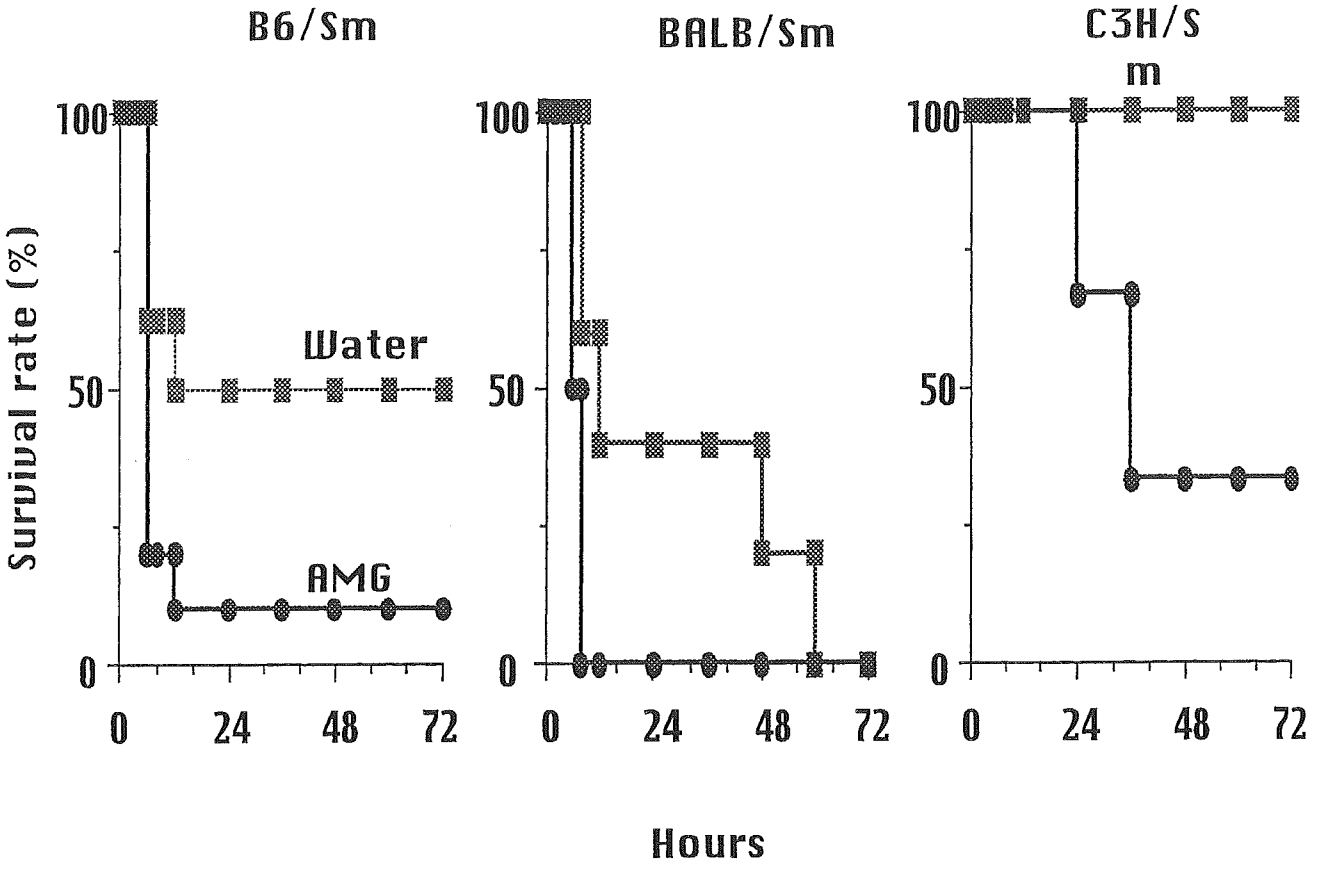

Fig. 10
Effect of aminoguanidine administration on the survival rate of 3 strains of mice infected with *Schistosoma mansoni* after LPS/D-GalN challenge

別紙4

研究成果の刊行に関する一覧表

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Establishment of *Schistosoma japonicum* calpain-specific mouse T cell hybridomas and identification of a T cell epitope that stimulates IFNγ production

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Received 22 April 2004; received in revised form 14 October 2004; accepted 19 October 2004
Available online 8 December 2004

Abstract

Calpain is a calcium-dependent cystein protease, and the homologues of schistosome are known as one of vaccine candidate molecules against schistosomiasis. Here, we established two IL-2 producing T cell hybridoma cell lines specific for *Schistosoma japonicum* calpain, to identify T cell epitope(s) on the molecule. Overlapping 15mer oligopeptides of calpain were synthesized and tested for their stimulatory abilities to the hybridomas. As a result, epitopes recognized by the two hybridoma lines were the same: EQLKIYAQRC. Spleen cells from calpain multiple antigenic peptide (MAP)-immunized BALB/c mice produced IFNγ upon stimulation with MAP or soluble worm antigen preparation (SWAP). The identification of the T cell epitope to stimulate Th1 response will contribute to the proper design of synthetic vaccines, evaluation of their protective potentials and elucidation of protective mechanisms in murine experimental schistosomiasis.

