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-

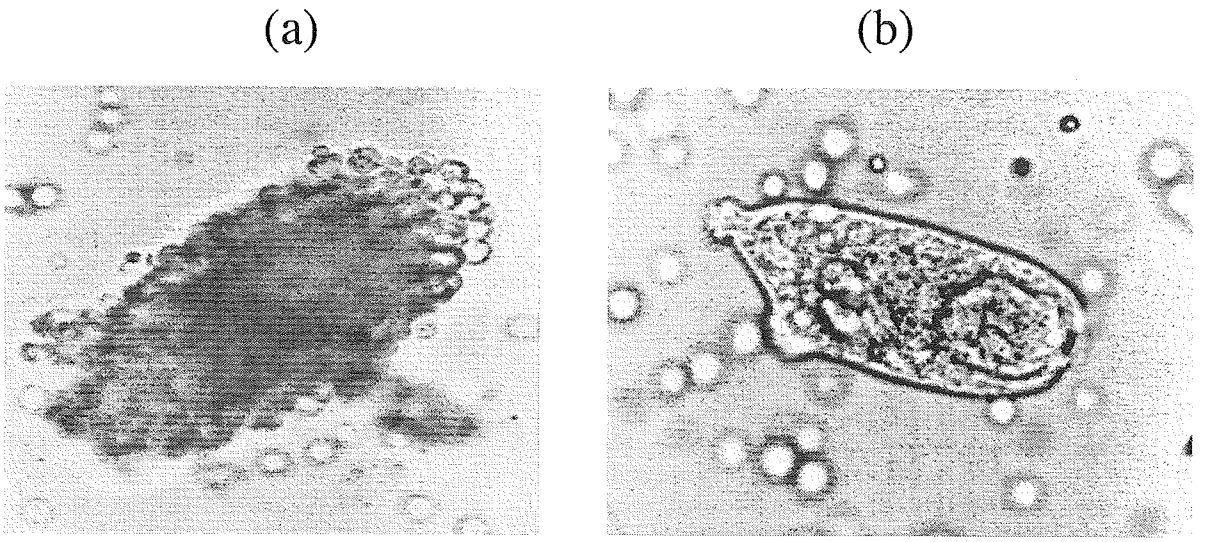

Fig. 1. Adhesion of host peritoneal exudate cells under the presence of calpain-immunized sera in vitro Murine peritoneal exudate cells showed tight adhesion around schistosomulae of *S. japonicum* only when calpain-immunized sera were added (a), however, no such adhesion was observed in the absence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

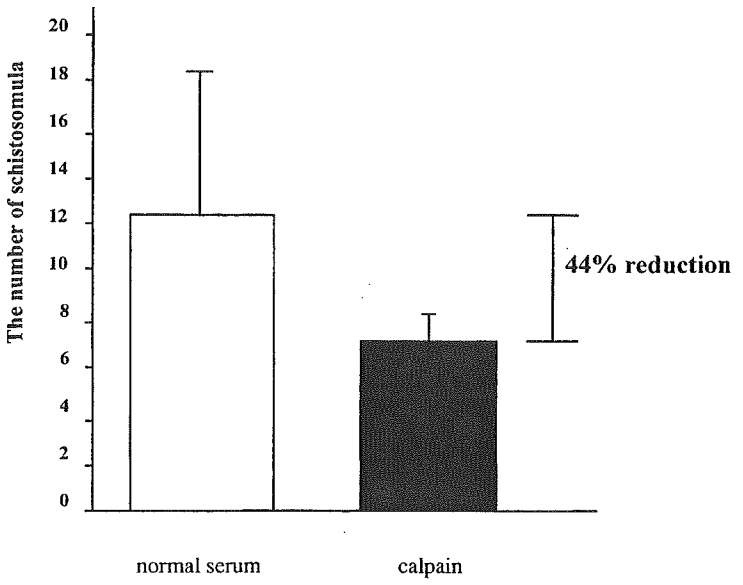

Fig. 2. Passively transferred anti-calpain mAb reduced number of lung-stage larvae of *S. japonicum* TK267, mAb specific to *S. japonicum* calpain was injected into BALB/c mice, and cercariae of *S. japonicum* was challenged. Calpain-injected group (Solid bar) showed reduced number of recovered larvae 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

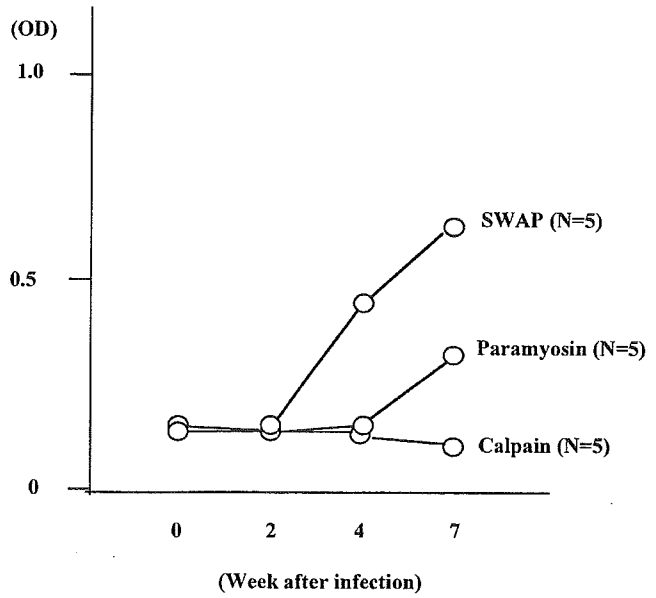

Fig. 3. Booster effects of infection for paramyosin-specific IgG production, but no for calpain in *S. japonicum* infected pigs. Pigs were infected with *S. japonicum* cercariae, and IgG levels to SWAS and vaccine candidates were monitored. Significant level of paramyosin-specific IgG was observed, while 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 [illegible], 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.

