 times per month, more common in middle-aged males (404/773). Ninety-six percent (81/84) of those who ate raw fish 10 or more times per month were male adults. The major mode of eating fish was as raw fish slices (720/773). The fish most frequently consumed was *Ctenopharyngodon idellus* (grass carp). Most of the subjects ate raw fish at home (689/773). In the restaurants surveyed (33/33), the most favorite dish for the consumers was raw fish slices, according to the cooks and managers.

The survey revealed that 8% of the interviewees (121/1, 521) used the same chopping block, knife and other utensils for both raw fish and cooked food. When questioned about a history of infection, 14% (217/1,521) had been examined, 8% (117/1,521) had received treatment. Thirty-six percent (544/1,521) of interviewees answered that they had no desire for an examination for *C. sinensis* infection. When give advice not eat raw fish/ 73% (1,103/1,521) thought that it was not feasible (Table 1).

A survey of contamination of the environment and fish ponds showed 25% (14/56) of the owners of fish ponds fed their fish with feces of domestic animals and 9% (5/56) with human feces. Within a radius of 50 meters, there were latrines around 36% (20/56) of the ponds, pigpens around 36% (21/56) of them, vegetable plots and other farming land where human nightsoil was used as fertilizer around 48% (27/56) of the ponds. During the rainy season, potentially infectious fecal material (from children, animals) and rubbish could be washed into 68% (38/56) of the ponds. Cleaning the ponds, which may reduce the host snail colonies, was carried out once every two years in 52% (29/56) of the ponds.

The infection of *C. sinensis* in domestic animals and fishes

The prevalences of clonorchis infection in cats, dogs and pigs from the three townships

Table 1
Some behaviors and attitudes regarding clonorchiasis in different age groups.

Age group	No. interviewed	No. with raw fish-eating habit (%)		No. without intent of taking examination (%)		No. believe impossible to follow advice of no raw fish-eating (%)	
0~	12	1	(8)	9	(75)	7	(58)
10~	291	57	(20)	128	(44)	194	(67)
20~	315	151	(48)	119	(38)	230	(73)
30~	326	204	(63)	83	(26)	252	(77)
40~	243	156	(64)	72	(30)	195	(80)
50~	156	93	(60)	59	(38)	105	(67)
60~	178	112	(63)	74	(42)	120	(67)
Total	1,521	773	(51)	544	(36)	1,103	(73)

were 70% (21/30), 50% (16/32) and 27% (8/30), respectively. Three popular species of fishes were caught from the ponds/canals in each of the townships, 10 fish of each species were examined. The metacercaria rate was 30% in *Rhodeus sinensi* and, *Mylopharyngodon piceus*, 43% in *Opsariichthys bidens* and 60% in *Toxabramis houdemeri*, with a total clonorchis infection rate of 40% (35/88).

DISCUSSION

As is well known, the prevalence of *C. sinensis* is affected by natural and social factors, like other parasites. The Guangxi Zhuang Autonomous Region is located in the subtropics with warm weather, abundant rain and rivers/canals, which are highly suitable for the intermediate hosts of *C. sinensis*. Hengxian County, as one of the counties of Guangxi Province, where the Zhuangzu are the majority people who live in that area, have a long history of eating raw fish, especially during festivals.

The survey indicates that more than half of the interviewees eat raw fish more than once per month. The majority of those who ate more frequently were middle-aged males, consistent with the higher prevalence of infection in adult males (Yu *et al*, 2003b). While about two- thirds of the interviewees did not know about clonorchiasis and its transmission route, 46% of those who knew about the fluke believed that the infection caused no harm or only slight harm. So some of them were infected with *C. sinensis* by eating raw fish because of poor knowledge, which the researcher called "unknown-initiative-infected model". Those who were aware of the harm caused by clonorchiasis infection but felt they could not change their habit of eating raw fish, were called by the researcher the "known-initiative-infected model". The former model usually causes a light prevalence, while the latter can cause a heavy prevalence (Zuo *et al*, 1999). Therefore to reduce clonorchis infection, health education is important for improving knowledge of the parasite and changing unhygienic behaviors especially in the former model population.

The fact that 8% of those surveyed used the same utensils for both raw fish and cooked food reveals possible contamination with metacercariae and infection through the utensils. In south China, in some areas, such as Hainan Province, *C. sinensis* is prevalent in the population, with infection rate of 0.4% (Xu *et al*, 1999), mostly due to contamination of utensils rather than eating raw fish (Yu *et al*, 2003a). This indicates that infection through utensil contamination with metacercare may be an important route for clonorchis infection in the human population. The traditional way or feeding fish with the feces of humans and domestic animals and building latrines/pigpens near ponds also contribute to contamination of water, and increases the infection rate in fishes. Meanwhile, the high prevalence of clonorchis infection in cats, dogs and pigs, and the high metacercariae rate in fishes

all contribute to a vicious circle of maintaining the life cycle of the parasite, which results in an increase in human infection.

In conclusion, study of the epidemiological factors of *C. sinensis* confirmed that poor knowledge and unhealthy behavior in humans, poor environmental hygiene, and inappropriate farming/fishery practices are an important cause of the increase of clonorchis in humans. Combined interventions include health education, environmental modification (proper building and the use of latrines and pigpens, improved hygiene), reform of traditional farming/fishery practices, mass screening and chemotherapy of humans, and the management of infected domestic animals need to be considered in developing a control strategy in the future.

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Mutagenicity evaluation of *Schistosoma* spp. extracts by the *umu*-test and V79/HGPRT gene mutation assay

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Abstract

Schistosomiasis has been suspected of being a risk factor for various types of cancers for sometime, e.g., bladder cancer, colorectal cancer and hepatic cancer. Among them, the etiological relationship between urinary schistosomiasis and bladder cancer is now widely accepted. However, mechanisms of the carcinogenesis are still unclear. Here, we tested the mutagenicity of the parasite extracts by the *umu*-test and hypoxanthine guanine phosphoribosyltransferase (HGPRT) gene mutation assay, which both overcome disadvantages of the Ames plate assay. Adult worm extracts and egg extracts of *Schistosoma haematobium* and *Schistosoma mansoni* were tested. Under our experimental conditions, neither worm nor egg extracts were shown to have any mutagenicity in both tests even in the presence of S9 mix. Our results suggest that there is very little possibility of immediate gene mutation due to the parasite-derived substances in schistosomiasis-related carcinogenesis.