Keywords: *Schistosoma japonicum*; Calpain; T cell epitope

1. Introduction

Schistosomiasis is one of the most prevalent parasitic diseases and is affecting 200 million people worldwide [1]. In order to control this disease, various control measures have been conducted for a long time. For instance, mass chemotherapy to control morbidity, snail control to reduce transmission, improvement of sanitary conditions to reduce influx of stool/urine eggs into water bodies. These efforts, however, succeeded in controlling or eliminating schistosomiasis only in some parts of endemic areas in the world; e.g., Japan, Caribbean islands and Tunissia [1]. One of the major difficulties in achieving successful control is rapid re-infection after chemotherapeutic treatment. For this reason, vaccine development is urgently needed. Until now, there have been reported many vaccine candidates; e.g., glutathione-*S*-transferase (GST) [2], triose phosphate isomerase [3], fatty-acid binding protein [4], myosin (IrV-5) [5], 22.6 kD antigen [6], glyceraldehyde-3P-dehydrogenase (G3PDH) [7], paramyosin [8–10] and calpain large subunit [11–15]. Among them, paramyosin [9] and calpain [12,15] are the molecules which have been reported to exhibit protective effects through Th1 responses. Especially, *Schistosoma mansoni* calpain has been identified as a target molecule of a protective Th1 cell clone [12].

Calpain large subunit is a cystein protease which contains four domains [11]. *Schistosoma japonicum* calpain ho-

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doi:10.1016/j.vaccine.2004.10.042

mologue was cloned and its immune stimulatory potential was demonstrated by the presence of calpain-specific antibodies in the sera of schistosomiasis patients [14]. In this same report, we also demonstrated that the sera from light-infection cases showed stronger reactivities to recombinant calpain than the sera from moderate/high infection cases. Additionally, vaccination with recombinant *S. japonicum* calpain fragment (A.A. 220–376) partially protected BALB/c mice against cercarial challenge [15]. The protective mechanism is still unclear, but it is at least partially due to Th1-biased immune responses [15].

In this study we established T cell hybridoma cell lines specific for *S. japonicum* calpain. The hybridomas produced IL-2 in the presence of syngeneic antigen presenting cells (APC) and a recombinant GST-calpain fusion protein. A T cell epitope EQLKIYAQRC was identified by comparison of stimulatory activities of synthetic oligopeptides and the epitope was shown to have Th1 response-inducing activity. Potential protective activities of the sequence are also discussed.

2. Materials and methods

2.1. Maintenance of S. japonicum (Japanese Yamanashi strain) life cycle

Outbred ICR mice (Japan SLC, Inc., Hamamatsu, Japan) were infected with 30–40 cercariae through shaved abdomen percutaneously. Six weeks later, the infected mice were perfused with physiological saline containing 0.45% tri-sodium citrate to recover adult worms. The collected worms were washed with physiological saline and frozen at −70 °C. Granulomatous livers of infected mice were minced and homogenized in a warring blender. The liver homogenate was centrifuged at 200 × *g* for 3 min repeatedly to make the supernatant clear. The egg-containing pellet was poured into aged tap water and kept under light for 1 h. Miracidia were collected and used for infecting intermediate host snails (*Oncomelania hupensis nosophora*), which were collected in the Kofu basin, ex-endemic area of schistosomiasis japonica. Each snail was infected with 4–5 miracidia. Four to six months later, the infected snails were crushed and emerging cercariae were used for the next infection of mice.

2.2. Soluble worm antigen preparation (SWAP)

Frozen worms were dispersed in phosphate buffered saline (PBS, pH 7.4) by ultrasonic treatment. The homogenate was centrifuged at 40,000 × *g* for 20 min. The supernatant was then collected and its protein concentration was determined. This antigen preparation was kept at −70 °C until use.

2.3. Preparation of recombinant calpain and its deletion fragments

The section of *S. japonicum* calpain (A.A. 220–376) that has been reported to be protective [15] was introduced to BamHI-EcoRI site of pGEX-2TK vector (Amersham Biosciences UK, Ltd., Little Chalfont, UK). Expression of the recombinant protein was induced by 0.1 mM IPTG. Harvested cells from 200 mL culture were lyzed with 4 mL of BugBuster® Protein Extraction Reagent (EMD Biosciences, Inc., San Diego, CA) containing 5 units/mL of Benzonase® Nuclease (EMD Biosciences, Inc.) by vigorous shaking at room temperature for 20 min. Then the lysate was centrifuged at 25,000 × *g* for 20 min. An insoluble pellet was washed with PBS containing 1% Triton X-100 twice to remove membrane proteins. The washed pellet (inclusion body) was then solubilized with 4 mL of denaturing solution (8 M Urea and 5 mM DTT in PBS) by pipetting and vigorous shaking for 20 min. By the addition of 3-fold volumes of PBS containing 5 mM DTT, urea concentration of the solution was adjusted to 2 M. The GST-calpain fusion protein was purified by using Glutathione-Sepharose 4B® gel (Amersham). Alternatively, after the fusion protein was applied to the gel, the GST portion and calpain portion were separated with thrombin. By this treatment, only the calpain portion was eluted. The purified proteins were dialyzed in PBS to remove urea and DTT. Protein concentration was measured by the Protein Assay Kit® (Bio-Rad Laboratories, Hercules, CA). For immunization of mice to produce T cell hybridomas, the purified calpain was used. For cytokine production experiments (including hybridoma screening), GST-calpain fusion protein was used to stimulate cells. To exclude the possibility that hybridomas reacted to GST portion of the fusion protein, purified GST was simultaneously used in the same experiments. As for cytokine production assay using GST-calpain deletion fragments, inclusion body suspensions in PBS were used for stimulation of hybridomas because it was difficult to obtain the recombinant proteins as soluble forms in PBS.

