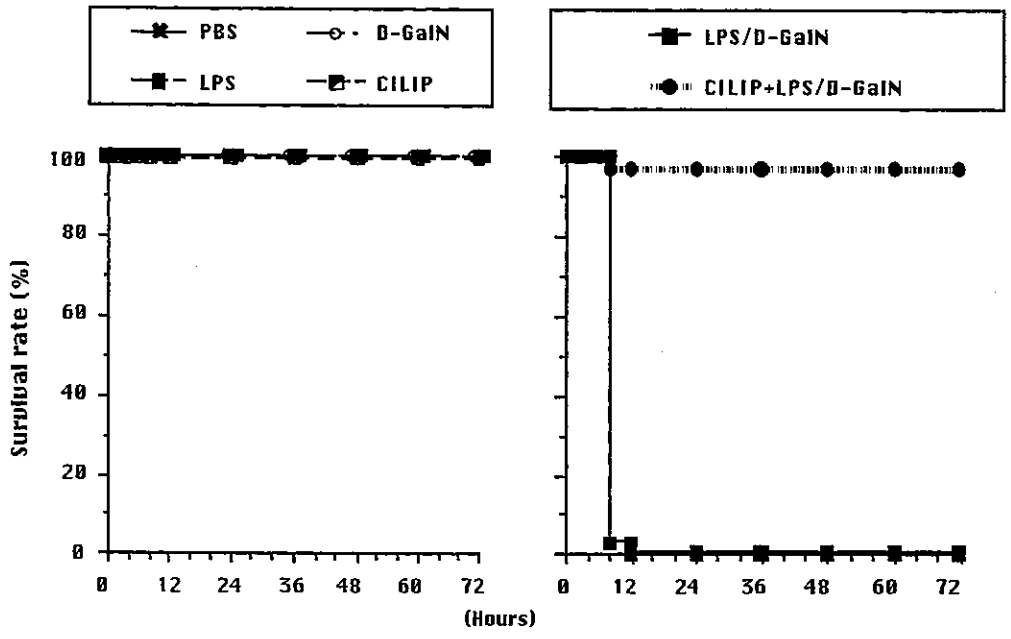

Fig. 1 Survival rate of mice treated with PBS, LPS, D-GalN, CILIP, LPS/D-GalN and CILIP+ LPS/D-GalN

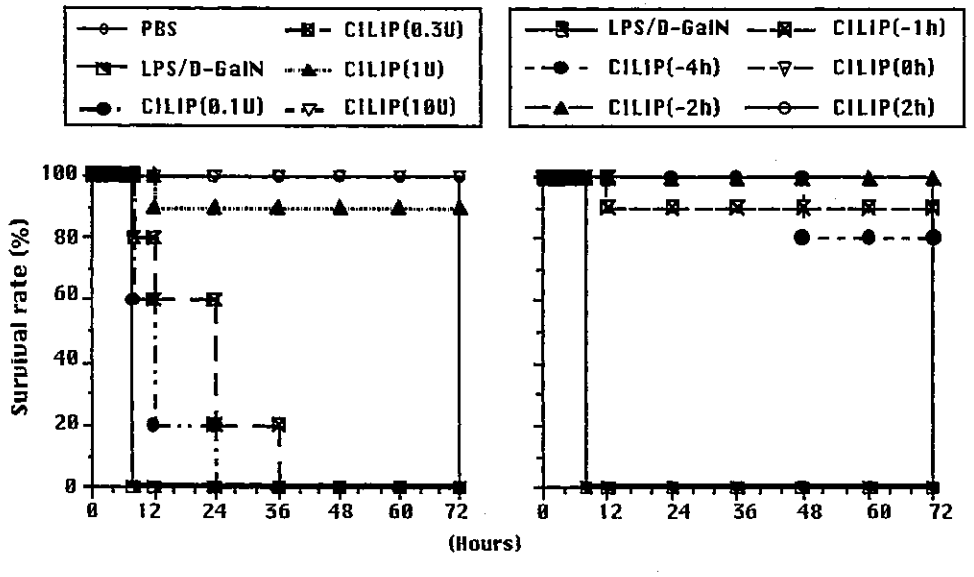

Fig. 2 Effect of CILIP treatment on survival rate of mice to lethal challenge with D-GalN/LPS

