④ ミクロスポーラ症

持続性の下痢を示すが，**米国における消化管の日和見感染症の原因として最も多い**とされている．ミクロスポーラとは類縁の複数種原虫の総称であり，イヌなどのペット動物，魚類，昆虫が保有しているほか，空気中や水中からも検出可能である．

⑤ トキソプラズマ症

細胞内寄生原虫であるトキソプラズマはネコの寄生虫であるが，肉食を通じて感染の機会が多く，米国政府は約40％が原虫陽性と推定している．したがって，米国人や米国在住の邦人ではトキソプラズマ感染を念頭におく必要がある．年間の新感染者は1,000人当たり2～6人と推定され，先天性トキソプラズマ症の頻度も日本と比べて高い．

⑥ イヌ/ネコ回虫症

イヌやネコの回虫による幼虫移行症で網膜下肉芽腫が問題となる寄生虫である．年間7～800例が確認されていて，国民の抗体陽性率は4～7％で，11歳以下の子供に多い．都市部以外の比較的低所得者層で頻度が高い．

⑦ その他

日本で見られる寄生虫症の状況とよく似ている．エキノコックス症は中西部で散見され，旋毛虫症も毎年数十例報告がある．また，**カリフォルニアやフロリダではマラリア感染が復活してきており，今後の監視が必要である．**

Ⅱ ヨーロッパでかかる寄生虫病

ヨーロッパも西欧と東欧，北欧と南欧など自然環境，社会環境が大きく違う．ヨーロッパへの渡航邦人やヨーロッパ出身者の寄生虫感染として特別のものは多くないが，HIV/AIDSに伴う寄生虫相の変化や東欧での政治的混乱の結果，寄生虫感染も増加したことなどが伝えられている．代表的なものを**表1**に示したが，最近の動向も北米より複雑である．

① トキソプラズマ症

北米と同様に食肉生産が盛んでトキソプラズマ感染が多い．ヨーロッパ圏内でも地域差は存在するが，パリでは局所的に住民の80％以上に原虫保有が確認される地域が存在する．日和見感染症として重要である．

② リーシュマニア症

南西ヨーロッパはリーシュマニア症の流行地である．サシチョウバエが媒介する原虫症で，イベリア半島を中心にフランス，イタリアにも存在する．最近ではHIVとの重複感染が新たな流行様態として注目されている[4]．

③ 広節裂頭条虫症

非加熱調理の鮭鱒類を摂食して，腸管内に巨大なサナダムシの寄生をみる．原因食品の市場流通域から北欧に多い．日本近海の日本海裂頭条虫とは別種である．北欧のサナダムシは時に悪性貧血を合併する．

④ 腸管寄生線虫症

ヨーロッパ各国での腸管寄生線虫の問題は在留邦人にとって最近まで問題ではなかった[5]．しかし，**東欧圏の一部では，衛生インフラが崩壊して，回虫，鉤虫などの腸管寄生線虫感染が再興しているとの警告もある．**

⑤ クリプトスポリジウム症

ヨーロッパのAIDS患者の*C. parvum*感染率は6.7％と報告されている．湖沼，河川以外に，水道水からも原虫が検出されており，「ヨーロッパの水道水は硬水だから下痢する」という日本人旅行者の一部が本症によるものであった可能性は否定できない．

⑥ その他

ロシアや南欧でマラリアの再興がみられ，モスクワでも蚊から感染した症例が発生している．欧州のバベシア症は*B. bovis*によるものもあり，予後は悪い．肉胞子虫は中部ヨーロッパの牛に感染がみられ，免疫低下宿主には病原性を示す．

Ⅲ 予防に必要な注意

北米，ヨーロッパで寄生虫感染に対する注意としては，日本国内での場合とほとんど変わることはない．一方で，北米やヨーロッパとはいえ，南部は亜熱帯性の気候条件であり，マラリアやリーシュマニア症などの熱帯寄生虫症が発生している．とくにシベリアを含むロシアでは都市部でもマラリア流行地になりつつある．また，欧米に流入する途上国から人たちが供血者になった結果，**輸血によるシャーガス病（アメリカトリパノソーマ症）やマラリアなどの感染事例が発生している**（**表2**）[6]．そのような社会背景も北米，ヨーロッパの特殊事情と考えてよいであろう．輸血歴は把握しておくべきである．