2.4. Immunization of mice and culture of lymph node (LN) cells

Female BALB/c mice were immunized with 5 μg of purified recombinant calpain fragment in emulsified condition with Freund complete adjuvant (FCA, Difco Laboratories, Detroit, MI). The emulsion was injected into the four footpads and at the base of the tail intradermally. One week later axillary, inguinal and popliteal LNs were collected and pooled. LN cells were then cultured at 3×10^6/mL for 4 days in the presence of 2 μg/mL recombinant calpain fragment and 20 units/mL of recombinant human IL-2 (Roche Diagnostics, Mannheim, Germany). Then the cells were harvested and supplied for fusion.

2.5. Fusion and screening of T cell hybridomas

The cultured LN cells and BW5147.G.1.4 (ATCC No. TIB-48) thymoma cells were mixed at 2:1 ratio. One milliliter of 50% polyethylene glycol 1500 solution (Roche Diagnostics) was added to the cell pellet under continuous gentle shaking at 37 °C. FCS-free DMEM medium (Sigma-Aldrich,

St. Louis, MO) was further added and centrifuged at 250 × *g* for 5 min. The cells were re-suspended in DMEM supplemented with 10%FCS, 100U/mL penicillin and 100 μg/mL streptomycin. The final density of the thymoma cell was approximately 1×10^6/mL. One hundred microlitres of the suspension was poured onto 96-well microplates and cultured in the medium supplemented with HAT (Sigma-Aldrich) for 2 weeks. Normal thymocytes of BALB/c mice were added to the culture as feeder cells for hybridoma growth. If cell proliferation was observed in some wells, the cells were transferred to 24-well plates and further expanded. Screening of the cells was performed by IL-2 producing activity in the presence of syngeneic spleen cells and GST-calpain fusion protein. To deny the possibility that the cells react to GST portion of the fusion protein, recombinant GST was also used for stimulation in the screening step. Cloning of positive cells was performed by repeated limiting dilutions. Established clones were tested again for their reactivity. Their surface markers (CD3, CD4 and CD8) were examined by flow cytometry.

2.6. Cytokine production assay of T cell hybridoma

For screening and subsequent experiments, 1×10^5 T cell hybridoma cells and 2×10^5 syngeneic BALB/c spleen cells were co-cultured in the presence of antigens (5 μg/mL of GST-calpain, 50 μg/mL of GST, 25 μg/mL oligopeptides or 50 μg/mL suspension of inclusion bodies) in 200 μL of DMEM supplemented with 10% FCS, 100 U/mL penicillin and 100 μg/mL streptomycin. Twenty-four hours later, culture supernatants were collected and stored at −70 °C until measurement of IL-2. The ELISA Development Kit® for mouse IL-2 (Techne Corp., Minneapolis, MN) was used to measure the levels of IL-2 in the supernatants. In some experiments for multiple cytokine assays, cultures were performed in 0.5–1.0 mL under the same cell density conditions as above.

2.7. Synthesis of oligopeptides and MAP

Pentadecamer oligopeptides (for epitope screening) and multiple antigenic peptides (MAP) of the epitope sequence (for cytokine assay) were synthesized by Biologica Co., (Nagoya, Japan) and Nikka Techno Service Co., Ltd. (Hitachi, Japan), respectively. For the construction of MAP, 5 amino acids of N-terminus and C-terminus flanking the identified core epitope (EQLKIYAQRC) were included; i.e., the sequence was PQDLI-EQLKIYAQRC-CLMGC. The lyophilized powders of synthetic peptides and MAP were dissolved or suspended in PBS and then used for stimulation of T cell hybridomas or MAP-immunized mouse spleen cells.