Keywords: Schistosome; Bladder cancer; Mutagenecity; The *umu*-test; HGPRT mutation assay

1. Introduction

In human urinary schistosomiasis, *Schistosoma haematobium* infection, epidemiological studies have indicated that this parasitic infection is related to a high incidence of bladder cancer in endemic areas [1,2], and etiological relationships between urinary schistosomiasis and bladder cancer are widely accepted [3,4]. Likewise, schistosomiasis japonica is suspected of being related to colorectal cancer and/or hepatic cancer [5]. Various possible mechanisms of carcinogenesis by the parasites have been presented. For instance, urine concentrations of *N*-nitroso compounds, which can act as an initiating agent of carcinogenesis, were increased in urinary schistosomiasis [6,7]. On the other hand, chronic inflammatory reactions and elevated cell proliferation due to schistosomiasis may be involved in the development of bladder cancer [8]. Effects of soluble egg antigens (SEA) on proliferation of endothelial cells and urothelial cells were also tested [9], and the investigators demonstrated that SEA induced proliferation of the cell lines and enhanced mRNA expression of human proliferating cell nuclear antigen (PCNA).

The Ames test [10], which detects reverse mutation of auxotroph bacteria of histidine, is widely used to evaluate mutagenic activities of various substances. A research group tested *Schistosoma japonicum* extracts by the Ames test. They observed no mutagenic activities in the adult worm and egg extracts [11]. However, there is a disadvantage in the Ames test because endogenous amino acids contained in crude samples, such as serum, urine or parasite extracts, interfere with this test. In fact, histidine in samples may have resulted in the pseudopositive increase of revertant colonies when human urine was tested in the assay [12]. Taking this into consideration, we opted for the *umu*-test [13], which does not detect reverse mutation following DNA repair but does detect *umu* gene (one of

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the SOS-response-related genes) expression that is immediately induced by DNA damage. Thus, the *umu*-test can be theoretically applied to histidine-containing crude samples or to cytotoxic substances that severely affect cell proliferation. In brief, the principle of the *umu*-test is as follows: when *Salmonella typhimurium* bacteria containing recombinant *umuC*'-'*lacZ* fusion genes are incubated with mutagenic substances, SOS responses, including up-regulation of *umuC* gene expression, occur and result in production of β-galactosidase activity in the bacteria. Consequently, DNA-damaging (mutagenic) activity can be monitored as an increase of β-galactosidase activity after the treatment.

In addition, as there are differences of genotoxic substance metabolism and DNA repairing system between bacteria and mammalian cells, we also opted for hypoxanthine guanine phosphoribosyltransferase (HGPRT) gene mutation assay using Chinese hamster-derived fibroblast cell line V79 [14–16] to detect mutagenicity in the mammalian system. Normal V79 cells (HGPRT+) can survive in normal MEM medium but cannot survive in 6-thioguanine (6-TG)-containing medium because HGPRT enzyme changes 6-TG into a cytotoxic metabolite, which is incorporated into host DNA resulting in the inhibition of further DNA synthesis. In contrast, HGPRT-mutated V79 cells, which cannot change 6-TG into the cytotoxic metabolite, can survive and proliferate in 6-TG-containing medium. Thus, it is easy to estimate mutagenic effects of substances by counting the 6-TG-resistant colonies after the treatment. Here, we tested mutagenic activity of schistosome-derived substances by using these assay systems. In these tests, we found that there was no evidence of mutagenicity in adult worm and egg extracts of both *S. haematobium* and *S. mansoni*. These results suggest that there is little possibility of the involvement of immediate gene mutation due to the parasite-derived substances in schistosomiasis-related cancer formation.

2. Materials and methods

2.1. Maintenance of parasites and recovery of adult worms and eggs

S. haematobium (Kenyan strain) was maintained by using female ICR mice or female hamsters as final hosts (SLC, Japan). *S. mansoni* (Puerto Rican strain) was maintained by using female ICR mice. As intermediate host snails, *Bulinus globosus* (Kenyan strain) and *Biomphalaria glabrata* (Puerto Rican strain) were used for *S. haematobium* and *S. mansoni*, respectively. Mice or hamsters were infected percutaneously under anesthesia with pentobarbital sodium. Each animal was infected with 300–500 cercariae of *S. haematobium* or with 200 cercariae of *S. mansoni*. Four months (*S. haematobium*) or 7 weeks (*S. mansoni*) after the infection, the portal system of the infected animals was perfused with physiological saline containing 0.45% trisodium citrate to collect adult worms. Thereafter, the livers of infected rodents, which contained the parasite eggs, were minced and homogenized in a warring blender. The liver homogenate was then digested with 0.5 mg/mL collagenase (Wako Pure Chemical Industries, Japan) and 1 mg/mL actinase E (Kaken Pharmaceutical, Japan) for 6 h at 37 °C. Eggs were purified by repetitive centrifugation at 150 *g* for 5 min. The adult worms and the eggs were finally washed with physiological saline and kept frozen at −70 °C until extract preparation. The experiments were performed under the control of the Ethics Committee of Animal Care and Experimentation in accordance with The Guiding of Principle for Animal Care Experimentation, The University of Occupational and Environmental Health, Japan and the Japanese Law for Animal Welfare and Care (No.221).