表2 アメリカ大陸における輸血用血液のアメリカパノソーマ原虫の混入

場所（国名）	検査数	陽性率
Los Angeles（米国）	998	0.1～1.1％
Cordoba（アルゼンチン）	283,962	7.6％
Sao Paulo（ブラジル）	56,902	2.9％
Santiago（チリ）	2,062	1.5％
Bogoda（コロンビア）	1,012	2.8％

（文献6）より改変）

寄生虫病は北米，ヨーロッパには存在しないのではなく，情報が乏しいに過ぎない，との認識を持ち，AIDSなどを背景にした日和見寄生虫感染症は十分に蔓延していることを渡航者と臨床医の双方が理解しておくことが予防の上で大切である．

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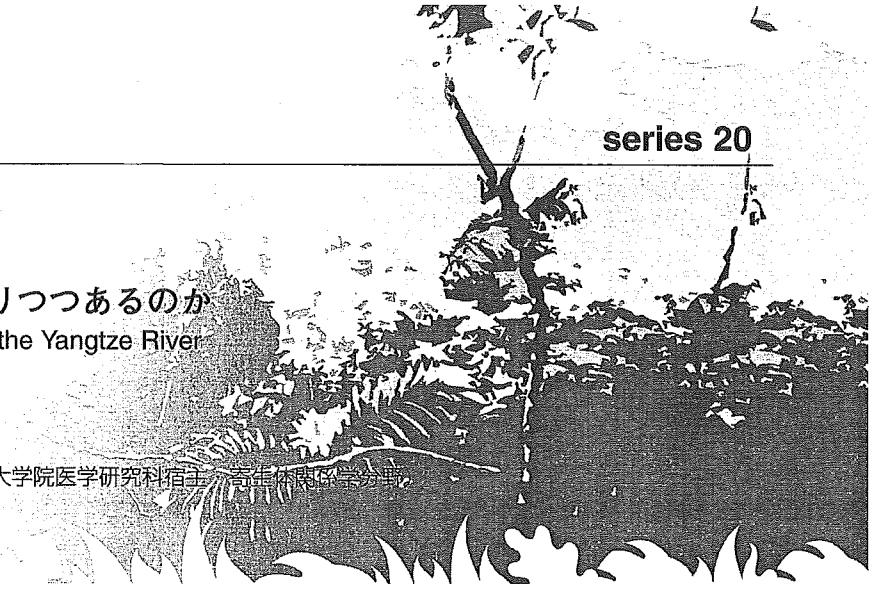
住血吸虫症

——揚子江流域の流行になにが起こりつつあるのか

Schistosomiasis——What is happening along the Yangtze River



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揚子江の歴史と日本住血吸虫症

中国における住血吸虫症は悠久な揚子江の流れとともにある．人類文明が勃興した地域のひとつであるが，おそらくはこの地域への人類の定着とともに住血吸虫症も起こったのであろう．中国国内の住血吸虫症流行地は揚子江中～下流域の低湿地に広がる湖沼型と四川省や雲南省にみられる山丘型の2型に分けられるが，揚子江流域には湖沼型流行地が湖南省，湖北省，江西省，安徽省，江蘇省など広範囲にわたって点在している[1]．この地域に古来，住血吸虫症が存在した確実な証拠は湖南省長沙市にある馬王堆遺跡から出土した女性の遺体から得られた．紀元前200年前後と推定されるその遺体はまるで前日に亡くなったといってよいほどの保存状態で出土し，病理解剖で心筋梗塞が死因であることも判明している．そして，その女性の直腸粘膜から日本住血吸虫卵が確認されたのである．