Acknowledgments

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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

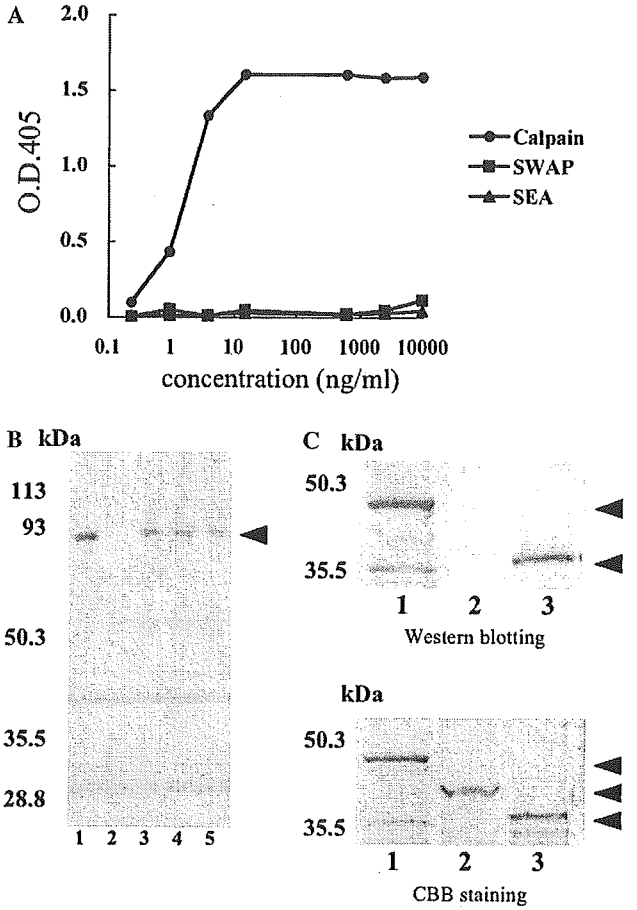

Fig. 1. Preparation and analysis of KG-2E11 by ELISA and Western blotting. (A) KG-2E11 generated from recombinant calpain-immunized BALB/c mice bound to calpain tightly, although SWAP was bound weakly and SEA was not by ELISA. (B) The soluble adult worm antigens (50 μg/well) were electrophoresed in SDS–PAGE. 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%

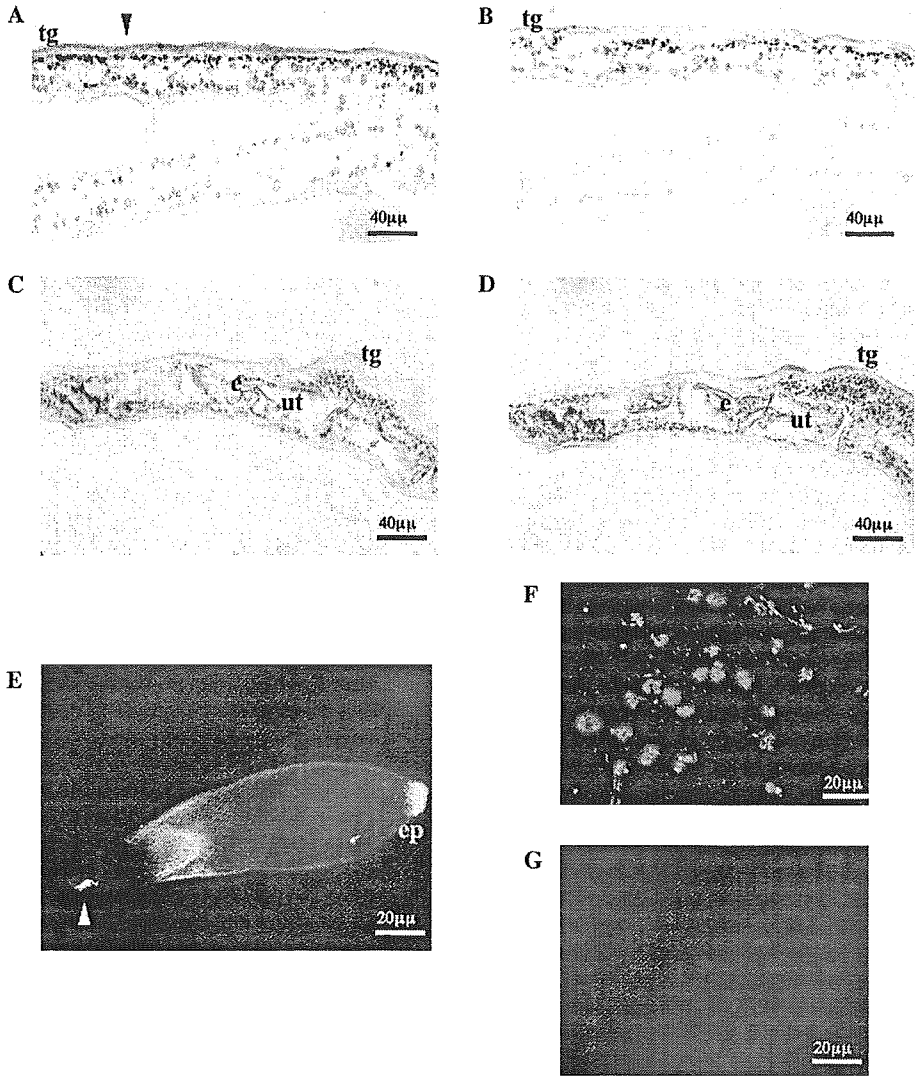

Fig. 2. Localization of calpain in *S. japonicum* with KG-2E11. KG-2E11 bound to the tegument (tg) of male worms (A), but did not bind to the tegument (tg) and the uterus (ut), including eggs (e), of female worms (C). Isotype control mAb was non-binding (B,D). Cercariae were stained with KG-2E11, and 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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Molecular evolutionary analyses implicate injection treatment for schistosomiasis in the initial hepatitis C epidemics in Japan