2.2. Preparation of parasite extracts

To prepare PBS extracts, the adult worms or the eggs were homogenized in PBS with ultrasonic treatment. As for the eggs, the Ultra-Turrax T8® homogenizer (IKA-Werke, Germany) was used to destroy eggshells before the ultrasonic treatment. The homogenates were centrifuged at 20,000 *g* for 20 min at 4 °C. Supernatants were collected, and their protein concentrations were measured by the Protein Assay Kit (Biorad, CA, USA). These preparations of adult worms and eggs were designated as soluble worm antigen preparation (SWAP) and soluble egg antigens (SEA), respectively.

As for the preparation of the lipid-soluble fraction, chloroform and methanol were added to the lyophilized adult worms or eggs. They were then homogenized and centrifuged in the same way as PBS extracts. After removal of the solvent through evaporation, the remaining substances were weighed and dissolved in demethylsulfoxide at 5 mg/mL.

2.3. Reagents

The *umu*-test kit (Umulac®) was purchased from the Japan Immunoresearch Laboratories. *S. typhimurium* bacteria for testing, S9 mix and positive control reagents, i.e., 2-(2-furyl)-3-(5-nitro-2-furyl)-acrylamide (AF-2) and 2-aminoanthracene (2-AA) were included in the kit. S9 mix for V79/HGPRT gene mutation assay was purchased from Oriental Yeast, Japan.

2.4. The umu-test

Conditions of the assay were based on the instruction of the kit. Briefly, *S. typhimurium* TA1535 containing pSK1002 plasmid, which has an *umuC*'-'*lacZ* fusion

gene, was precultured in the culture medium for 2 h at 37 °C. During the incubation time, 10 μL of test samples and positive control reagents was diluted and poured into wells of flat bottom 96-well microplates. Then 100 μL of the precultured bacterial suspension was added to around half of the wells. S9 mix-containing bacterial suspension was added to the remaining wells. The plates were further incubated for 2 h at 37 °C. After incubation, 100 μL of substrate solution (X-gal solution) was added to each well. One hour later, 100 μL of stop solution (SDS solution) was added, and optical densities were measured at 630 nm.

2.5. V79/HGPRT gene mutation assay

Chinese hamster lung fibroblast cell line V79 (JCRB 0603) was purchased from the Japanese Collection of Research Bioresources Cell Bank (Sennan, Japan). V79 cells were plated onto 6-well plates at 5×10^5/well. After 24 h of incubation, materials for testing were added and further incubated. Some of the cultures were metabolically activated by the addition of S9 mix. Culture supernatants containing tested materials were removed after 4 h, and the cells were briefly rinsed with PBS and harvested by treatment with PBS containing 0.25% trypsin and 0.1% EDTA. The cells of each treatment group were plated onto 100-mm culture dishes at 5×10^5/100 mm dish (one dish each, for mutation assay) and 200/60 mm dish (three dishes each, for plating efficiency test 1 [PE1]) in 10% FCS/MEM. Three days later, the former cells were harvested, 1/10 of the total cells were replated and further incubated for 4 days. The latter cells were cultured without passage for 6–7 days, and colonies were stained with Giemsa solution and counted (PE1).

Seven days posttreatment, the cells for mutation assay were harvested and replated in 6-TG-containing medium (10 μg/mL) at 2×10^5/100 mm dish. Each treatment group was composed of five dishes. Simultaneously, the cells of each treatment were plated at 200/60 mm dish for plating efficiency test 2 ([PE2]; three dishes each) in 6-TG free medium. The colonies of former and latter cells were stained with Giemsa at 10 days and 6–7 days postplating, respectively.

3. Results

3.1. Responses of positive control reagents (mutagens) in the umu-test

Dose responses of positive controls are shown in Table 1. AF-2 showed a dose-dependent increase of OD value when the concentration was lower than 0.1 μg/mL (the concentrations shown in Table 1 and 2 are the ones before the addition of bacterial suspension; that is, the concentrations in the culture with the bacteria were 1/11, and the ones in the substrate reaction were 1/21 of the values in the Tables).

Table 1
The effect of mutagens on β-galactosidase activity in the *umu*-test

Treatment	Concentration (μg/mL)	S9 mix	β-galactosidase (OD630 nm)
PBS		–	0.214±0.016[a]
Solvent	10% DMSO	–	0.077
AF-2	0.0012	–	0.120
	0.0037	–	0.275
	0.011	–	0.399
	0.033	–	0.508
	0.1	–	1.298
	0.3	–	1.217
	0.9	–	0.465
2-AA	0.04	–	0.199
	0.12	–	0.162
	0.37	–	0.224
	1.1	–	0.161
	3.3	–	0.181
	10	–	0.179
	30	–	0.157
PBS		+	0.182±0.039[b]
Solvent	10% DMSO	+	0.197±0.033[c]
2-AA	0.04	+	0.225
	0.12	+	0.365
	0.37	+	0.550
	1.1	+	0.983
	3.3	+	1.453
	10	+	1.878
	30	+	1.580

Representative data of similar experiments performed.
[a,b,c]The values of controls (PBS and solvent) are mean and S.D. of duplicate assays.

In the absence of S9 mix, treatment with 2-AA did not increase OD value (Table 1). However, a prominent increase of OD was observed in the presence of S9 mix.

3.2. Absence of mutagenicity of schistosome extracts in the umu-test

Results in the absence and presence of S9 mix are shown in Table 2a and b, respectively. The concentration of the samples is represented as protein concentrations. There were no dose-dependent changes of OD values both in the absence and presence of S9 mix. No changes of OD values were observed when chloroform/methanol extracts of the adults and the eggs were tested in a similar way (data not shown).

3.3. Effects of schistosome extracts on plating efficiencies of V79 cells

The effects of schistosome extracts on plating efficiencies (both at the end of treatment [PE1] and at the end of the mutation expression period [PE2]) of V79 cells are shown in Table 3. Although some of the treatment groups showed slightly lower or higher plating efficiencies compared with

control, dose-dependent toxicity of schistosome extracts was not observed in the concentrations used here. Thus, schistosome extracts were not so toxic to V79 cells under our experimental conditions.