「三国誌」のハイライトのひとつ，“赤壁の戦い”は西暦208年の出来事で，湖北省と湖南省の境の揚子江の一角がその古戦場跡である．曹操率いる魏の水軍が大敗を喫したことで知られるが，そこはまさに住血吸虫症の濃厚流行地として知られた場所である．史家の多くは，住血吸虫症とは無縁の北方出身の魏の兵士が揚子江に接して急性住血吸虫症を病んだために戦力が大幅に低下したことが敗因のひとつと論じている．近年に眼を向けると毛沢東と住血吸虫症の戦いも歴史的な逸話になりつつある．毛沢東は湖南省韶山の出身で，青年時代は流行地である長沙市内の師範学校で学んでいる．その影響か住血吸虫症には関心が高かったらしく，革命後はただちに住血吸虫症制圧を党の重要政策に据えた．上海近郊の青浦にある住血吸虫記念館の資料によれば，この病気で全村壊滅の悲劇があったことは事実のようである．恐怖の病気であった住血吸虫症に対する彼の想いが「送瘟神」という詩に謳われている．毛沢東はそのなかで古来人民を苦しめた住血吸虫症がいまや制圧に至ったことを高らかに讃えているが，実際には彼の存命中に揚子江流域で，制圧はさほど成功していなかったのである．

著者は1988年以来，揚子江流域，とくに湖南省の住血吸虫症対策にかかわってきた．21世紀に入った現在，揚子江の変わらぬ流れのなかで，そこに住む人びとと住血吸虫の移ろいの行く末を展望してみるのが本稿の目的である．

困難を極める日本住血吸虫症の制圧

中国に存在する住血吸虫症は日本住血吸虫によるものである．いわゆるintestinal schistosomiasisがその病型であり，急性期に消化器症状，慢性期には肝線維症などが流行地住民にみられた．末期症状としては肝硬変も併発して死に至る（図1）．住血吸虫症は水中で幼虫が皮膚から侵入することで感染が成立する．幼虫は淡水産の貝のなかで発育するので，疾病制圧において取られる戦略は終宿主の治療，中間宿主貝の殺滅，および生活用水/糞便の衛生管理である．最近では“終宿主の治療”が住血吸虫症対策の柱となっているが，一方で中