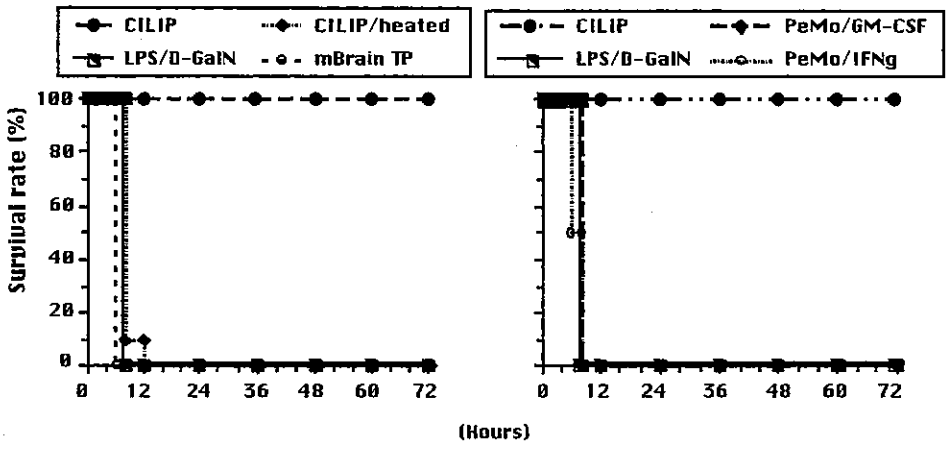

Fig. 3 Survival rate of mice treated with heat-inactivated CILIP, mouse brain thromboplastin, peritoneal macrophages stimulated by IFNg or GM-CSF to lethal challenge with D-GalN/LPS

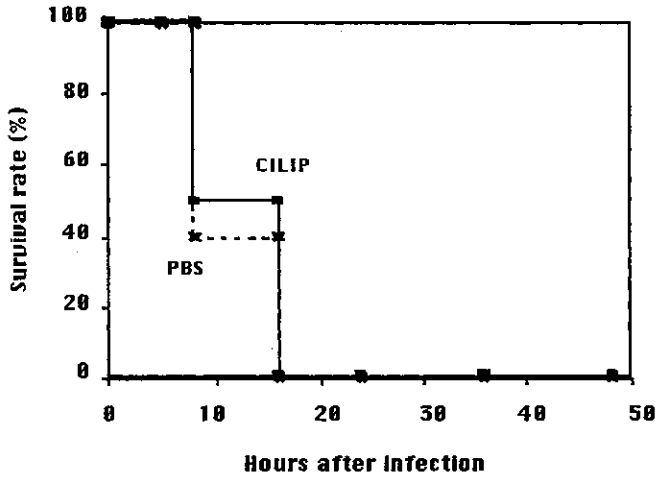

Fig. 4 Effect of CILIP treatment on survival rate of mice to lethal infection with *Pseudomonas aeruginosa*

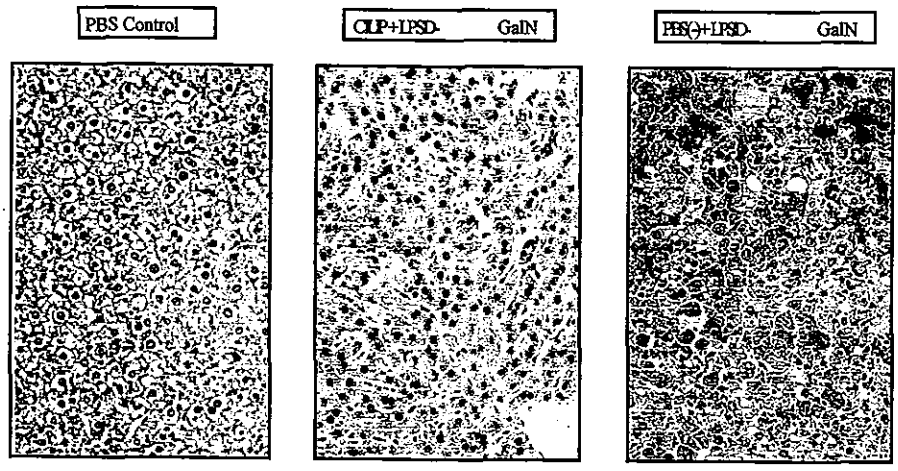

Fig. 5 Histopathological changes in the livers of mice 6 hours after injection with lethal dose of LPS/D-GalN