2.8. Immunization of mice with MAP and cytokine production assay

Lyophilized MAP powder was suspended and homogenized in PBS by ultrasonic treatment because it was not soluble in PBS. MAP suspension (100 μg/mL) was mixed with an equal volume of FCA and emulsified. Two hundred microlitres of the emulsion (containing 10 μg of MAP) was injected into two sites of dorsal skin of BALB/c mice subcutaneously. Three weeks later, similarly prepared Freund incomplete adjuvant (FIA, Difco) emulsion of MAP was injected as a booster immunization. Three weeks after the booster immunization, the mice were used for the cytokine production experiments. Spleen cells (3×10^6) of the mice were cultured with antigens in RPMI 1640 supplemented with 10%FCS, 100 U/mL penicillin, 100 μg/mL streptomycin and 50 μM 2-mercaptoethanol in 0.5 mL culture. Concentrations of MAP, oligopeptides and SWAP used for stimulation were 20, 20 and 100 μg/mL, respectively. Forty-eight hours later, supernatant was collected and kept at −70 °C until use. Cytokines were measured using the Ready-Set-Go! Kit® (eBioscience, San Diego, CA) or the ELISA Development Kit® for mouse cytokines (Techne).

3. Results

3.1. Establishment of T cell hybridomas specific for S. japonicum calpain

Two lines of calpain-specific T cell hybridomas were established. The profiles of CD expression and cytokine production of them are summarized in Table 1a and b, respectively. When stimulated with GST-calpain fusion protein, both cell lines produced IL-2 in the presence of syngeneic spleen cells but did not produce IL-2 in the presence of non-syngeneic spleen cells (Table 1b). Both hybridomas did not produce detectable levels of IFNγ and produced a very little amount of IL-4 (Table 1b).

Table 1
Characteristics of calpain-specific T cell hybridomas

(a) Expression of CD molecules

Clone	CD3	CD4	CD8
BALB/SjCalp.1	+	+	−
BALB/SjCalp.71	+	−	−

(b) Cytokine production

Clone	APC	GST-calpain	IL-2	IFNγ	IL-4
BALB/SjCalp.1	BALB/c	−	<16	<16	<4
	BALB/c	+	1697 ± 71	<16	19 ± 2
	C3H/He	+	<16	NT[a]	NT
	C57BL/6	+	<16	NT	NT
BALB/SjCalp.71	BALB/c	−	<16	<16	<4
	BALB/c	+	1268 ± 125	<16	17 ± 2
	C3H/He	+	<16	NT	NT
	C57BL/6	+	<16	NT	NT

The hybridoma clones were stimulated in the presence of APC and GST-calpain (5 μg/mL). Representative data of similar experiments performed. Error values are S.D. of triplicate assays.

[a] NT: not tested. The units of cytokines are pg/mL.

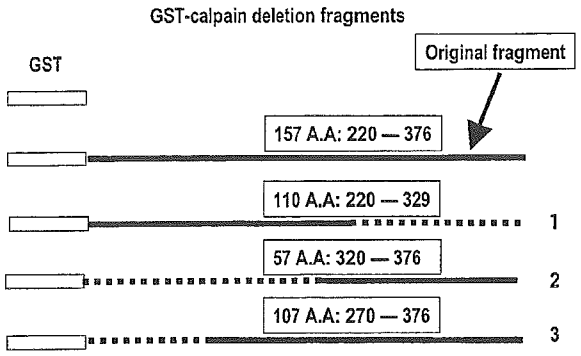

Fig. 1. Schematic presentation of GST-calpain (original fragment) and three deletion fragments (No. 1–3). Fragment No. 1 and No. 2 overlapped 10 amino acids. All fragments were expressed as fusion proteins with GST and used for cytokine assay without removal of GST portion.

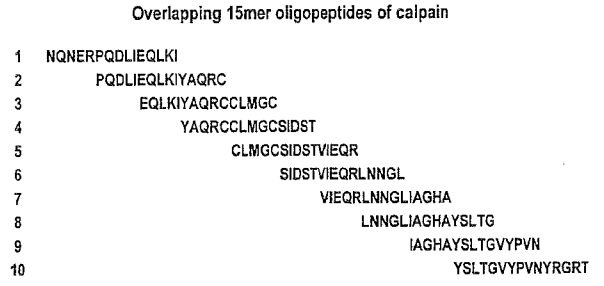

Fig. 2. Schematic presentation of synthetic 15mers covering the central portion (A.A. 270–329) of the original calpain fragment. All oligopeptides overlapped 10 amino acids with the following oligopeptides.

3.2. *Identification of a T cell epitope recognized by the T cell hybridomas*

Firstly, we narrowed the region of the calpain fragment that contains T cell epitope(s). We produced three GST-calpain deletion fragments as shown in Fig. 1. Then the stimulatory activity of each fragment for IL-2 production was examined. As summarized in Table 2, only fragments 1 and 3 had activity to stimulate both hybridomas. We concluded that there must be epitopes in the central portion (A.A.270–329) of the calpain original fragment. Based on this result, we synthesized overlapping 15mer oligopeptides covering the central portion of the calpain fragment and stimulatory activities of each oligopeptides were examined. The oligopeptide No. 2 (PQDLIEQLKIYAQRC) and No. 3 (EQLKIYAQRCCLMGC) stimulated both T cell hybridomas to produce IL-2 (Fig. 2 and Table 3). Therefore, the core epitope of the calpain fragment recognized by the hybridomas was determined to be EQLKIYAQRC.