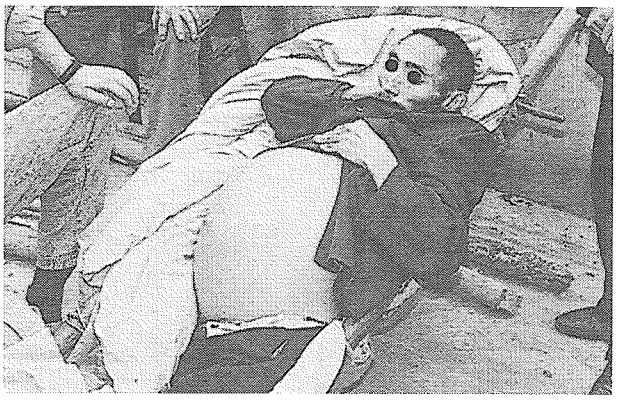

図 1　肝硬変を併発した日本住血吸虫感染者

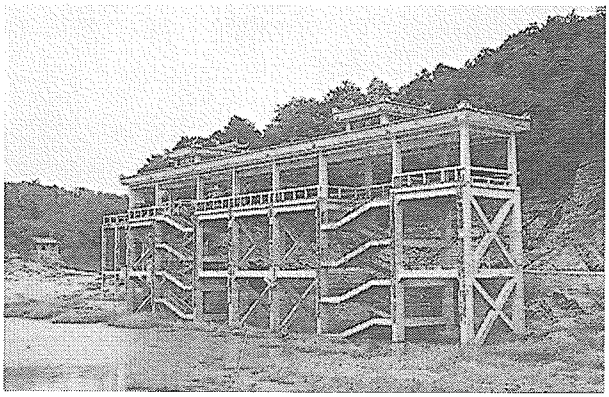

図 2　揚子江流域洞庭湖の観光地
君山の船着き場は渇水期には使用できない

間宿主対策は殺貝剤を散布することで環境汚染の問題がつねに附随してくるため，最近では対策戦略の中心たりえない．

揚子江流域の住血吸虫症対策を困難なものにしているおもな要因は 2 つある．ひとつはヒト以外の動物が終宿主として流行の維持に大きな貢献をしていることである[2]．揚子江流域では水牛が農耕を担う重要な家畜であるが，水牛は日本住血吸虫に対する感染感受性が高い．すなわち，日本住血吸虫症は人獣共通感染症であり，水牛のほかにブタ，ウシ，ヒツジなども日本住血吸虫の終宿主になっている．したがって，終宿主の治療ということはヒトの治療だけでは不十分で，水牛などの保虫宿主も同時に治療しないとヒトの感染を遮断する効果は十分に得られないことになる．しかし，実際には水牛など放牧された家畜一頭一頭の診断と治療を正確に実施することは不可能である．ちなみに，同じ日本住血吸虫症流行地でもフィリピンの水牛はけっして重要な保虫宿主ではない．生物学的に説明を試みているが，中国とフィリピンとで水牛の住血吸虫感染感受性が違うことの機序はいぜんとして不明である．

第 2 の問題は揚子江の自然条件である．揚子江は乾季と雨季とで水位が大きく変化する．その水位差はゆうに 10 m を超え，流域の景観は季節によってまったく異なるものとなる(図 2)．そのことは中間宿主貝の生息面積に直接影響する．乾季には揚子江流域には広大な草地帯が出現し，それが中間宿主である *Oncomelania* 属貝の好適繁殖地となっている．季節によって“有病地”が変化するため，行政対応に大きな支障が生じることになる．揚子江の大洪水がもたらす影響も深刻である．洪水によって中間宿主貝が予測をこえる範囲にまで拡散する結果，あらたな有病地が確認されるようになった．

三峡ダムの予想される功罪

中国有数の景勝地である三峡にダムを建設する計画が正式に承認されたのは 1992 年である．その目的は揚子江中流域の治水や大規模電力開発などで，2009 年に完成をめざす中国の巨大国家プロジェクトである．住血吸虫症の流行にいかなる影響が出るというのであろうか．ダム建設による住血吸虫症流行の拡大ということではエジプトのアスワンハイダム建設の事例を想起される向きもあろうが，日本住血吸虫症に与える影響は事情が異なる．アスワンハイダムでは人造湖が出現して，そこに中間宿主貝が大繁殖したことが問題であったのだが，三峡ダムで問題にしているのは実は下流域に及ぼす効果なのである．揚子江が季節によって水位の上下を繰り返していたものが，ダムによって季節を通じて安定した草地帯が流域に出現することになる．すなわち，下流域に広大な貝の棲息地が確保されることを意味するからである．

その影響についてさまざまなシミュレーションがなされている．現状では流行拡大が懸念される地域はダムに比較的近い湖北省，湖南省に限られ，一方で江西省では幸いに影響がないという予測が

ある．また，三峡ダム建設の目的のひとつに治水効果が上げられていたが，年中行事のような揚子江の大水害が治まるという説と，大自然は人間のちっぽけなダム建設くらいでは変化することはありえない，という説が交わされており，われわれは当然のことながら，その答えを知らない．しかし，確実にいえるのはダム建設によって中国の詩人達に謳われた天下の景観が失われることであり，そのことこそが最大の影響であるのかもしれない．