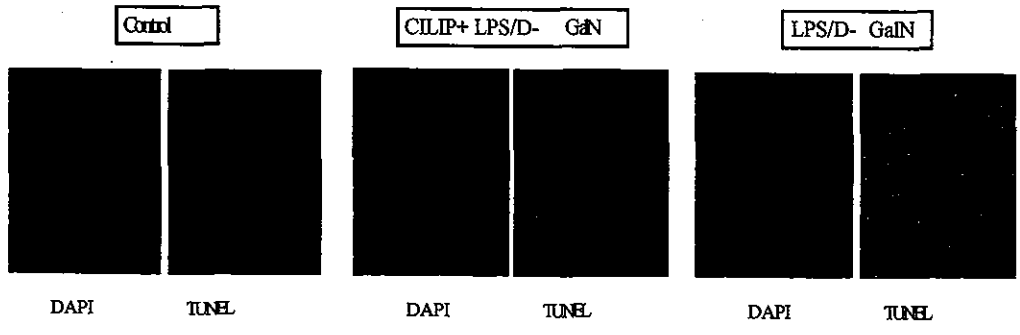

Fig. 6 Apoptotic changes in the livers of mice 6 hours after injection with lethal dose of LPS/D-GalN

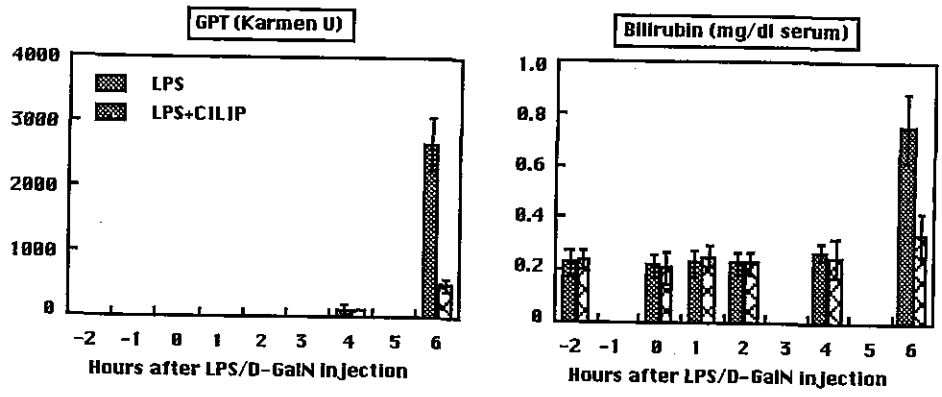

Fig. 7 Changes in serum GPT activities and bilirubin levels in mice after intravenous injection with LPS/D-GalN