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***Background/Aims*: The mortality due to hepatocellular carcinoma (HCC) has ranged widely in various areas of Japan since 30 years ago and the incidence was particularly high in once *Schistosoma japonicum* (*Sj*)-endemic areas. Our aim was to estimate the spread time of hepatitis C virus (HCV) infection in the past with possible relevance to a higher incidence of HCC in once *Sj*-endemic than *Sj*-nonendemic areas.**

***Methods*: During 2001, 131 strains of HCV-1b were obtained from patients in three previously *Sj*-endemic areas, as well as *Sj*-nonendemic areas in Japan and a cross-sectional study was conducted on them with molecular evolutionary analyses.**

***Results*: A phylogenetic tree reconstructed on HCV-1b sequences in the NS5B region disclosed 2 independent clusters for *Sj*-positive and -negative groups with a high bootstrap value. The estimated effective number of HCV-infections indicated a transition from quiescence to rapid exponential growth in the 1920s among patients with schistosomiasis, which is 20 years earlier than that among patients without schistosomiasis.**

***Conclusions*: The estimated spread time in previously *Sj*-endemic areas in Japan coincides with injection treatment for *Sj* since 1921. A high incidence of HCC there would be attributed to a long duration of HCV infection since 1920s.**

***Keywords*: Hepatitis C virus; *Schistosoma japonicum*; Molecular evolutionary analysis; Hepatocellular carcinoma**

1. Introduction

Recently, the molecular clock has been successfully applied to long-term serial serum samples containing hepatitis C virus (HCV) from the US and Japan and estimated the spread time of HCV in the 1930s in Japan, which is 30 years earlier than that in the US in the 1960s [1]. Insofar as a long duration of HCV infection is the most important factor for the development of hepatocellular

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Abbreviations HCV, hepatitis C virus; Anti-HCV, antibody to HCV; HCC, hepatocellular carcinoma; *Sj*, *Schistosoma japonicum*.

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carcinoma (HCC), it can be predicted that the incidence of HCC will increase in the US over the next 2–3 decades. Thus, a combination of classical epidemiological approaches and molecular evolutionary analyses would be particularly useful in the study of contagious diseases, typified by HCV infection.

The way how individuals contracted HCV infection has remained unclear in Japan. Recently, a Japanese report (Ministry of Health, Labour and Welfare: Distribution of age-adjusted mortality rate from liver cancer by prefecture between 1971 and 1975, Tokyo, 2001) indicated that the mortality due to HCC has already varied widely in various areas of Japan since 30 years ago; the incidence of HCC was much higher in Saga/Fukuoka, Hiroshima and Yamanashi Prefectures, which were once endemic for schistosomiasis japonica in the long past. Hence, a high incidence of HCC in the 1970s would be related to HCV transmitted by injection treatment for *Schistosoma japonicum* (*Sj*) conducted since 1921 in these areas. In fact, shared needles and syringes for intravenous injection treatment with antimonyl potassium tartrate or sodium antimony tartrate posed a significant risk for HCV transmission in endemic areas [2]. Indeed, the prevalence of antibody to HCV (anti-HCV) is high (36.5; 95% CI=28.1–44.9%) in patients with chronic schistosomiasis [2] and therefore, HCV infection is considered responsible for the development of HCC in patients with chronic schistosomiasis.

Since, once popular intravenous injection for schistosomiasis was a risk factor for HCV transmission, the spread time of HCV in the areas once endemic for *Sj* in Japan would deserve determination. In this study, molecular evolutionary analyses using principles of both population genetics and mathematical epidemiology [3] were applied to HCV-infected patients with and without a past history of chronic schistosomiasis in once *Sj*-endemic areas.

2. Meterials and methods

2.1. Sample collection

In Japan during 2001, 181 random serum samples positive for anti-HCV were obtained from patients with chronic liver disease in widely separated areas previously endemic for *Sj*, including Kofu in Yamanashi (n=75), Katayama in Hiroshima (n=50) and Chikugo in Saga/Fukuoka Prefectures (n=56). Schistosomiasis was diagnosed by ultrasonographic (US) and/or computer tomographic (CT) modalities or serological examinations [4]. Two kinds of serological tests, which can detect past history of schistosomiasis, were available in this study. In brief, IgG antibodies binding to two different *schistosome antigens, Sj* adult worm antigen and *Sj* egg antigen, were detected using an enzyme-linked immunosorbent assay (ELISA). As it is now accepted that ELISA titer of egg antigen-specific IgG is reliable for case-detection rather than IgG for adult worm antigen [4–6], the results based on the egg antigen-specific IgG were accepted in this study. Samples of more than 0.25 of optical density at 415 nm were determined to be positive, as previously confirmed [4–6]. The serum samples were tested for anti-HCV by Lumipulse II Ortho HCV (Ortho-Clinical Diagnostics K.K., Tokyo, Japan). As patients with *Sj* treatments were estimated to be old, relatively older patients were selected in the *Sj*-endemic areas to match age factor that might influence duration of HCV infection or HCC incidence. For a cross-sectional study, 30 serum samples were obtained from patients infected with HCV in Aichi Prefecture where *Sj* has not been endemic. The age- and sex- matced patients were also selected from the *Sj*-nonendemic areas excluding influece of these factors on HCC incidence. The study protocol conformed to the 1975 Declaration of Helsinki and was approved by Ethic Committees of institutions. Every patient gave a written informed consent to participate in the virological research of HCV. Information of injection treatment for *Sj* was obtained by means of self-administrated questionnaires or structured interviews. None had been treated with interferon therapy for HCV infection. HCC incidence was estimated by historical information from patients themselves and/or medical records during 2001. HCC was diagnosed by liver biopsy or combination of imaging modalities such as US, enhanced CT and angiography.