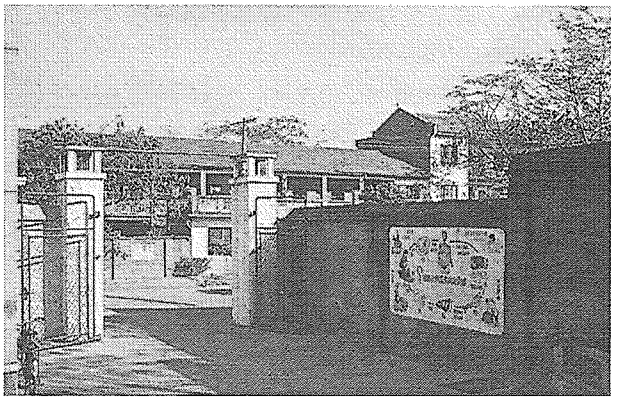

図 3　湖南省内にある住血吸虫症防治ステーション
保健所と病院を合わせたような機能をもつ

世界銀行融資と揚子江流域流行地の住血吸虫症対策

中国の日本住血吸虫症流行は，一時的とはいえ，過去 10 年間に流行の制圧に向けてたしかに前進がみられた．前述のとおり中国政府は 1992 年から世界銀行の融資を受けて日本住血吸虫症対策の一大国家プロジェクトに取り組んだ．そして，1999 年の統計では中国国内の流行地は 409 郡を数えるが，うち 58.2％で流行がほぼ終息したと判定している．流行状況は“流行継続”，“流行制圧”そして“流行駆逐”の 3 つに区分されるが，“流行継続”から“流行制圧”に改善するだけでも行政的には大きな負担軽減が見込まれる．流行地では WHO が推奨する住血吸虫症対策法がとられ実効が上がったのである．世界銀行経由の潤沢な予算も相まって当時取りうる最善のアプローチであった[3]．住民の検査と治療はすべて無料であった．そのために住民検診は高いコンプライアンスで実施され，当初の目論みどおりの効果が得られる結果となった．さらに，重要なことは検査実施者の給料支払いの事実である．世界銀行の融資は末端の検査実施者にも労働対価の支払いが保証されるシステムを確立することになった．このように対策実施者，被験者ともにハッピーな 8 年間であった．

世界銀行の融資が終了した 2000 年をもって中国では状況が変化した．予算不足のために住民検診は被験者自身によるコスト負担が定められ，また特効薬プラジカンテルの処方にも受益者負担の原則が導入されたのである．中国経済の発展に伴って上海や広州などの沿岸部の繁栄が伝えられるが，内陸部との経済格差は想像以上のものがあり，著者自身が調査にあたった湖南省の農村地帯では農家の平均年収が 1,000 元程度(日本円で 15,000 円くらい)であった．そこに治療薬の自己負担が導入された結果，プラジカンテルの代金として 1 回 20 元を感染陽性者が支払うようになった．けっして軽い負担とはいえない金額である．有料の検査を受けて有料の治療を受けるというシステムであれば，今後ますます受診者が減少することは確実である．

さらに並行して生じた問題は検査実施者の給料未支給の問題である．揚子江流域の各省は住血吸虫症対策の行政的スキームを構築している．末端には“対策ステーション”が村に設置してあり，日本の保健所を小規模にしたものといえば想像は容易であろう(図 3)．著者は 2002 年の時点で，職員の給料遅配は日常的になっていると聞いた．検査室の実態も機器，消耗品など，厳しい予算カットの結果，実施するためには住民からの料金徴集の徹底が必要で，住民は自発的に受診を希望しないようになっていた．2004 年現在，すでに正確な疫学情報が把握できなくなったとして本年秋から第三次の住血吸虫症全国調査の実施を中国政府は決定している．

揚子江流域から住血吸虫症をなくすために

毛沢東が願ってやまなかった揚子江流域からの住血吸虫根絶はいかにして達成できるであろうか．感染症制圧をもっとも効率的に推進できるの

表 1 住血吸虫ワクチン開発に関するカイロ会議（1997 年 5 月）のまとめ

① 研究からワクチン実用化に進む段階に至った
② GST，パラミオシン，MAP-4/TPI，Sm 14 については臨床試験の後，GMP グレードで抗原の大量精製を行うべきである
③ IrV5 と MAP-3/Sm 23 の DNA ワクチン化に向けた研究を進めるべきである
④ 上記の他に有望なワクチン抗原を考慮するべきである

はワクチンである．住血吸虫は治療してもすぐに再感染するように，いわゆる“二度なし”免疫は成立しない．しかし，日本でも流行地で生育した人と非流行地から移入した人とで住血吸虫のかかりやすさや病気の重篤度が違うことがあったように，日本住血吸虫感染にも部分免疫は成立しうると考えられる[4]．そこで，部分的ではあっても宿主の防御免疫を賦活してやれば，集団の感染濃度が低下し，やがてその虫を駆逐できるであろう，というのが住血吸虫をはじめとする蠕虫ワクチンの考え方である．住血吸虫ワクチンとしては WHO や地域ネットワークなどを通じて開発戦略が討議されてきた（「サイドメモ」参照）．これまでに表に示したような抗原がワクチン候補として同定されている（表 1）．