3.3. *IFNγ producing activity of synthetic MAP-immunized mouse spleen cells*

IFNγ is known to be a key cytokine in protective immunity against schistosome in mice [17–19]. As cytokine profiles of T cell hybridomas do not reflect those of original T cells [16], it is necessary to know the IFNγ producing potential of the identified T cell epitope. Accordingly, we synthesized MAP, immunized BALB/c mice with it and examined IFNγ production by spleen cells from immunized mice. As shown in Table 4, spleen cells from the immunized mice produced IFNγ upon stimulation of the MAP. IFNγ production was also observed when the same cells were stimulated with the oligopeptide No. 2 or No. 3, which contains the core epitope sequence (Fig. 2, Table 4). Importantly, soluble worm antigen preparation (SWAP) stimulated the sensitized spleen cells to produce IFNγ. In addition, low levels of Th2 cytokine (IL-4 and IL-10) production were observed in the antigen-stimulated spleen cell culture supernatants.

4. Discussion

There are a large number of reports on vaccine-induced immunity in murine experimental schistosomiasis. Many of them focused on vaccine-induced immunity induced by attenuated cercariae. The crucial importance of IFNγ and Th1 immune responses have been shown in such reports on *S. mansoni* [17–19]. Likewise, the importance of IFNγ was suggested in protection against *S. japonicum* infection [20]. Also in human schistosomiasis mansoni, IFNγ was produced in a larger amount by PBMC in endemic normal individuals [21]. Therefore, it is meaningful to develop vaccines that exhibit their protective immunity through Th1 responses, in addition to developing antibody-based vaccines.

Calpain of *S. mansoni* was identified as a target molecule of protective Th1 clone of C57BL/6 mice [12]. In this context,

Table 2
IL-2 production by hybridoma cells upon stimulation with calpain deletion fragments

Clone	Soluble forms[a]		Inclusion body suspensions[b]			
	GST	GST-calpain	GST-calpain	GST-calpain deletion fragments		
				1	2	3
BALB/SjCalp.1	37 ± 37	>2000	961 ± 69	890 ± 132	20 ± 16	993 ± 170
BALB/SjCalp.71	77 ± 30	>2000	541 ± 45	468 ± 50	<16	465 ± 67

The unit of IL-2 is pg/mL. Each deletion fragment is shown in Fig. 1. Representative data of similar experiments performed. Error values are S.D. of triplicate assays.

[a] T cell hybridoma clones were stimulated with soluble forms of 50 and 5 μg/mL of GST and GST-calpain, respectively.

[b] As for inclusion bodies, 50 μg/mL suspensions were used for stimulation.

Table 3
IL-2 production by hybridoma cells upon stimulation with each oligopeptide

Clone	GST	GST-calpain	GST-calpain oliogopeptides									
			1	2	3	4	5	6	7	8	9	10
BALB/SjCalp.1	22 ± 0	1600 ± 76	24 ± 8	>2000	1637 ± 124	23 ± 1	23 ± 16	<16	19 ± 4	23 ± 4	18 ± 2	25 ± 4
BALB/SjCalp.71	<16	1313 ± 119	<16	>2000	927 ± 73	<16	<16	<16	<16	24 ± 18	34 ± 20	<16

The unit of IL-2 is pg/mL. The sequence of each oligopeptide is shown in Fig. 2. T cell hybridoma clones were stimulated with 50, 5, and 25 μg/mL of GST, GST-calpain and oligopeptiodes, respectively. Representative data of similar experiments performed. Error values are S.D. of duplicate assays.

Table 4
Cytokine production by spleen cells from MAP-immunized BALB/c mice

Immunization	Cytokine	No Ag	MAP	Peptide 2	Peptide 3	SWAP
PBS + FCA/FIA	IFNγ	<78	<78	<78	<78	407 ± 241
	IL-4	11 ± 2	9 ± 2	8 ± 2	8 ± 2	20 ± 2
	IL-10	29 ± 13	<16	33 ± 17	16 ± 26	<16
MAP + FCA/FIA	IFNγ	<78	4903 ± 487	3064 ± 550	2676 ± 791	3807 ± 1431
	IL-4	<4	49 ± 1	59 ± 5	54 ± 2	54 ± 5
	IL-10	20 ± 20	46 ± 19	96 ± 33	44 ± 12	40 ± 25

The unit of cytokines are pg/mL. See Fig. 2 for sequences of peptide 2 and 3. The spleen cells were stimulated with MAP, oligopeptides (each 20 μg/mL) or SWAP (100 μg/mL). Representative data of similar experiments performed. Error values are S.D. of triplicate assays.

we cloned an *S. japonicum* homologue of calpain large subunit [14] and demonstrated that a recombinant calpain fragment induced Th1-biased protective immunity in BALB/c mice in our previous studies [15]. Based on these results, here we tried to identify T cell epitope(s) of the protective calpain fragment of *S. japonicum* by the establishment of T cell hybridomas from BALB/c mice. Then we confirmed IFNγ producing potential of the epitope.