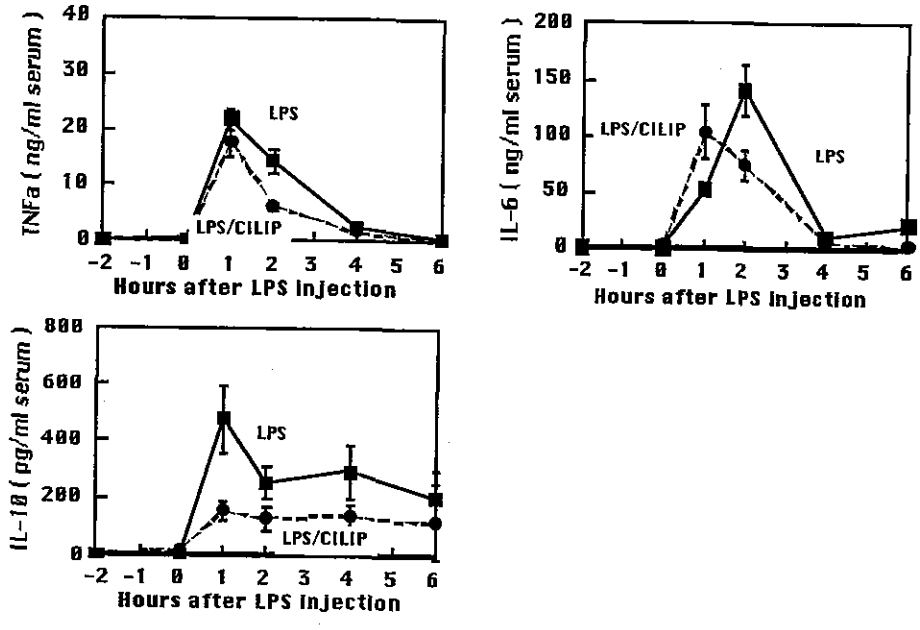

Fig. 8 Changes in serum TNFa, IL-6 and IL-10 levels in mice after intravenous injection with LPS/D-GalN

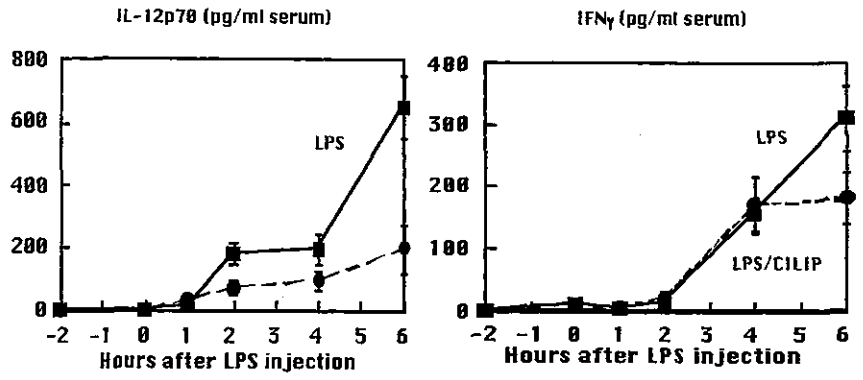

Fig. 9 Changes in serum IL-12p70 and IFNγ levels in mice after intravenous injection with LPS/D-GalN

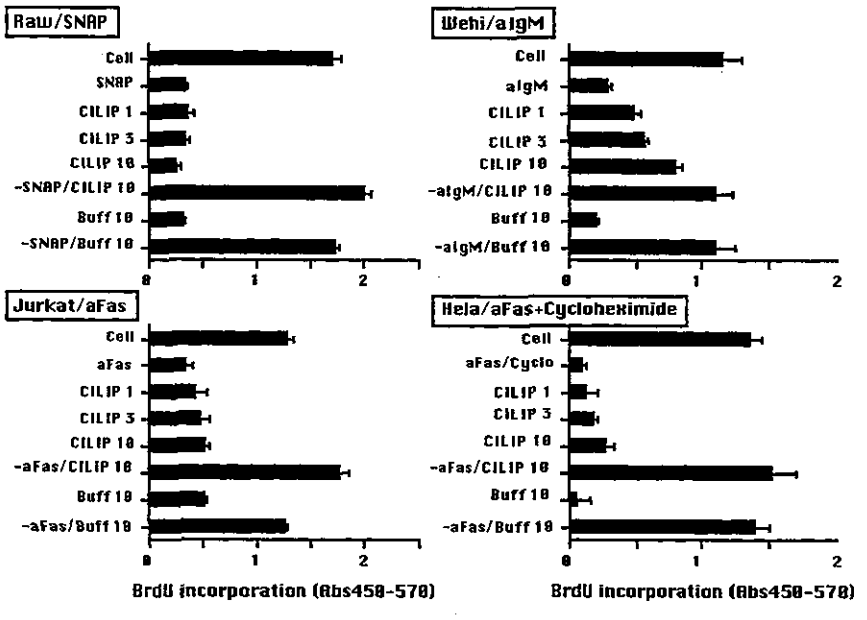

Fig. 10 Effect of CILIP administration on the viability of Raw, Wehi, Jurkat and Hela cells after treatment with apoptosis-inducingstimuli

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Research on calpain of *Schistosoma japonicum* as a vaccine candidate