日本住血吸虫症は他の住血吸虫に比べてワクチン開発の好適な標的である．その理由はこの寄生虫病が人獣共通感染症であるため，ヒトでの実用化試験が倫理的に困難であっても水牛など家畜動物に効果が認められれば，疾病制圧に一定の貢献がなされることになるからである．日本住血吸虫ワクチンとしていくつかのものがマウスの実験で効果が確認されており，ブタなどの大形家畜動物を用いた試験に進みつつある[5]．この分野では中国，日本，オーストラリアでワクチン実用化のための共同研究が進められている．パラミオシンは現時点でもっとも期待の大きいワクチン標的抗原であるが，長崎大学・平山らは江西省との共同研究でブタにおける効果を検討している．約 40%の感染防御効果が観察されているが，用いるアジュバントの選択など検討が必要である．オーストラリア QIMR の MacManus らは中国の水牛でパラミオシンによるワクチン効果を検討した．残念ながら有意な防御効果は観察できていないが，水牛の免疫に必要な抗原量などを知る予備試験としては情報を得ている．著者らは湖南省においてカルパインのワクチン効果をブタで検討した．マウスで観察されたような有意な防御効果を観察することはできなかったが，明らかな産卵抑制効果があったことは発病阻止ワクチンとしての期待は残された．いずれにせよ，家畜動物を用いたワクチン研究はコストがかかりすぎる点が問題である．そこで今後の方針としては，ワクチン開発のためのワークショップを通じて候補分子を絞り込み，国際協力体制の下で十分な数の家畜動物を用いて試験していくことの合意が得られている．近い将来は中国における住血吸虫症対策としてヒトを治療し，家畜動物にワクチンを用いることで，住血吸虫症根絶をめざしたいと考えている．

サイドメモ

住血吸虫ワクチン

蠕虫ワクチンの意義は他のワクチン開発戦略と根本的なところで異なっている．細菌，ウイルス，原虫に対するワクチンは，sterile immunity，すなわち 1 個体たりとも侵入を許さないことが要求される．それは細菌や原虫は宿主体内で増殖するからであるが，蠕虫は宿主体内で例外を除いては増殖することができない．その結果，蠕虫ワクチンでは部分的な感染防御が誘導できれば，宿主の寄生個体数を減少させる効果が期待されることになる．WHO では住血吸虫ワクチンに対して“40%の感染防御効果”を開発の必要条件として設定した．表 1 はそれをクリアしたワクチン候補であるが，それ以外にも候補分子が同定されつつある．住血吸虫排除の機序は十分にはわかっていないため，どのような免疫エフェクターを導いたらよいか，議論があるところであるが，通常の蛋白ワクチンに加えて，DNA ワクチンも各種実験動物で試みられている．

おわりに

揚子江流域を含むアジアの日本住血吸虫症対策は感染率を軽減することには成功したが，これをさらにゼロにするための事業展開がはかられている．しかし，中国においても住血吸虫症は“neglected

diseases"に位置づけられる傾向にあったことは今後の本症再興の可能性を示唆するものである．一般的にいって中国の住血吸虫症対策はしだいに手薄になりつつあるように思われる．悠久の歴史のなかで揚子江の変わらぬ流れを考えるとき，広大な大地から住血吸虫症をなくすことは不可能なのかもしれないという気になる．近未来の歴史のなかで，たとえば三峡ダム運用を機に住血吸虫流行が拡大したという記述が加わることのないように願っているが，敵はけっして容易な寄生虫でないことは確かである．

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「医学のあゆみ」のあと

本誌の創刊は昭和21年（1946年）3月1日であり，東京大学名誉教授緒方富雄の発想による．

創刊のことばに「“医学のあゆみ”は世界の医学のあゆみをあらゆる姿で紹介する雑誌である．また日本の医学のあゆみを，なにかのかたちで促進したいと念ずる雑誌である．医学のあゆみは，すべての科学のあゆみとおなじように，自由でとらわれない．そのようにわれわれの“医学のあゆみ”は自由でとらわれないつもりである」と．