2.2. Genotyping and sequencing

Nucleic acids were extracted using a SepaGean RV-R Nucleic acid extracting kit (Sanko Junyaku Co., Ltd., Tokyo, Japan) in accordance with the manufacturer's protocol. They were reverse-transcribed to cDNA using SuperScript II Rnase H^- Reverse Transcriptase (Invirogen Corp., Carlsbad, California, USA) and random hexamer primer (Takara Shuzo Co. Ltd, Tokyo, Japan) by the method described previously [7].

A sequence spanning 339 nucleotides (nt) in the NS5B region was amplified by polymerase chain reaction (PCR) with primers described previously [1]. PCR products were directly sequenced with Prism Big Dye (Applied Biosystems, Foster City, California, USA) in an ABI 3100 DNA automated sequencer. To reduce the number of artificial substitutions arising in PCR, PLATINUM Pfx DNA Polymerase (Invirogen Corp.) with a very high fidelity was used. The sequences determined were utilized to confirm HCV genotypes and construct phylogenetic trees.

2.3. Test for clustering between Sj-positive and -negative groups

The phylogenetic tree was first constructed to examine the evolutionary history for *Sj*-positive and *Sj*-negative groups by the neighbor joining method [8]. Furthermore, to test whether either *Sj*-positive or *Sj*-negative group have evolved independently or not, we conducted an interior branch test for the neighbor-joining tree [9]. Thereafter, a t-test was conducted for the interior branch length and its standard error, which is computed using the bootstrap procedure.

2.4. Demographic model

A reconstructed tree was built on the NS5B sequence of 339 nt by a heuristic maximum-likelihood topology search with stepwise-addition and the nearest neighbor-interchange algorithms. Tree likelihood scores were calculated using HKY85 with the molecular clock enforced by PAUP version 4.0b8.

As estimates of the demographic history, a nonparametric function $N(t)$, known also as the skyline plot, was obtained by transforming coalescent intervals of an observed genealogy into a piecewise plot that represents an effective number of infections through time [3,10]. A parametric maximum-likelihood was estimated by several models with the computer software Genie v3.5 to build a statistical framework for inferring the demographic history of a population on phylogenies reconstructed on sampled DNA sequences [10]. This model assumes a continuous epidemic process in which the viral transmission parameters remain constant through time. Model fitting was evaluated by likelihood ratio tests of the parametric maximum-likelihood estimates [11,12].

2.5. Statistical method

Data for continuous variables were demonstrated as the mean ± standard deviation. The Fishers' exact test, Chi square test with Yates' correction and one-way ANOVA followed by the Scheffe's multiple comparison test were used to evaluate differences in the mean age, sex ratio

and incidence of HCC between groups, respectively. Differences with P values less than 0.05 were considered significant.

3. Results

Of 181 anti-HCV positive samples, 113 were classified into HCV genotype 1b (HCV-1b), which is predominant in Japan. Fifty-two of 181 samples (29%) were negative for HCV RNA or incomplete for sequencing and the remaining 16 samples (9%) of genotype 2a were excluded in this study due to a minor population. Of the HCV-1b strains, 47 were recovered from patients in Yamanashi, 31 in Hiroshima and 35 in Saga/Fukuoka Prefectures. Along with 18 HCV-1b strains in Aichi Prefecture serving as controls, a cross-sectional study was conducted on them with molecular evolutionary analyses. The patients in areas previously endemic for *Sj* revealed a significantly higher prevalence of chronic schistosomiasis [24/47 (51%) in Yamanashi (Kofu area), 21/31 (68%) in Hiroshima (Katayama area) and 19/35 (54%) in Saga/Fukuoka (Chikugo area)] than that in Aichi Prefecture (0/18 [0%], $P<0.0001$). There were no significant differences in the mean age or sex ratio among patients from these four areas (Fig. 1). Although the mean age of *Sj*-positive patients was just higher than that of *Sj*-negative patients in once *Sj*-endemic areas or matched-control patients in Aichi Prefecture, there were also no significant differences between these groups (Table 1).

