2. 慢性の下痢で便寄生虫検査をおこなわなかった糞線虫症例

糞線虫症では時に死亡例が報告されている．2001 年に報告された例では，77 歳女性が特にきっかけがなく腹部膨満感と全身浮腫および低蛋白血症が出現し，蛋白漏出性胃腸症を疑って治療していたが，大腸内視鏡の際に発見された炎症性変化に対しては抗生剤が投与されていた．この症例では，脂肪便検査の際にラブジチス型幼虫が発見され，上部内視鏡検査で粘膜内に多数の虫体がみとめられた．イベルメクチンを投与したが肺炎を併発して死亡した[3]．

その他これまでに症例報告されているものでは，免疫抑制剤投与後に下痢が出現したが抗生剤の投与のみで対応し，消化器症状が改善しないために大学病院に転院し，胃粘膜生検で多数の糞線虫成虫をみとめた例[4]，あるいは体重減少，食欲不振，腹痛，下痢などがあったが諸病院へ入退院を繰り返して診断がつかず，拒食症（疑）とすらされていたが，翌年になって下痢が増強，嘔吐や浮腫も加わって入院し，内視鏡検査にて十二指腸粘膜に虫体を多数認めて診断に至った例などが報告されている[5]．

いずれの例においても，もう少し早く寄生虫を念頭に置いた便検査が実施されていれば，異なった経過をとったであろうことは容易に推測される．

寄生虫疾患の見落としを減らすシステム

寄生虫疾患は，ほとんどの種について検査法が確立しており，どの施設がどの寄生虫検査を実施可能なのかも検索可能である（表 2）．したがって，最も重要な点は寄生虫感染症が鑑別診断に含まれることである．しかしながら，卒前卒後教育における寄生虫疾患の占める割合は小さく，日常的に頻繁に遭遇する疾患でもない．したがって，寄生虫疾患の見落としを防ぐためには，皆が知識がないのが当たり前という点から出発しないといけないだろう．

表 2　主な寄生虫検査対応施設

原虫性疾患
クリプトスポリジウム、ランブル鞭毛虫、赤痢アメーバ：金沢大医学部寄生虫
赤痢アメーバ：国立感染研寄生動物部、慶応大医学部熱帯医学
トキソプラズマ：千葉大医学部寄生虫、金沢大医学部寄生虫
リーシュマニア：琉球大医学部皮膚科、金沢大医学部寄生虫
マラリア原虫：国際医療センター研究所熱帯医学マラリア研究部など
蠕虫性疾患
抗体スクリーニング：宮崎大医学部寄生虫
切片・虫体からの遺伝子検査：国立感染研寄生動物部
形態学的鑑別：大分大学医学部寄生虫、目黒寄生虫館
エキノコックス、嚢虫：旭川医大寄生虫
リンパ系フィラリア：愛知医大寄生虫
イヌ回虫、旋尾線虫、住血吸虫：東京医科歯科大寄生虫

それではどうすればよいのか．一つのヒントは「寄生虫抗体スクリーニング検査」にあるように思われる．この方式では，寄生虫のことはほとんど知らなくても，「好酸球増多」というキーワードに反応して検体を送付すれば，寄生虫感染の関与についてある程度の情報が得られる．「抗生剤の効かない肺炎」「原因不明の炎症」についても同様である．診療科によっては，これに「脊髄炎」「ぶどう膜炎」「皮膚爬行疹」のような症状も含まれるだろうが，基本的に細かい知識は要求されない．

寄生虫疾患の見落としを防ぐためには，血清診断以外にターゲットを絞らないスクリーニング的な検査法を設定すべきではないだろうか．そこで最もカバーすべき分野は，腸管内寄生虫症であろう．腸管内寄生虫に対しては抗体があまり産生されないため，抗体スクリーニングの検査項目には入っていない．しかしながら上述した糞線虫症のように重大な結果を招くこともあり得る．

幸いなことに，仮に慢性の下痢が続いているとして，その原因が寄生虫であれば，寄生虫由来の細胞成分は必ず便中に排出される．虫卵，幼虫，シスト，栄養体などである．また，寄生虫抗原も排出されている．したがって，「寄生虫抗体スクリーニング検査」のように，「寄生虫抗原または遺伝子スクリーニング検査」を構築できれば，「原因不明の下痢」というキーワードに反応して提出された検体に対して，寄生虫感染の有無についてある程度の情報を得ることができるようになるだろう（図1）．