B6判（月刊）で出発した本誌は，昭和26年9月号からA5判に，昭和33年6月から週刊となり，判型もB5判と大きく発展してきた．

常に世界の医学のあゆみ，日本の医学のあゆみを紹介し続けながら，特に昭和35年4月以降は「短報」欄を設け，質の高い研究の速報を行っている．

本誌創刊当時の日本の医学は抗生物質の導入により大きく変革し，はじめて訪れた自由と平和を基盤に活力に充ちていた．1980～1990年にかけ世界の医学はコンピュータを駆使しながら，バイオテクノロジーを牽引車としてめざましい進歩・改革を行ってきた．

真理を追求し，疾病を駆逐し患者の幸せを求める医の原点は常にかわらぬし，本誌創刊の精神は脈々とうけつがれている．

第1期・第2期の編集委員の名をかかげ永く感謝の碑とする．

第1期

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この他に，第1期の編集委員として途中でおやめになった方に故・渋沢喜守雄，故・秋元寿恵夫，故・卜部美代志，故・若林　勲，また第2期の編集委員として途中でおやめになった方に星　猛，林　四郎，故・木川源則，故・植田　穣がいる．

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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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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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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.

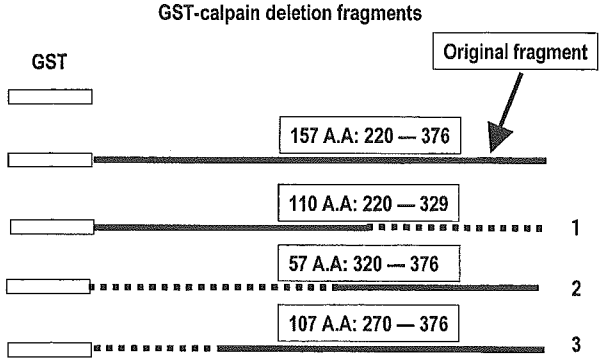

Fig. 1. Schematic presentation of GST-calpain (original fragment) and three deletion fragments (No. 1–3). Fragment No. 1 and No. 2 overlapped 10 amino acids. All fragments were expressed as fusion proteins with GST and used for cytokine assay without removal of GST portion.

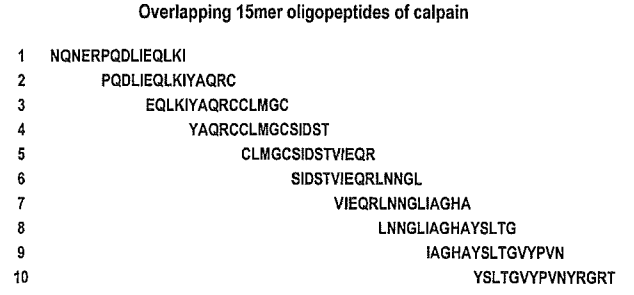

Fig. 2. Schematic presentation of synthetic 15mers covering the central portion (A.A. 270–329) of the original calpain fragment. All oligopeptides overlapped 10 amino acids with the following oligopeptides.

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

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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