We immunized BALB/c mice with purified recombinant calpain fragment and established two lines of T cell hybridoma. Both lines produced IL-2 upon stimulation with GST-calpain fusion protein but did not produce IL-2 upon stimulation with GST (Tables 2 and 3), indicating that these hybridomas were specific for the calpain portion of the fusion protein. Then, their CD expression and cytokine profiles were briefly tested. As expected, both were positive in CD3 molecule (Table 1a). As for CD4/CD8 molecules, BALB/SjCalp.1 was CD4 -positive and BALB/SjCalp.71 was double negative (Table 1a). According to the brief cytokine production assay, only IL-2 was prominently produced by both hybridomas (Table 1b). IFNγ was not produced at a detectable level and IL-4 was produced at a very little amount (Table 1b). As these results may be due to the timing of supernatant collection that is appropriate for IL-2 assay (24 h), we measured IFNγ levels at 48 h. However, we did not observe the increase of IFNγ levels in the 48 h supernatants (data not shown). At all events, prediction of cytokine profiles of original T cells by those of T cell hybridomas is not possible. The reason is that fusion with BW5147.G.1.4 thymoma cells affects cytokine profiles of original T cells [16].

To narrow the region of epitope(s), we constructed deletion fragments of the calpain fused to GST (Fig. 1). As it was very difficult to solubilize the fusion proteins of GST-calpain deletion fragments in PBS, we used inclusion body suspensions (mostly constituted with recombinant proteins) of each recombinant *E. coli* lysate for stimulation of the T cell hybridomas. As shown in Table 2, only fragment No. 2 failed to stimulate the T cell hybridomas. These results indicated that epitope(s) should be located at the central portion (Fig. 1, A.A.270–329) of the original calpain fragment. The lower levels of IL-2 when stimulated with inclusion body suspensions of GST-calpain were probably due to the antigen conditions different from those of soluble form of the same antigen. In addition, the amounts of IL-2 produced by these T cell hybridomas usually fluctuated very much between independent experiments, even cultured in the same condition. We do not know the reason; however, this is probably because of the unstable cell conditions of the hybridomas.

Based on the result of Fig. 2, 10-amino acid-overlapping 15mer oligopeptides were synthesized covering the full length of the central portion (Fig. 2). Among them, only No. 2 and No. 3 oligopeptides stimulated both hybridomas to produce IL-2 (Table 3). This means that the epitope recognized by the two hybridomas were common; i.e., EQLKIYAQRC, A.A.280–289 (Fig. 2, Table 3).

As noted above, the cytokine profiles of T cell hybridomas do not reflect the profiles of the original T cells [16]. For this reason, it was necessary to check IFNγ production by lymphocytes of calpain-immunized or MAP-immunized mice, to determine the Th1 response-inducing potential of the epitope. As shown in Table 4, spleen cells from synthetic MAP-immunized BALB/c mice produced IFNγ upon stimulation with MAP (immunogen) or oligopeptides No. 2 and 3 containing the epitope sequence EQLKIYAQRC. Additionally, the spleen cells produced IFNγ when stimulated with SWAP (Table 4), implying that calpain molecules contained in the SWAP sample stimulated MAP-sensitized T cells. This result showed high possibility that if mice were immunized with MAP, IFNγ would be produced in vivo following challenge infection. As IFNγ is known to be a crucial cytokine

for protective immunity in mice [17,19], it is expected that immunized mice would be protected from challenge infection of normal cercariae. However, the levels of vaccine-induced protection against schistosome do not depend on IFNγ production levels only, but rather depend on the balance of Th1/Th2 cytokines [20,22]. Here, Th2 cytokines (IL-4 and IL-10) were also produced when the sensitized spleen cells were cultured with the immunogen (MAP) or SWAP, but their levels were relatively low (Table 4). This is consistent with our previous report [15], which demonstrated *S. japonicum* calpain preferentially induced Th1-biased immune response.

Yang et al. [23] reported inefficacy of multi-epitope vaccination to protect mice against challenge infection of *S. mansoni* although their vaccine constructs contained T cell epitope of calpain [12] in C57BL/6 mice. It might be necessary to design appropriate immunization protocols to induce protective Th1 responses, in addition to identification of Th1-inducing T cell epitopes.

In conclusion, here we have identified a T cell epitope of *S. japonicum* calpain that can induce Th1 response in BALB/c mice. Further experimental studies to improve adjuvant formulation and immunization protocols in mice, as previously reported with calpain [24–27], will contribute to vaccine development against schistosomiasis.

Acknowledgements

This work was supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (No. 15390137), the Ministry of Health, Labor and Welfare of Japan (H15-Shinko-8) and the US-Japan Cooperative Medical Science Program.

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Journal of Hepatology 42 (2005) 47–53

Journal of Hepatology

www.elsevier.com/locate/jhep

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

Received 17 June 2004; received in revised form 1 September 2004; accepted 6 September 2004; available online 22 October 2004

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

0168-8278/$30.00
doi:10.1016/j.jhep.2004.09.023

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$\pm$ 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).