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Abstract

Vaccine development by the use of calpain of *Schistosoma japonicum* has been tried in our laboratory. We cloned cDNA encoding the heavy chain of *S. japonicum* calpain, and prepared recombinant molecule of a possible vaccine region of the heavy chain. When BALB/c mice were immunized with our recombinant calpain of *S. japonicum* with Freund's complete adjuvant, we observed significant reduction in worm burden (41.2% reduction, $P<0.05$), and also significant anti-fecundity effects. In this sense, calpain of *S. japonicum* seems to have infection control as well as anti-disease effects. Mechanisms of vaccine effects of calpain remain to be clarified, however, several effecter mechanisms are suspected. In immunized mice, raised level of iNos expression was observed, while adhesion of peritoneal exudates cells were also observed in the presence of calpain-immunized sera, suggesting the possibilities of both cellular and humoral protective mechanisms. We examined tissue distribution of calpain in various developmental stages of *S. japonicum*. Strong signal was observed around excretory grand of cercariae, and they secreted calpain during their migratory movement tested in vitro. Together with the findings, calpain seems to induce larvicidal effects in the immunized mice. We observed time-course kinetics of antibody production against vaccine candidates in experimental *S. japonicum* infection in pigs. Although significant levels of antibody production were observed for paramyosin and GST, no significant antibody production was observed for calpain. This suggests that calpain is less immunogenic, and route of immunization and/or choice of adjuvant are important in future trials of calpain vaccine.

Keywords: *Schistosoma japonicum*; Calpain; Vaccine; Anti-fecundity; Immunogenicity

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Table 1
No significant 'Infection inhibition', but significant 'anti-fecundity effect' of calpain immunization in pigs

	Worm burden	Egg number/female worm	Total egg number
Adjuvant alone	15.0%	682 666	30 867 739
GST alone	30.9%	548 811	40 999 166
GST-calpain	24.0%	149 228	7 999 166*

* $P<0.05$.

1. Introduction

Schistosomiasis is endemic in tropical and subtropical countries and more than 200 million people are exposed to the risk of infection [1]. After the development of praziquantel, mortality due to schistosomiasis drastically fell down, however, socioeconomical loss is still a problem when we think about the morbidity of schistosomiasis in productive age groups. Vaccine is a promising strategy to overcome the disease, and accumulated results suggest a probability of vaccine development against schistosomiasis [2–5]. Schistosomiasis japonica is the most promising target of vaccine development; schistosomiasis japonica is a zoonosis, and domestic animals are important reservoir hosts [6]. For the first step of vaccine development, domestic animals, such as pigs and water buffaloes, should be the targets. In comparison with the case of human trial, which has been on-going for GST of *S. mansoni*, it is much easier to carry out in domestic animals.

Vaccine development targeting schistosomiasis japonica has been conducted in Japan. There are two candidate molecules tested; paramyosin and calpain [7–10]. The formaer candidate is described in the other part of this issue, and we introduce calpain of *S. japonicum* as a promising vaccine candidate. Characteristics of the efficacies, effecter mechanisms, and the immunological properties will be discussed.

2. Calpain of *S. japonicum*

Calpain is a Ca^{2+}-dependent cystein protease of which function is still not fully understood in vivo [11,12]. Protective effects of schistosome calpain was reported by Hotta-Mitchell et al. in *S. mansoni* infection. Calpain-sensitized T cells were shown to carry protective effects, and DNA immunization with a gene encoding calpain also induced significant protective immunity in mice [13,14]. We cloned the gene encoding calpain in *S. japonicum* by the method of homology probing, and we clarified a full length gene coding for calpain of Yamanashi strain of *S. japonicum*. Although calpain is thought to be a conservative protein, the identity with human calpain was 39% in amino acid sequences [9]. When we compared with calpain of the Puerto Rican strain of *S. mansoni*, the identities were 99.1% in nucleic acid sequence and 98.8% in amino acid sequence.

By the use of cloned cDNA, we prepared recombinant calpain molecule of F219 to G376. After we obtained GST-fusion protein in *E. coli*, GST was cut out by the treatment of thrombin. The recombinant molecule was used for production of monoclonal antibodies specific to *S. japonicum* calpain.