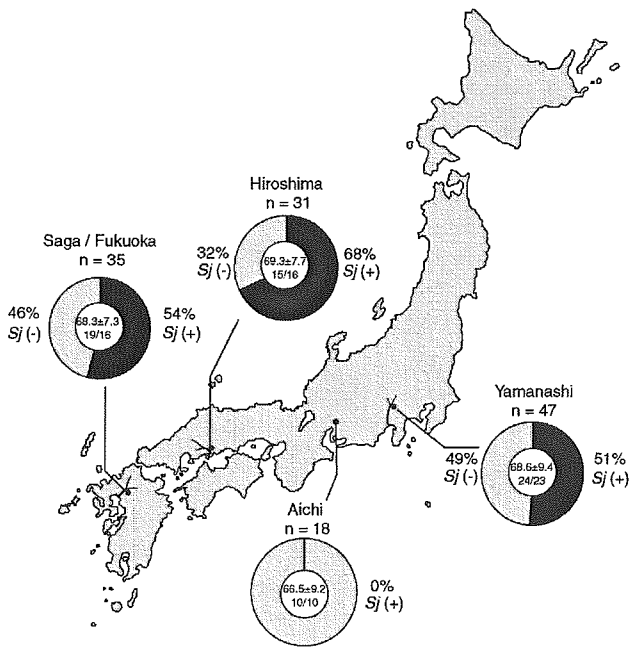

Fig. 1. Geographic distribution of *Schistosoma japonicum* (*Sj*) and characteristics of patients infected with HCV. *Sj* (+) and *Sj* (−) denote, respectively, presence and absence of infection with *Sj* diagnosed by ultrasonographic and/or computer tomographic methods or serological examinations. Pie graphs include the age (mean± standard deviation) and sex ratio (male/female).

Table 1
Characteristics of patients with and without schistosomiasis

	Schistosoma japonicum		Controls (Aichi)
	Positive ($n=64$)	Negative ($n=49$)	($n=18$)
Mean age			
Total	69.9±7.7	67.4±8.7	66.5±9.2
Yamanashi	69.9±7.2	67.3±11.2	
Hiroshima	71.2±8.7	67.6±6.5	
Saga/Fukuoka	69.0±7.7	67.5±7.1	
Sex (male/female)			
Total	34/30	24/25	9/9
Yamanashi	13/11	11/12	
Hiroshima	10/11	5/5	
Saga/Fukuoka	11/8	8/8	
Incidence of HCC	25/55 (45%)	11/48 (23%)	3/18 (17%)

The incidence of HCC in *Sj*-positive patients was significantly higher than that in *Sj*-negative patients ($P=0.0226$) or controls ($P=0.0488$).
Abbreviations: HCC, hepatocellular carcinoma.

For cross-sectional study on the viral population size between HCV-infected patients with and without a past history of schistosomiasis, a phylogenetic tree for HCV-1b strains in the *Sj*-positive and -negative patients was constructed with use of the maximum-likelihood method enforced by the molecular clock as introduced in our previous report [1] and an independent study by Pybus et al. [3]; a substitution rate of 5.3×10^{-4} per site per year [1,3] was assumed for HCV. The phylogenetic tree disclosed 2 independent clusters for *Sj*-positive and -negative groups, with a high bootstrap value (81%) by the interior branch testing (Fig. 2), which is comparative with past epidemiological backgrounds in Japan. From distinct evolutionary histories in the two populations, the effective number of HCV-1b infections through time, N (t), were assessed by the skyline plot. The parameters for several models in Genie v3.5 [3,10] were also examined. Time t was then transformed to year using the same rate, assuming the collecting time (year 2001) as the present. Fig. 3 shows the skyline plots and population growth for *Sj*-positive and -negative patients, according to a specific demographic model in Genie v3.5 with three parameters, piecewise expansion growth model, that was evaluated by the likelihood ratio testing [11,12]. Molecular evolutionary results thus obtained supported our previous study in which the divergence time of the most recent common ancestor of HCV-1b in each area in Japan was estimated before 1850 [1]. Our estimates of the effective number of HCV-infections showed a transition from constant size to rapid exponential growth in the 1920s among patients with chronic schistosomiasis in endemic areas, which is 20 years earlier than that among patients without schistosomiasis in the 1940s. Information on HCC was available for 121 of the 131 patients with HCV-1b. Although they were relatively small in number, the incidence of HCC was significantly higher in *Sj*-positive than -negative patients ($P=0.0226$) or controls ($P=0.0488$) (Table 1).