3.4. Absence of mutagenicity of schistosome extracts in V79/HGPRT gene mutation assay

Table 3 summarizes the results of HGPRT gene mutation assay of schistosome extracts. In the absence of S9 mix, background level of mutant frequency was less than 10 over 1×10^6 V79 cells. Treatment with highly mutagenic reagent MNNG (1 μg/mL) resulted in a dramatic increase of the mutant frequency; that is, more than 500 mutant colonies were observed in 1×10^6 cell cultures. In contrast, treatment with benzo[α]pyrene (2 μg/mL) did not change the frequency of mutation. None of the treatment with schistosome extracts (both SWAP and SEA of both *S. haematobium* and *S .mansoni*) resulted in increase of mutation frequency. Simultaneously, we tested schistosome samples in the presence of S9 mix. Under our experimental conditions, treatment with benzo[α]pyrene (2 μg/mL) dramatically increased mutation frequency compared with control (Table 3b), indicating that S9 mix used in this test was sufficient in the enzymatic activity. In some of the experiments shown in Table 3b, slight increases of mutant frequency in worm extracts treated groups were observed. However, such increases were not reproducible, and in addition, the levels of increases were much lower than the cutoff point: >20 over control [14].

Table 2
The effect of schistosome extracts on β-galactosidase activity in the *umu*-test

Treatment	Concentration (mg/mL)	β-galactosidase (OD630 nm)
(a) S9 mix−		
PBS		0.214±0.016
S. haematobium SWAP	1	0.128±0.081
	3	0.148±0.053
	10	0.139±0.083
	20	0.116±0.018
S. haematobium SEA	0.3	0.209±0.011
	1	0.180±0.049
	1.5	0.197±0.006
S. mansoni SWAP	1	0.147±0.035
	3	0.150±0.016
	10	0.113±0.019
	20	0.157±0.006
S. mansoni SEA	0.3	0.204±0.030
	1	0.188±0.047
	1.5	0.174±0.053
(b) S9 mix+		
PBS		0.182±0.039
S. haematobium SWAP	1	0.151±0.053
	3	0.152±0.060
	10	0.144±0.057
	20	0.186±0.051
S. haematobium SEA	0.3	0.225±0.028
	1	0.185±0.013
	1.5	0.186±0.020
S. mansoni SWAP	1	0.159±0.004
	3	0.182±0.000
	10	0.155±0.004
	20	0.173±0.006
S. mansoni SEA	0.3	0.210±0.014
	1	0.163±0.020
	1.5	0.192±0.019

Representative data of similar experiments performed.
The values are mean and S.D. of duplicate assays.

Table 3
The effect of schistosome extracts on gene mutations in the V79/HGPRT mutation assay

Treatment	Concentration (mg/mL)	PE1 (%)[a]	PE2 (%)[b]	(Mutants/10^6)[c]
(a) S9 mix−				
Control		78.6±32.7	78.9±15.5	3.3±2.7
Solvent	DMSO 0.5%	82.7±29.6	68.6±7.2	1.4±1.0
MNNG	0.001	61.3±17.0	48.6±13.3	928.4±305.5
Benzo[α]pyrene	0.002	91.3±36.2	72.6±18.6	0.8±1.4
S. haematobium	0.3	57.2±1.4	75.3±15.6	4.1±0.8
SWAP	1	71.7±26.8	78.2±18.7	2.7±2.8
	3	66.0±31.3	78.2±21.8	7.7±5.5
	6	107.5±18.7	71.9±6.2	9.3±5.7
S. haematobium	0.5	84.8±23.7	69.5±18.4	1.8±2.5
SEA				
S. mansoni	1	73.9±44.8	85.3±24.8	5.0±6.1
SWAP	3	82.2±37.3	85.9±23.0	1.5±1.7
	6	84.2±14.8	59.7±16.3	5.9±6.4
S. mansoni	0.5	88.5	54.5	1.8
SEA				
(b) S9 mix+				
Control		82.4±14.9	85.3±16.3	5.8±4.3
Solvent	DMSO 0.5%	87.8±23.8	88.6±14.5	1.8±2.3
Benzo[α]pyrene	0.002	96.9±24.9	68.5±11.7	108.8±64.0
S. haematobium	0.3	75.6±18.5	68.3±11.4	7.3±8.5
SWAP	1	92.9±26.5	82.5±11.5	1.8±3.1
	3	85.8±23.7	76.7±17.7	11.9±8.4
	6	90.5±7.4	78.9±2.3	10.3±12.9
S. haematobium	0.5	98.2±14.1	70.3±12.7	0.0±0.0
SEA				
S. mansoni	1	71.5±32.9	74.2±6.0	4.6±2.5
SWAP	3	76.6±14.9	85.9±8.8	8.1±8.5
	6	84.8±17.7	77.9±29.6	4.4±6.2
S. mansoni	0.5	96.8	67.8	10.3
SEA				

Combined results of four independent similar experiments.
The values are mean and S.D. of two to four experiments.
[a,b]PE1 and PE2 are the plating efficiencies of the cells immediately after the test material treatment and after the mutation expression period, respectively (see Materials and methods).
[c]The numbers of mutant colonies are expressed as mutants per 10^6 surviving cells.

4. Discussion

Mechanisms of bladder carcinogenesis in urinary schistosomiasis patients are still obscure although many exper-

imental studies showing etiological relationships between them have been performed. For instance, a research group observed proliferative epithelial lesions including papillary transitional cell carcinoma and squamous metaplasia in *S. haematobium*-infected nonhuman primates, i.e., the talapoin monkey, capuchin monkey, African baboon, etc. [17]. Not only in the primate models but also in rodent infection models (hamsters, marsh rats and jirds), hyperplasia and squamous metaplasia were observed in *S. haematobium*-infected animals [18,19]. Additionally, carcinogenesis caused by low doses of carcinogen (*N*-butyl-*N*-(4-hydroxybutyl)nitrosamine [BBN]) was accelerated in *S. haematobium*-infected baboons [20]. Some researchers have demonstrated that schistosome-infected animals had modifications of mutagen metabolism, which may accelerate carcinogenesis [21–23]. Possible mechanisms of bladder carcinogenesis have been reviewed by Badawi et al. [4], e.g., generation of carcinogenic substances by abnormal tryptophan metabolism, production of *N*-nitroso compounds by secondarily infecting bacteria and hydrolysis of carcinogen–glucuronide conjugates by β-glucuronidase. To confirm the presence of mutagens in the urine from bilharzial bladders, a research group tested the urine by the Ames *Salmonella* plate assay [12]. However, the group could not show any convincing evidence of the presence of mutagenicity in the urine samples because of interference of histidine in the urine.