3. Vaccine effects by immunization with *S. japonicum* calpain

Recombinant molecule of *S. japonicum* calpain was used for immunization of BALB/c mice with Freund Complete adjuvant. Two weeks after the final immunization, mice were infected with 30 cercariae of *S. japonicum*. In comparisons with adjuvant control mice, we observed significant reduction in worm burden (41.1%, $P<0.05$). Furthermore, significant reduction in egg production per female worm was also observed (6057 vs. 3762) ($P<0.05$). Mean granuloma size in the liver of the immunized mice was also significantly smaller than that in adjuvant control group. In our preliminary trial experiment using pigs, significant reduction in fecundity in the immunized group was observed (Table 1) (manuscript in prepara-

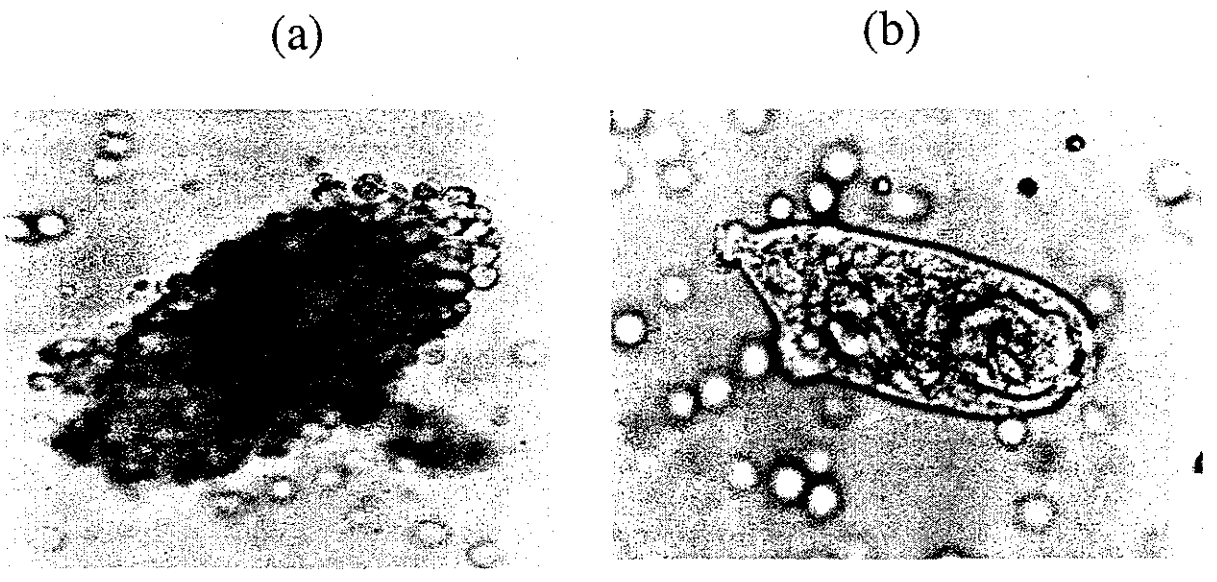

Fig. 1. Adhesion of host peritoneal exudate cells under the presence of calpain-immunized sera in vitro Murine peritoneal exudate cells showed tight adhesion around schistosomulae of *S. japonicum* only when calpain-immunized sera were added (a), however, no such adhesion was observed in the absence of the immune sera (b).

tion). This suggests that calpain has both infection control and disease preventing effects.

Immunological parameters were also tested during the challenge infection. It is accepted that schistosome infection induces strong host responses of type 2 categories [15–17]. Biological mechanisms for the phenomenon have been suggested [18,19]. However, calpain-immunized mice showed significantly higher levels of IFN-γ production in response to the egg antigen of *S. japonicum* in vitro. Furthermore, splenic cells from the immunized mice showed higher expression of iNOS than adjuvant controls [10]. These findings suggest that immunization with calpain might induce host responses polarized to type 1, although no direct evidence is yet available that such polarized Th1 response is directly related to protective effects. When we treated schistosomulae of *S. japonicum* with the immunized sera in vitro, we observed enhanced adhesion of peritoneal exudates cells from naïve mice (Fig. 1). This suggests that antibody-mediated mechanisms could be involved in the vaccine efficacy of calpain.