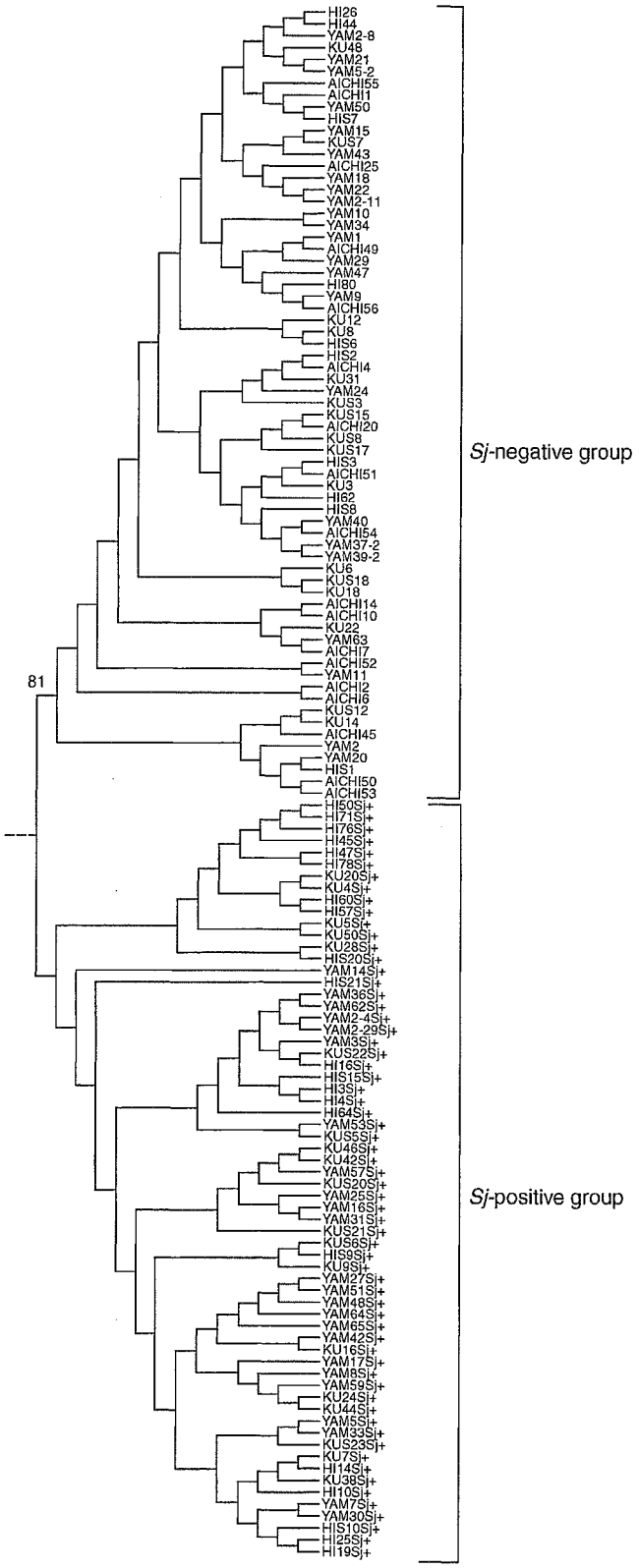

Fig. 2. A phylogenetic tree constructed on NS5B sequences of HCV-1b strains in *Schistosoma japonicum* (*Sj*)-positive ($n=64$) and -negative ($n=67$) groups. The numbers in the tree indicate bootstrap reliability by the interior branch test. *Sj*+ indicates *Sj*-positive strains. YAM; Yamanashi, HI/HIS; Hiroshima, KU/KUS; Saga/Fukuoka, Aichi; control strains.

4. Discussion

The specific demographic model based on the neutral theory [3,11,12], which has a constant size in the past and changes to exponential growth until the present, is applied to investigate the Japanese endemic of HCV. By means of the molecular evolutionary analyses, the spread time of HCV in *Sj*-positive patients was estimated 20 years earlier than that in *Sj*-negative patients from three areas in Japan where *Sj* was previously endemic (Yamanashi, Hiroshima, Saga/Fukuoka Prefectures). The spread time of HCV much earlier in *Sj*-positive than -negative patients indicates that the previous intravenous injection treatment with antimony compounds (antimonyl potassium tartarate or antimony sodium tartarate) on patients with schistosomiasis since 1921 [2] would have been a significant risk factor for HCV transmission in endemic areas through re-used needles and syringes. Indeed, it might be possible that HCV transmission from *Sj*-positive patients to *Sj*-negative patients occurs in the once *Sj*-endemic areas, but we could not find such strains in this study. One of the reasons is that residents in the village around the river, where schistosomiasis had been the most prevalent, might have been isolated from those in the other areas of the same Prefecture in the past due to the endemic disease 'schistosomiasis'. Interestingly, most Japanese strains from *Sj*-nonendemic areas in the database clustered with the *Sj*-negative group of the present study. Hence, factors other than the injection treatment for *Sj*, such as intravenous stimulants popular during and after World War II [13] and medical treatments including transfusion with blood units from paid donors in the past, would have imposed the risk for HCV transmission in most areas in Japan [14]. In addition, there would have been opportunities for HCV transmission through inadequately sterilized needles and syringes in general practices, which have contributed to a large reservoir of chronic HCV infection in Japan during the 1950s [13]. Such inadequately sterilized medical injections were still common in the less-developed world in the 20th century. WHO estimates that unsafe injections result in 2.3–4.7 million new HCV infections worldwide every year [15].

Although the spread time of HCV in *Sj*-positive group was earlier than that in *Sj*-negative group, there was no significant difference of mean age between the 2 groups. Two possibilities are considered. One is a sampling bias; as patients with *Sj* treatments were estimated to be old, relatively older patients were selected in the *Sj*-endemic areas to match age factor that might influence duration of HCV infection or HCC incidence. Second, the ages that patients had been infected with HCV were different between the 2 groups; the treatments for *Sj* in Japan were mainly conducted among relatively younger people including school children after screening of *Sj* [4,16,17], while the