Besides the many studies mentioned above, there are only a few reports regarding immediate involvement of parasite-derived substances in the carcinogenesis. Mutagenic activities of the *S. japonicum* adult worm and egg extracts were tested in the Ames test [11], and it was shown that there was no detectable mutagenicity in the extracts. In another study, *S. haematobium* egg extract accelerated proliferation of an urothelial cell line [9]. In the present study, we tested schistosome adult worm and egg extracts by the *umu*-test [13]. As shown in Table 2, PBS extracts of schistosome showed no mutagenic activity in this test. In addition, chloroform/methanol extracts of schistosome also showed no such activity (data not shown). There were no obvious differences in the responses between *S. haematobium* and *S. mansoni*.

Some of potentially mutagenic substances (for instances, 2-AA and benzo[α]pyrene) become mutagenic only after metabolic activation in the liver. To evaluate such substances, rat S9 mix is usually used for the metabolic activation in vitro. S9 is the microsome fraction of liver homogenate of rats pretreated with phenobarbital and 5,6-benzoflavone. S9 mix is the mixture of S9 and essential cofactors for the enzymatic activities. In our present study, the addition of S9 mix did not affect the results (Table 2), indicating that there was no mutagenicity induced by metabolic activation.

As mammalian metabolic and genetic systems are very different from prokaryotic systems, we also tested mutagenicity of PBS extracts of schistosome adults and eggs in V79/HGPRT gene mutation assay [14–16]. This assay detects direct gene mutation caused by test materials as an increase of mutant colonies and can be performed in vitro without microscopic examination training needed for other mammalian assay systems, i.e., chromosome aberration test or micronucleus test. As shown in Table 3, the extracts of adult worms or eggs of *S. haematobium* or *S. mansoni* have shown no or very small increases of mutant colonies after the treatment, while treatment with positive control mutagens (MNNG in the absence of S9 mix and benzo[α]pyrene in the presence of S9 mix) dramatically increased mutant colonies. Although slight increases of mutant colony count were observed in some of the treatments with schistosome extracts (e.g., high concentrations of *S. haematobium* SWAP), such increases were not reproducible between the independent experiments performed. In addition, the levels of observed increases were below the cutoff point suggested by the mammalian cell gene mutation assays working group report [14], which is >20 mutant increase over control in the case of V79 cells. Therefore, we concluded that the extracts of worms and eggs of schistsomes did not have mutagenicity to mammalian cells.

In conclusion, there is little possibility of immediate gene mutation due to the substances from the adult worms or from the eggs in bladder cancer formation. Further investigation is needed to elucidate mechanisms of carcinogenesis by schistosome infections. Testing of promoter activity of schistosome extracts by BALB/3T3 transformation assay is currently underway in our laboratory.

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Research brief

Schistosoma japonicum: localization of calpain in the penetration glands and secretions of cercariae

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Abstract

A monoclonal antibody was generated against the large subunit of *Schistosoma japonicum* calpain to study the localization and possible function of the molecule in vivo. Mice were immunized with recombinant *S. japonicum* calpain and polyclonal antisera and a monoclonal antibody specific to schistosome calpain was obtained. In immunohistochemistry, a monoclonal antibody against *S. japonicum* calpain, KG-2E11, bound weakly to calpain expressed at the surface of adult worm tegument, however, it bound strongly to the cercarial secretions ("footprints") of *S. japonicum*, emitted from the penetration glands. The present study indicates that calpain is multifunctional as it is expressed at various locations in different developmental stages. Calpain-based vaccines could thus possibly induce protective immunity against cercariae and the following early developing stages.

Index descriptors and abbreviations: IFN-γ, interferon-gamma; CBB, Coomassie brilliant blue; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; SEA, soluble egg antigen; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SWAP, soluble adult worm antigen.
Keywords: *Schistosoma japonicum*; Cercariae; Calpain; Tegument; Penetration gland

Schistosomiasis affects over 200 million people, and almost 600 million people are exposed to the disease with significant morbidity and mortality (WHO, 1998). One species, *Schistosoma japonicum*, inhabits the mesenteric portal circulation of mammalian host animals. Although chemotherapeutic agents are available for treatment, vaccine strategy is still a high priority because of a number of problems. One of such problems is a high re-infection rate of schistosomiasis in endemic areas, because re-infection is high following anthelmintic treatment. Continuous treatment with a single drug might raise a possibility of drug-resistant schistosomes. Furthermore, vaccine development may lead to the reduction of ongoing medical costs, and contribute to economic development (Fallon et al., 1996; Redman et al., 1996).

Calpain, a calcium dependent neutral cystein protease, was identified in schistosomes (Andresen et al., 1991; Scott and McManus, 2000; Zhang et al., 2000). In *Schistosoma mansoni*, calpain appears to be expressed in the surface syncythial epithelium and underlying musculature (Siddiqui et al., 1993). It has been demonstrated that calpain may be necessary for C3b- and 5-HT-induced acceleration of surface membrane synthesis (Siddiqui et al., 1993), while physiological functions of mammalian calpain seem to be related to the transduction of extracellular signals and intracellular signaling pathway mediated by Ca^{2+} (Sorimachi et al., 1997).