4. Tissue distribution of calpain in *S. japonicum*

We obtained a monoclonal antibody binding to calpain of *S. japonicum*. The specificity was confirmed by Western blotting analysis, and the linear epitope was determined to be $_{229}$TQW$_{xxxxxxx}$WGDSHEW$_{xxx}$WCD$_{xxx}$WRE$_{358}$ by testing the method of solid-phase overlapping synthesized oligopeptides [20]. By the use of this monoclonal antibody (TK261), we stained male and female adult worms and cercariae of *S. japonicum* in immunohistochemistry or fluorescent antibody technique. In the adult worms, positive signals were observed in the mesenchymal tissues, but lack of signal on the tegumental surface. Cercariae showed strong signals around the excretory gland, and in case of mechanical schistosomulae, the tail portion was stained positively. To test the possibility that cercariae/schistosomulae excrete calpain from the excretory gland, we incubated cercariae on a slide glass, and then the slide glass was stained with TK261 monoclonal antibody. On the surface of the slide glass, we observed numerous

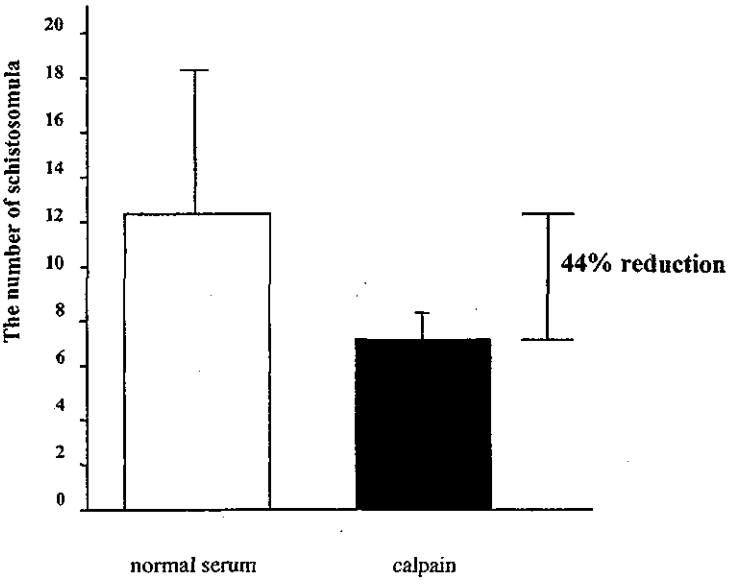

Fig. 2. Passively transferred anti-calpain mAb reduced number of lung-stage larvae of *S. japonicum* TK267, mAb specific to *S. japonicum* calpain was injected into BALB/c mice, and cercariae of *S. japonicum* was challenged. Calpain-injected group (Solid bar) showed reduced number of recovered larvae from the lungs, however, the difference was not statistically significant.

numbers of 'kissing marks' possibly excreted by the cercariae. It was already reported that cercariae excrete a protein(s) from the excretory gland during the migration [21,22], however, the protein(s) has, so far, not been identified. Our observation clearly showed that calpain is one of the component(s) secreted by cercariae. Physiological functions of calpain are still not fully understood, however, it was suggested that calpain had critical roles in tissue damage and repair [23]. Considering that cercariae invade into host tissue during the infection and migration, excreted calpain could have essential roles for the infectivity of cercariae. To test this possibility, we injected mice with TK261 mAb at the time point of cercarial infection, and we examined the number of schistosomulae in the mice. We observed reduction in number of recovered larvae from the lung of mAb-injected mice (Fig. 2), although it was a statistically marginal significance ($P=0.055$).

5. Immunogenicity of calpain in natural infection in pigs

Vaccine effects are generally boosted by natural infection after the immunization. This is important to enhance the protective effects of the vaccine. Since vaccine materials are derived from the pathogens, we expect that the host immune system is stimulated by the pathogen antigens including our vaccine molecule. We infected pigs with 500 cercariae of *S. japonicum*, and we examined time-course kinetics of antibody production against vaccine molecules; paramyosine, GST, and calpain. After the cercarial inoculation, IgG production was observed for soluble adult worm antigen (SWAP) from week 4. As for the vaccine candidates tested, significant level of IgG to paramyosin was observed 7 weeks after the infection, and GST-reactive IgG was also detected. However, there was no detectable IgG binding to calpain even 7 weeks after the infection (Fig. 3).