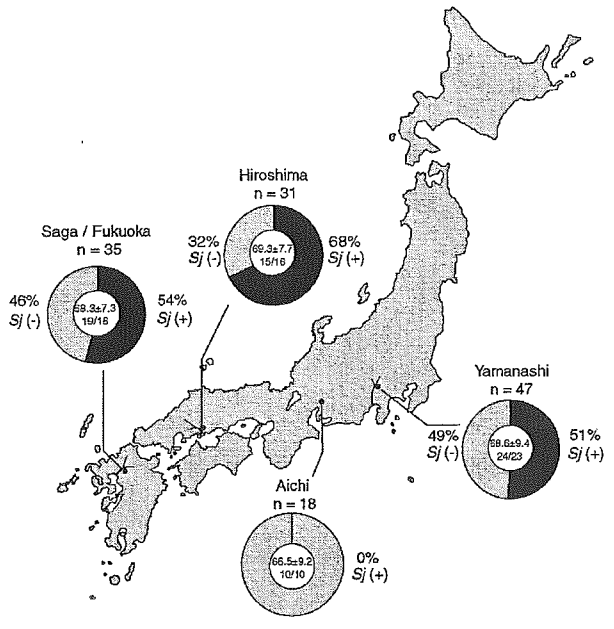

Fig. 1. Geographic distribution of *Schistosoma japonicum* (*Sj*) and characteristics of patients infected with HCV. *Sj* (+) and *Sj* (−) denote, respectively, presence and absence of infection with *Sj* diagnosed by ultrasonographic and/or computer tomographic methods or serological examinations. Pie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

Table 1
Characteristics of patients with and without schistosomiasis

	Schistosoma japonicum		Controls
	Positive ($n=64$)	Negative ($n=49$)	(Aichi) ($n=18$)
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).

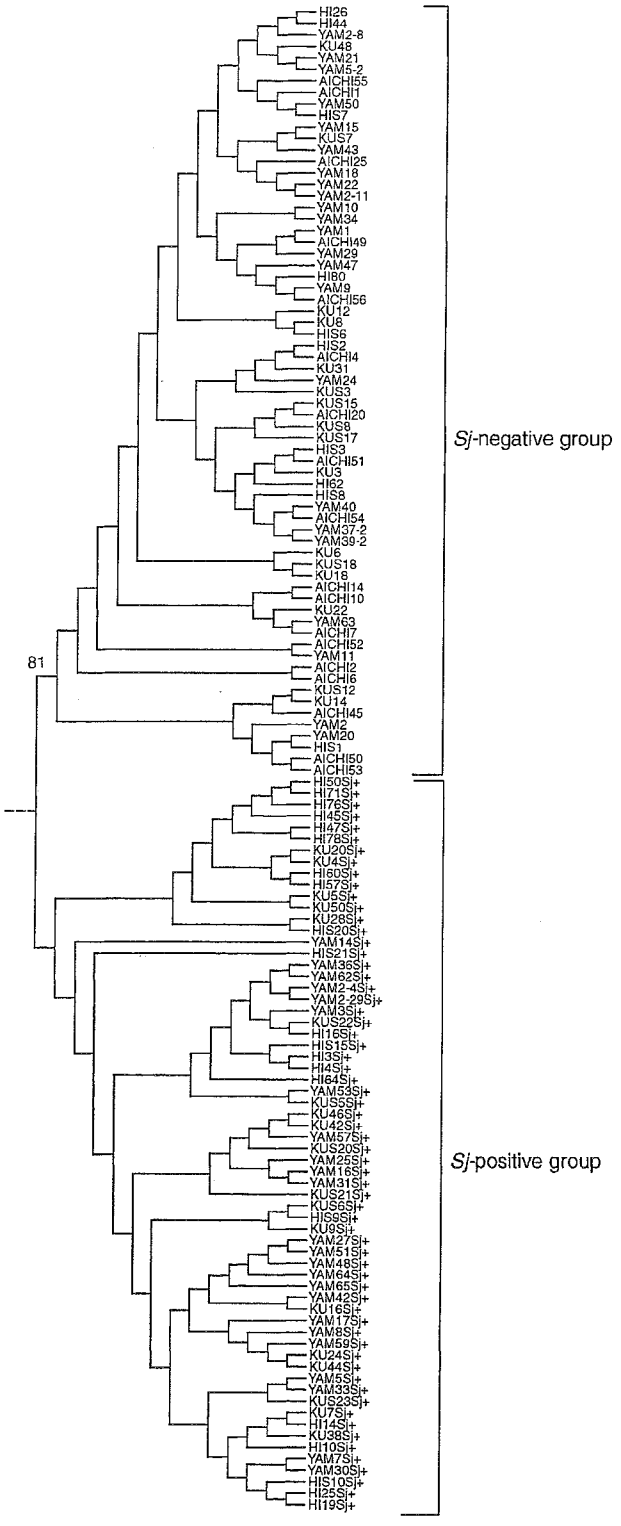

Fig. 2. A phylogenetic tree constructed on NS5B sequences of HCV-1b strains in *Schistosoma japonicum* (*Sj*)-positive ($n=64$) and -negative ($n=67$) groups. The numbers in the tree indicate bootstrap reliability by the interior branch test. *Sj*+ indicates *Sj*-positive strains. YAM; Yamanashi, HI/HIS;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