Calpain is a good vaccine candidate for *S. mansoni* as well as *S. japonicum*. When mice have been infected by *S. mansoni*, calpain has been reported to induce strong protective immunity (Hota-Mitchell et al., 1997, 1999; Jankovic et al., 1996). Baculovirus-expressed recombinant calpain heavy chain induced 29–39% protection (Hota-Mitchell et al., 1997), cell transfer with a T cell clone recognizing the large subunit of *S. mansoni* calpain conferred 65% protection (Jankovic et al., 1996), and immunization with a plasmid expressing the large subunit with the full 5′ untranslated region induced 60% protection (Hota-Mitchell et al., 1999). Recently, the high effects of DNA-based vaccination combined with several cytokine vectors have also been reported (Siddiqui et al., 2003a,b). In *S. japonicum*, we found that immunization with recombinant calpain in Freund's adjuvant induced 37–41% reduction in worm burden, and also reduced fecundity of female adult

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worms (Zhang et al., 2001). Spleen cells of immunized mice produced an elevated level of IFN-γ in response to antigen stimulation. In our preliminary experiment in pigs, we also observed a significant anti-fecundity effect (Ohta et al., 2004).

In spite of the accumulating evidence for the efficacies of calpain as an anti-schistosome vaccine, it is still not clear how protective immunity is induced in calpain-immunized animals. To understand how anti-calpain immunity works, we examined the localization of calpain in different developmental stages of *S. japonicum*.

Schistosoma japonicum isolated in Yamanashi, Japan, was maintained by standard laboratory procedure in female BALB/c mice (SLC, Hamamatsu, Japan) and their snail hosts, *Oncomelania hupensis nosophora*. Worms were recovered by the perfusion method from the hepatic portal system of mice at 8 weeks after infection with 40 cercariae (Smithers and Terry, 1965). Antigens used in the present study were SWAP, SEA, and recombinant calpain molecules of *S. japonicum*. The methods for preparation of SWAP and SEA have been described previously (Rosane et al., 1996). Recombinant calpain was prepared as previously described (Zhang et al., 2001). This recombinant calpain is the region of 220–376 amino acid of the large subunit, and used for immunization and several assays. Furthermore, we prepared two fragments of recombinant calpain: (1) the region of 220–330 amino acid and (2) the region of 300–376 amino acid of the large subunit. These two fragments were used to determine a monoclonal antibody recognizing portion. In brief, all coding sequences were inserted to pGEX-2TK vector (Pharmacia, Uppsala, Sweden). This vector was then transformed into BL21 cells. The recombinant proteins were induced by addition of isopropanol β-D-thiogalactoside to a final concentration of 0.1 mM for 6 h. After induction, BL21 cells were collected and lysed with BugBuster reagent plus Benzonase (Novagen, Madison, USA). The insoluble fraction was then solubilized in sample buffer for SDS–PAGE to use in CBB staining and Western blotting.

Female BALB/c mice were intradermally immunized with recombinant calpain plus complete Freund's adjuvant for the first immunization, and Freund's incomplete adjuvant for subsequent immunizations. The calpain was administered at 2-week intervals and blood samples were collected before and after the immunizations. The presence of specific antibodies was detected via ELISA (see below). For the generation of monoclonal antibodies, spleen cells of immunized mice were fused with P3×63.Ag8.653 myeloma cells using polyethylene glycol 1500 (Boehringer–Mannheim, Germany). Hybridomas that secrete antibodies to calpain were selected and cloned by limiting dilution. Ascites was produced in BALB/c mice by injecting 5×10^6 hybridoma cells. Isotype was determined in ELISA using anti-isotype mAbs (Southern Biotechnology Associates, Birmingham, USA). For ELISA, 5 μg/ml of antigens was coated in microtiter plates (Nunc, Roskilde, Denmark). Wells were blocked with Tris-buffered saline-containing 1% casein, and incubated with antibodies. After washing, anti-mouse IgG (H + L) (KPL, Gaithersburg, USA) was added and incubated for 1 h. ABTS (2,2′-azino-di[3-ethyl-benzthiazoline sulfonate]) was used as substrate and optical densities were read in a Microplate Reader (Bio-Rad, Hercules, USA). Recombinant proteins and SWAP were fractionated on 10% SDS–polyacrylamide gels (TEFCO, Tokyo, Japan) and electrophoretically transferred to PVDF membrane (Bio-Rad). The membrane was blocked by 3% BSA in PBS, and then incubated with antibodies in Tris-buffered saline-containing 1% casein. After that, membranes were washed and incubated with peroxidase-conjugated anti-mouse IgG (H + L) (KPL). Detection of positive bands was done using Konica immuno-stain kit (Konica, Tokyo, Japan).

Adult worms of *S. japonicum* at 8 weeks after infection were prepared for use as frozen sections. Worms were incubated in RPMI 1640 (Sigma–Aldrich) supplemented with 10% FBS (Gibco-BRL, Grand Islands, USA) at 37 °C overnight to wash away the host components. After washing worms embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan) were snap frozen, and were sliced with a cryostat (Lica, Nassloch, Germany) at a thickness of 6 μm. Sections