Seroepidemiological study was also carried out in the endemic population to test whether calpain is enoughly immunogenic in humans. In comparison with IgG levels to egg antigen, calpain-binding IgG was low in the residents in endemic areas. Although individuals with light infection had higher IgG level than that in heavily infected ones, the difference was still marginal (mean OD for light

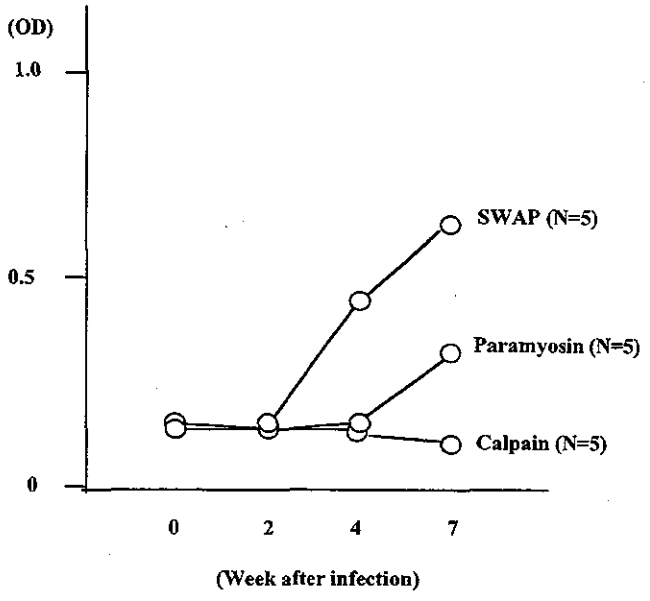

Fig. 3. Booster effects of infection for paramyosin-specific IgG production, but no for calpain in *S. japonicum* infected pigs. Pigs were infected with *S. japonicum* cercariae, and IgG levels to SWAS and vaccine candidates were monitored. Significant level of paramyosin-specific IgG was observed, while there was no detectable level of calpain-specific IgG even at the point of week 7.

infection group 0.42 vs. 0.28 for heavy infection group).

Together with those, calpain seems to be not so immunogenic during natural infection both in domestic animals and humans. This suggests that vaccine strategy taken for calpain should be different from other candidate molecules. Vaccination schedule, injection route, and the choice of adjuvant might be different from other candidate molecules. Further study is needed.

6. Future prospects

WHO designated several vaccine candidates for schistosomiasis control, however, calpain is not listed there [24]. It is therefore needed to uncover the mechanisms of calpain-mediated protective immunity, if developing calpain vaccine is thought to be one of the prioritized. Calpain seems to be unique; both infection control and preventing disease onset are expected. For the infection control effects, we are trying to analyze possible mechanisms. Possibility of calapin vaccine was demonstrated in the $CD4^+$ T cell-mediated immunity in experimental *S. mansoni* infection [13]. Therefore, it is underway to characterize murine T cell hybridomas specific to calpain of *S. japonicum* (Osada et al., in preparation). Antibody-mediated protection was also possible in calpain-immunized mice. We identified a linear epitope recognized by the mAb, TK261. Those findings should be sent back to molecular characterization of calpain. Not enough information is available about genetic polymorphism of calpain in *Schistosoma* sp. When we compared partial amino acid sequences of calpain between the Japanese and Chinese-Hunan strains, identity was almost 100%. We do not have information about the other Chinese geographical strains of *S. japonicum*.

When we think about the low immuogenicity of calpain, it is necessary to consider the route of immunization, and the immunization schedule. We are thinking intranasal immunization of calpain. It was already established that some antigens, if not all, induce strong systemic immune responses without adjuvant in mice [25] (Ohno et al., submitted). Intranasal sensitization is non-invasive, and is good for frequent immunization without use of needles. Adjuvant is also a point to be considered. It is reported that Th1-skewed responses are somehow protective [26], and our recent trial using a CpG oligonucleotide showed its applicability in the vaccine strategy [27].

Finally, the tentative targets for vaccine development in schistosomiasis japonica are domestic animals. It is not easy to carry out because of difficulty in handling and the cost of animals. It was agreed in Asian countries to have collaboration for vaccine development targeting domestic animals [28]. Through such collaborative schemes, it might be possible to start practical use of schistosome vaccine in domestic animals, and this enables us to move to human trials in the near future.

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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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