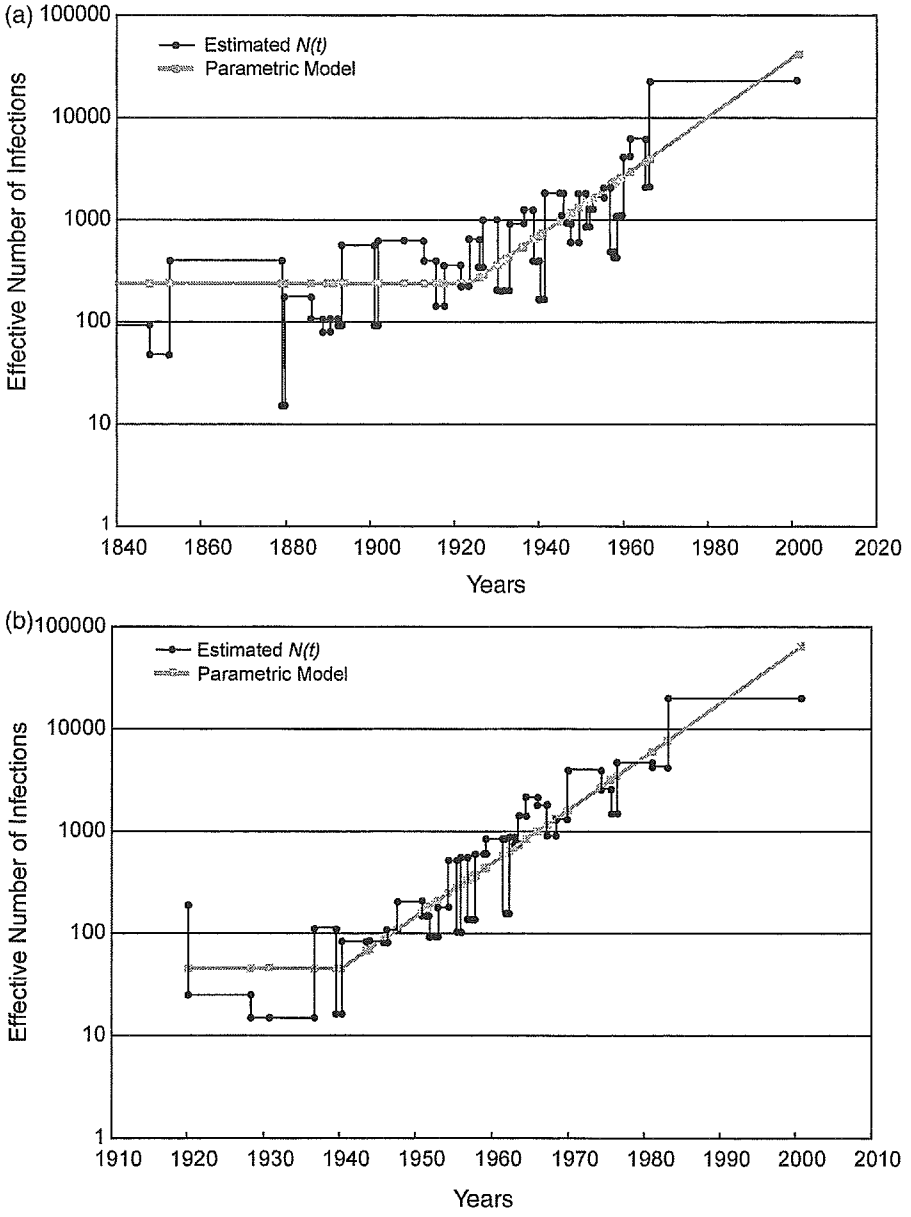

Fig. 3. The maximum-likelihood estimates of $N(t)$ on the effective number of infections with HCV genotype 1b in Japan for *Schistosoma japonicum* (*Sj*)-positive group (a) and *Sj*-negative group (b) separated in the phylogenetic tree (Fig. 2). The parametric model is indicated by the grey line and stepwise plots by the black line that represents corresponding nonparametric estimates of $N(t)$ (number as a function of time). Genetic distances are transformed into a time scale of year using estimates of the molecular clock in the NS5B region.

other risk factors such as blood transfusion were found in older people excluding at least children.

A disease possibly caused by schistosomal infection in Japan is documented in a book written some 300 years ago. In 1847, the clinical picture of this disease was precisely described by Yoshinao Fujii in the book 'Katayama-ki' that documented an endemic disease in Katayama area as Katayama's disease (equivalent to schistosomiasis). Water-borne epidemics of schistosomiasis prevailed in inhabitants around rivers (the tributaries of the Fuji river in Yamanashi, the Takaya river in Hiroshima and the Chikugo river in Saga/Fukuoka) in Japan, mediated by small shellfish (Miyairi-kai) serving as the natural host. More than 200,000 individuals were estimated to have been infected with *Sj* in Yamanashi Prefecture alone during 1965 through 1990 [16] and approximately 1,000,000 patients in the entire Japan since 1920s [17]. To cope with these endemics, more than 10 million intravenous injections with antimony compounds had been given in Japan since 1921 [17]. Thus, Japan would have started ahead of any other countries, in terms of HCV spread in association with schistosomiasis, wherein intravenous drugs were invented. Although acute schistosomal infection has disappeared in Japan since long ago, there are still elderly people with

chronic schistosomiasis in previously endemic areas, some of whom are developing HCC [2,14]. Substantial transmission among regions is supported by the lack of regional clustering of HCV sequences in this study.

A similar situation is reported in the Nile delta in Egypt where schistosomiasis once prevailed mediated by small shellfish [18] and the national campaigns for injection treatment with antimonyl potassium tartarate (tartar emetic) from the 1961 until 1986 are suspected to have given rise to the highest endemicity of HCV in the world ever, involving >20% of the national population there [19]. The prevalence of anti-HCV is extremely high (>70%) in patients with schistosomiasis there [18,20,21]. Highly prevalent HCV infection in the general Egyptian population accounts for most HCC cases in Egypt [22]. A question may arise whether schistosomiasis alone is responsible for the development of HCC. Patients co-infected with HCV and *Schistosoma mansoni* (*Sm*) may have a high incidence of viral persistence, accelerated fibrosis and development of HCC [23,24]. A recent population-based study between two large populations with district histories of *Sm* and hepatitis C infections, however, failed to indicate any interaction between *Sm* infection and the prevalence or severity of hepatitis C [25]. Moreover, no significant histological differences were found between anti-HCV-positive Egyptian patients with and without schistosoma [26]. Hence, the long duration of persistent HCV infection would be a more important factor for the development of HCC than the pathogeneticity of *Sm* itself.