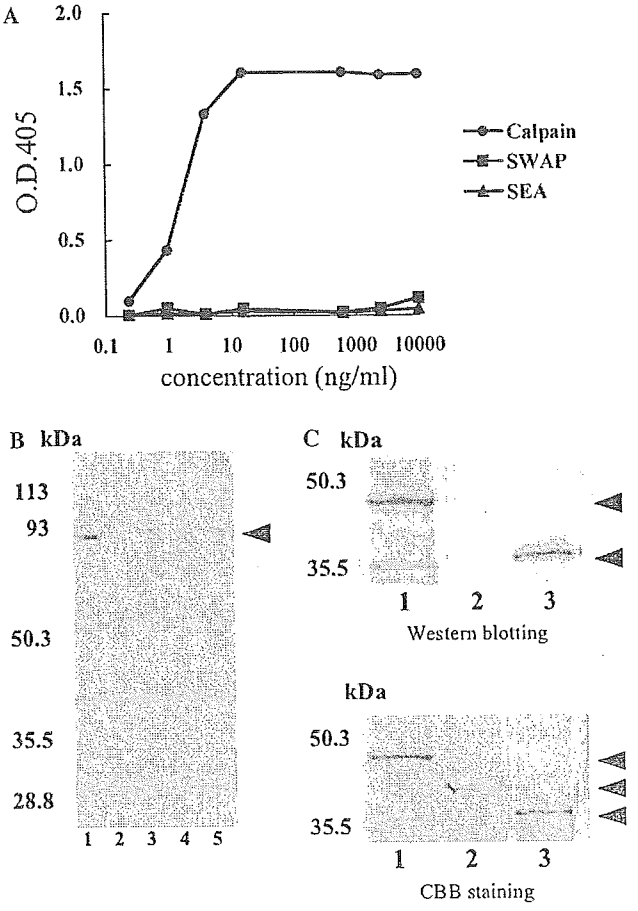

Fig. 1. Preparation and analysis of KG-2E11 by ELISA and Western blotting. (A) KG-2E11 generated from recombinant calpain-immunized BALB/c mice bound to calpain tightly, although SWAP was bound weakly and SEA was not by ELISA. (B) The soluble adult worm antigens (50 μg/well) were electrophoresed in SDS–PAGE. The bands on the gel were transferred to PVDF membrane and the membrane was treated with KG-2E11 (lane 1), normal mouse serum (lane 2) and several anti-calpain antisera, each of which was individual serum of a calpain-immunized mouse (lanes 3–5). All lanes except for lane 2 specifically recognized a molecule of 80 kDa, of which molecular mass was equivalent to a large subunit of *S. japonicum* calpain. (C) Determination of the epitope recognized with KG-2E11 was done by Western blotting analysis using three types of recombinant calpain. Recombinant calpain; the region of 220–376 amino acid (lane 1); the region of 220–330 amino acid (lane 2); and the region of 300–376 amino acid (lane 3) of calpain heavy chain were electrophoresed in SDS–PAGE. Positive bindings to KG-2E11 were observed in lane 1 and lane 3. We confirmed by CBB staining that each protein was transferred to PVDF membrane successfully.

were dried and preserved in acetone for 15 min at −20 °C. Immunostaining was done using a HistoScan kit (Biomeda, Foster City, USA). After incubation in blocking solution containing normal rat immunoglobulin (Sigma–Aldrich) for 30 min at room temperature, sections were incubated together with KG-2E11 mAb diluted 1:100 with blocking buffer for 3–9 h at 4 °C. An isotype control mAb (Dako, Glostrup, Denmark) was used as a negative control. After washing, each section was incubated with anti-mouse secondary antibodies (KPL) for 1 h at room temperature, and as a next step was treated with anti-goat polyclonal antibodies for 30 min (Biomeda). After treatment of antibodies, endogenous peroxidase was blocked by the use of 0.3%

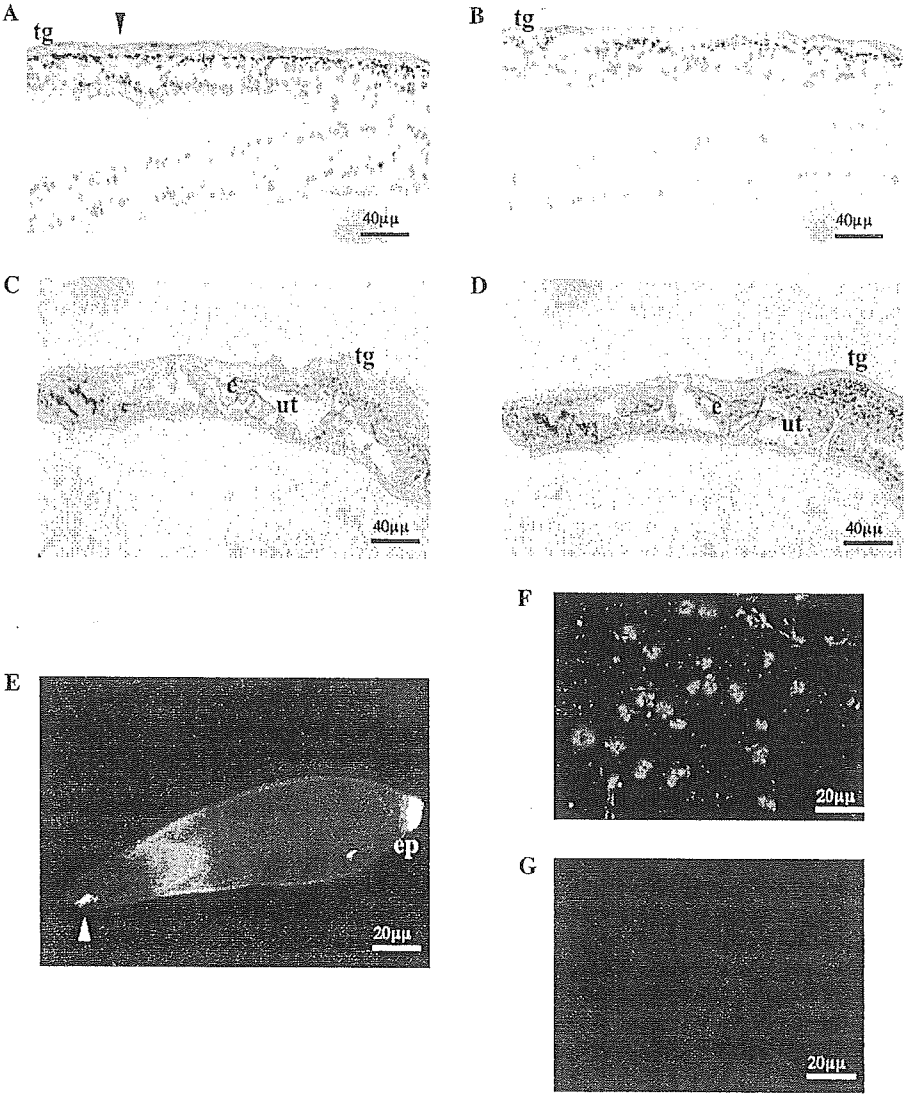

Fig. 2. Localization of calpain in *S. japonicum* with KG-2E11. KG-2E11 bound to the tegument (tg) of male worms (A), but did not bind to the tegument (tg) and the uterus (ut), including eggs (e), of female worms (C). Isotype control mAb was non-binding (B,D). Cercariae were stained with KG-2E11, and then the outlets of penetration glands (arrowhead) and the excretory pore (ep) were strongly positive (E). The secretions from cercariae were strongly stained by KG-2E11 (F), but they were non-binding by isotype control mAb (G).