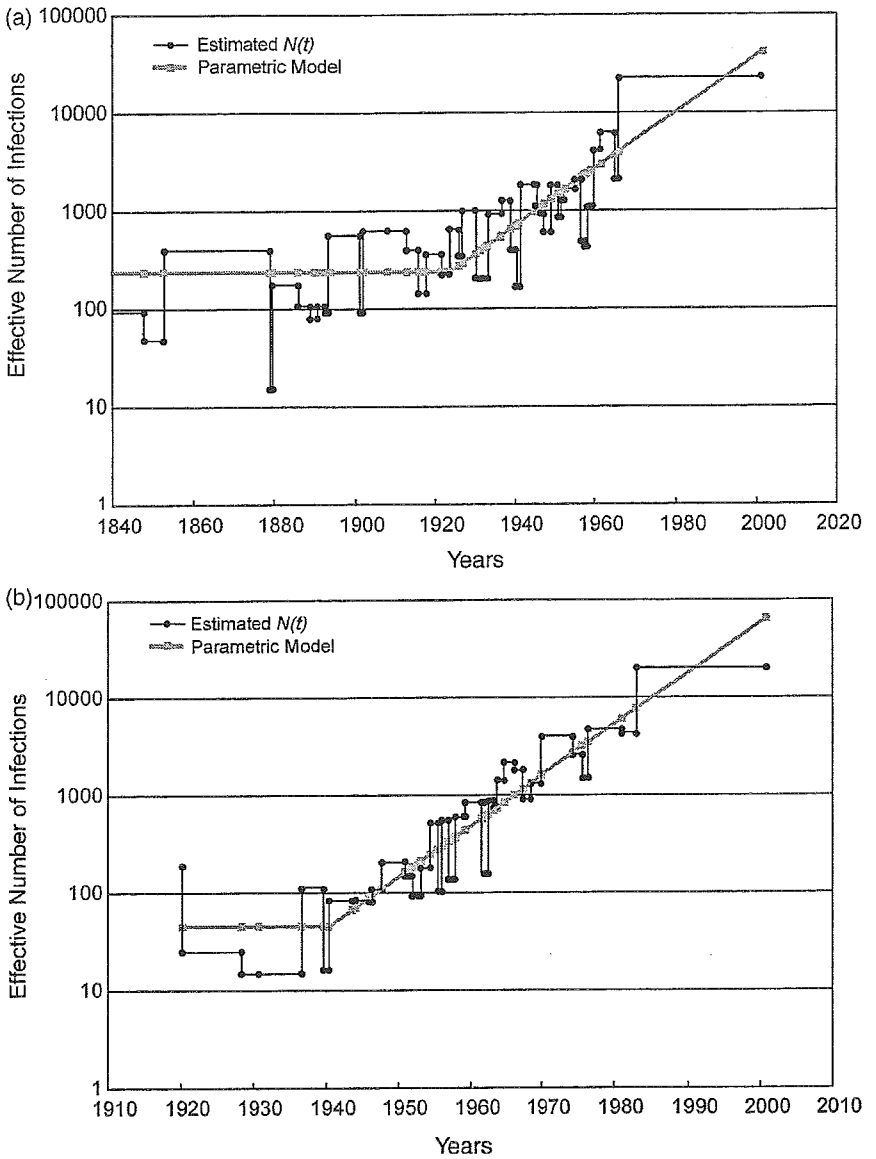

Fig. 3. The maximum-likelihood estimates of $N(t)$ on the effective number of infections with HCV genotype 1b in Japan for *Schistosoma japonicum* (*Sj*)-positive group (a) and *Sj*-negative group (b) separated in the phylogenetic tree (Fig. 2). The parametric model is indicated by the grey line and 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.

Acknowledgements

We greatly appreciate Dr Oliver G. Pybus (Department of Zoology, University of Oxford, Oxford, UK) for his enlightening advice on molecular evolutionary analyses using Genie v3.5. We thank Ms Yoshiko Kobayashi (Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan) for technical assistance and Dr Tatsuya Ide (Kurume University School of Medicine, Fukuoka, Japan) and Dr Hirotaka Kono (Hiroshima University Graduate School of Biomedical Sciences, School of Medicine, Hiroshima, Japan) for providing us with valuable materials. The work was supported by a grant-in-aid from the Ministry of Health, Labour and Welfare of Japan (H13-kanen-2), grants-in-aid for Young Scientists (A) from the Ministry of Education, Culture, Science and Sports of Japan (16689016), The Hori Information Science Promotion Foundation and Miyakawa Memorial Research Foundation.

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

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Received 23 May 2005; accepted 5 October 2005
Available online 11 November 2005

Abstract

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

Keywords: *Schistosoma mansoni*; Artesunate; Prophylaxis; Iron-deficiency; Fecundity

1. Introduction

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

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

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

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

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doi:10.1016/j.parint.2005.10.001