Estimating the effective number of HCV infections has been very informative in looking back epidemic spreads of HCV infection in the Unites States [1] and Egypt [12,27]. In addition, it would also be useful in predicting the population size and extent of HCV infection. Studies to foresee future spreads of HCV would be required to cope with and prevent healthcare problems where de novo infections are increasing. The advantage of molecular evolutionary analyses, its ability to accurately estimate the dynamics of HCV based on a limited number of isolates in particular [3], will extend its application anywhere in the world where clinical sequelae of persistent HCV infection pose increasing burdens on the public health of nations.

In conclusion, the evolutionary analyses indicated that the estimated spread time in previously *Sj*-endemic areas in Japan coincides with injection treatment for *Sj* conducted since 1921. The high incidence of HCC in *Sj*-endemic areas is most likely attributed to long duration of HCV infection there transmitted through injection treatments.

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特集

北米・ヨーロッパでかかる寄生虫症

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SUMMARY

・北米・ヨーロッパでは先進国型寄生虫感染がみられる.
・最近の欧米の疾病構造の特徴から,寄生虫感染症の正確な実態把握ができていない.
・AIDS や高齢者などの日和見寄生虫感染症が多く見られる.
・発展途上国からの人間や物流など交流増加により熱帯寄生虫症も持ち込まれたり,輸血を通じた感染が起こっている.
・ミクロスポーラ症やサイクロスポーラ症などの新興寄生虫感染症のリスクは北米やヨーロッパへの渡航でも低くない.
・一部でマラリア流行の再興が起こってきた.

はじめに

日本人にとって，北米，ヨーロッパに渡航する際に寄生虫感染の可能性を考えることは少ないし，日常診療でそのような地域への渡航歴から寄生虫病を鑑別疾患に想起することは例外的な場合に限られるであろう．しかし，北米やヨーロッパでどのような寄生虫病が存在し，その感染の可能性はどの程度のものか，あるいは世界のヒトや物流の集散地として，持ち込まれた輸入病原体に邦人が感染する可能性はどうか，などの情報を把握しておくことは時に有用であろう．本稿では北米やヨーロッパに渡航する際に感染する可能性のある寄生虫病について概略を整理するとともに，最近の欧米の寄生虫感染の動向や特殊な感染経路などについて説明することを試みた．

I　北米の寄生虫症

北米とはメキシコ，アメリカ合衆国およびカナダを含むが，本稿では情報整備が進んでいるアメリカ合衆国の情況を中心に論じることにしたい．それだけでも亜熱帯から寒帯まで気候風土の異なる地域を包含しており，全体を一つに論じることは困難で，存在する寄生虫相も多様である．

ほかの先進諸国と同様に寄生虫感染症の実態は明らかでない．米国CDCではELISAによる寄生虫感染スクリーニングサービスを実施しているが，年間の依頼件数は5,000件以内で決して多くない．さらに確定診断の依頼は2,000件以下に留まっている．実際に寄生虫症が少ないのか，医師が寄生虫症を鑑別診断に考えないのかの判断はできないが，疫学調査結果によれば米国の寄生虫感染の実数が少ないとは考えられない．確実に把握されている寄生虫症を**表1**にまとめたが，注目されるものについて以下に説明を加えたい．

① クリプトスポリジウム症

本症は牛の腸管寄生原虫である*Cryptosporidium parvum*による代表的な水系感染症である．1993年にMilwaukee市全域で40万人以上の集団発生があり，重症下痢症による死亡者まで出た[1]．水道水からのクリプトスポリジウム検出率は17～55％に上っていたため，水道水の安全基準が見直された．しかし，**自然水の原虫汚染は依然として高く，自然公園や都市公園の噴水などから集団発生する事例が確認されている**[2]．

② バベシア症

マダニによって媒介されるバベシア症は，米国北東部を中心に*Babesia microti*による症例が年間数百例発生している．媒介マダニはライム病媒介種と同一であるため，米国東北部で発生したマダニ咬症では両方の検査が必要であろう．通常，重症化することはないが，免疫機能が低下した人，高齢者および脾臓摘出者では治療抵抗性となり死亡例もある．

③ サイクロスポーラ症

新興寄生虫症であり，米国で汚染輸入フルーツ（ラズベリー）による下痢症の集団発生がみられた[3]．米国内の本原虫の分布，ヒト以外の保虫宿主の存在などよくわかっていない．

表1　欧米で感染の可能性のある寄生虫症

寄生虫症	北　米	ヨーロッパ
原虫		
マラリア	△（南部で定着？）	△（南部，ロシアで再興）
トリパノソーマ	＊（輸血でシャーガス病）	—
リーシュマニア	—	△（南西ヨーロッパ）
トキソプラズマ	◎	◎
クリプトスポリジウム	○（水道水，自然水）	○（水道水，自然水）
ランブル鞭毛虫	○（水道水，自然水）	○（水道水，自然水）
サイクロスポーラ	△（輸入食品）	？（情報なし）
ミクロスポーラ	△（日和見感染）	？（情報なし）
バベシア	△（東北部で*B. microti*）	？（*B. bovis*も？）
その他		○肉胞子虫（食肉）
蠕虫		
腸管寄生線虫	—	？（東欧で再興か）
イヌ/ネコ回虫	△（小児の感染）	？（情報なし）
旋毛虫	△（年間40例程度）	△（集団発生も）
肝蛭	—	—（畜産地域に）
広節裂頭条虫	△（カナダ，アラスカ）	△（北欧で）
エキノコックス	△（中西部以北で発生）	△（中～北部で）

◎：高頻度で見られるもの
○：感染の危険性が低くないと思われるもの
△：感染の可能性は高くないが確実に発生しているもの
—：存在しないか，ほとんど問題がないもの
＊：特殊なケースで問題となるもの