H_2O_2 in methanol. Each section was then incubated with avidin–peroxidase for 15 min, stained with AEC staining solution for 10 min, and a counter staining with hematoxylin was done. For staining whole bodies, cercariae were fixed in acetone for 10 min at −20 °C. After washing with PBS, blocking was done by putting cercariae in 1% casein Tris buffer overnight at 4 °C. For staining "footprints" of *S. japonicum* cercariae, freshly released from crushed snails, were directly placed on silane-coated slide glasses for 1 h at room temperature. After these slide glasses were completely dried, and they were fixed in acetone for 10 min at −20 °C. KG-2E11 mAb was treated against both cercarial bodies and footprints for 3 h at room temperature. After incubation with biotin-conjugated antibodies (Vector laboratories, Burlingame, USA), both cercarial bodies and footprints were reacted with avidin-conjugated fluorescein (Vector laboratories) for 30 min at room temperature.

Mice were immunized with recombinant *S. japonicum* calpain to generate specific monoclonal antibodies. We obtained only one single stable clone, KG-2E11, which bound to recombinant calpain tightly (IgG2b, κ). In ELISA, KG-2E11 showed only weak binding to *S. japonicum* SWAP, and no detectable binding to SEA (Fig. 1A). Calpain, thus, seemed to be a minor component of whole adult worm proteins. However, it recognized an 80 kDa protein in SWAP, which corresponded to a large calpain subunit (Fig. 1B). KG-2E11 bound to the C-terminal portion of recombinant *S. japonicum* calpain in the tested two fragments of the heavy chain (Fig. 1C). This suggested that the KG-2E11 epitope is located in the region of 330–376 amino acid of calpain heavy chain.

Immunohistochemistry of adult worms with KG-2E11 showed that this mAb bound to the tegument of the male adult worms (Figs. 2A and B), whereas, it did not bind to the female tegument (Figs. 2C and D). Schistosomes had calpain molecules in the surface of the tegument in the present study as was reported previously (Siddiqui et al., 1993). On the other hand, KG-2E11 bound strongly to the outlets of cercarial penetration glands in *S. japonicum* cercariae (Fig. 2E). This suggested that calpain seemed to be secreted from cercariae. To confirm that calpain was secreted from the cercarial penetration glands, we incubated cercariae on slide glasses to let them secrete gland contents on the surface of the slide glasses. It has been reported that cercariae secrete mucoid substances and leave secretion spots ("footprints") on the bottom of the dishes (Linder, 1985). Many spots were observed

as footprints in the substances secreted from cercariae when we tested KG-2E11 binding (Fig. 2F). No positive binding was observed for isotype control mAb (Fig. 2G), indicating that the binding was not in a non-specific manner. Although, we still do not have solid evidence that the secreting substances contain native or partial fraction(s) of calpain, this could be a probable demonstration that cercariae are directly exposed to calpain-driven host immunity. Several researchers have reported that schistosome cercariae secrete substances containing proteases (Chavez-Olortegui et al., 1992; Fishelson et al., 1992; Landsperger et al., 1982; Stirewalt and Austin, 1973). Especially, the serin protease was localized in both pre- and post-acetabular glands of cercariae of *S. mansoni* (Fishelson et al., 1992; Marikovsky et al., 1990). Calpain is one of calcium-activated cystein proteases. At the time of penetration calpain may work as dermal alterations directly or indirectly dependent on calcium activation. It is important to investigate calpain secreted from cercaria to understand the mechanism of penetration. On the other hand, our observations that a large amount of calpain seems to be localized in the footprints strongly suggest important roles of calpain during survival and/or growth of the larval stage of schistosomes. Calpain release from newly transformed schistosomula of *S. mansoni* has been reported (Jankovic et al., 1996), and we have supported the expression of calpain in mechanical transformed schistosomula in *S. japonicum* (Ohta et al., 2004). Moreover, we found an elevated production of inducible nitric oxide synthase mRNA in the lungs of mice immunized with recombinant calpain (Zhang et al., 2001). This means that calpain from schistosomula moving on through the lung of host stimulates the effector cells of immunized-host. We suggest that the expression of calpain is found in all stages from cercaria, through the penetration, to adult worm. However, the functions of calpain in each stage are still unknown completely. The expression and secretion of calpain in cercariae is an important finding because cercaria and schistosomula seem to be targets for protective immunity characterized as a reduction of worm burden. It has been proposed that calpain-reactive Th1 cells recognize enzymes released from early migrating larvae (Jankovic et al., 1996), and that antigens derived from lung-stage schistosomulae stimulate secretion of IFN-γ (Mountford et al., 1995). The production of type1 cytokines may cause a limited migration of schistosomulae (Wilson et al., 1986) and the killing of the larvae through the production of toxic nitrogen oxides (James et al., 1984; Oswald et al., 1994). To our knowledge, this is the first demonstration that calpain is localized in the penetration glands of cercariae, and is secreted from cercariae. The same situation might be supposed in schistosomula, although we still have no direct evidence. These findings could be clues for understanding the reasons why worm burden is reduced in mice immunized with calpain.

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