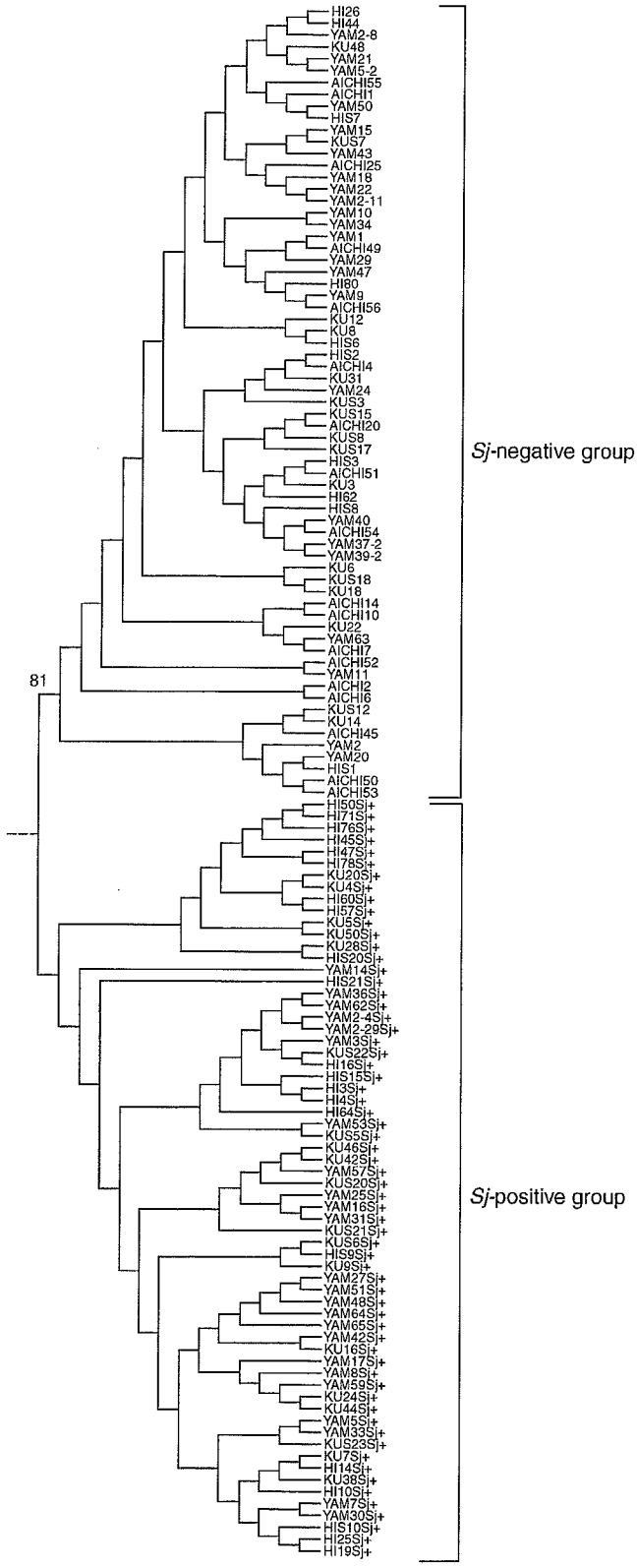

Fig. 2. A phylogenetic tree constructed on NS5B sequences of HCV-1b strains in *Schistosoma japonicum* (*Sj*)-positive ($n=64$) and -negative ($n=67$) groups. The numbers in the tree indicate bootstrap reliability by the interior branch test. *Sj*+ indicates *Sj*-positive strains. YAM; Yamanashi, HI/HIS;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

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).

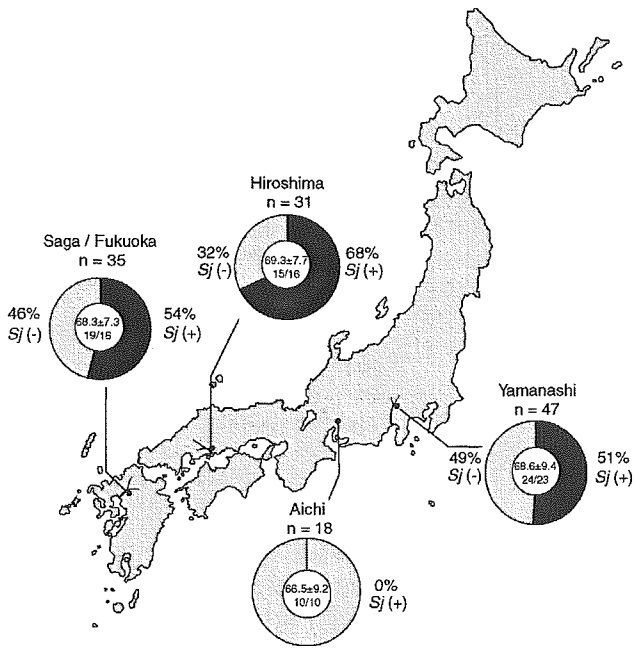

Fig. 1. Geographic distribution of *Schistosoma japonicum* (*Sj*) and characteristics of patients infected with HCV. *Sj* (+) and *Sj* (−) denote, respectively, presence and absence of infection with *Sj* diagnosed by ultrasonographic and/or computer tomographic methods or serological examinations. Pie 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).

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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