おそらく実現可能性という点から考えると，PCR法をベースにした「寄生虫遺伝子スクリーニング検査」の方が現実的であろう．抗原検出のためにはそれぞれの抗原に対して特異抗体を作製する必要があるが，PCR法ならプライマの塩基配列を注意深く選べば，すぐにでも実行可能である．便検査において，虫卵，幼虫，シストなどを従来法によって検出しようとすると，あらかじめ標的に合った検査法をオーダーする必要がある．しかしながら遺伝子スクリーニングであれば，発注側にはとくに要求される知識はない．

今後への提案

これまで，寄生虫学会ではさまざまな診断法が

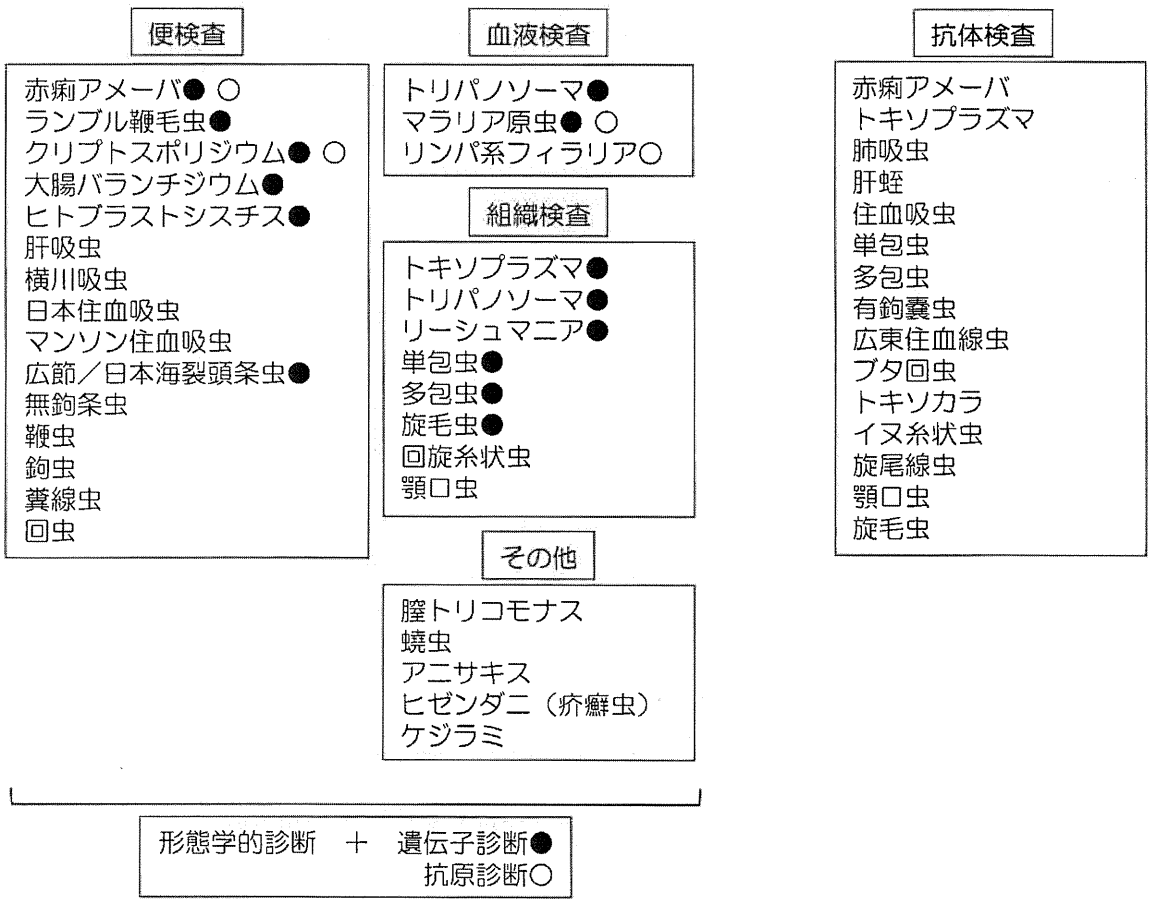

図1　寄生虫疾患診断のための各種検査法

開発され報告されてきた．組換え抗原を用いた抗体検査法，モノクローナル抗体を用いた抗原検出法，種特異的塩基配列を標的にした PCR 法などである．これらは，それぞれが特定の寄生虫を検出するために開発されてきたものであるが，今ここでこれらの知見を総合しリソースを集中させ，寄生虫疾患総合診断システムの構築を目指してはどうだろうか（図 2）．

抗体検査では組換え抗原の開発に力を入れ，糞便抗原検出スクリーニング検査が困難なら糞便遺伝子検出の標準化を推進する．いずれにしても，検体数が膨大になっても結果にばらつきのない検査システムであることが重要である．そのような検査が保険適用になり気軽にオーダーできるようになれば，寄生虫疾患そのものもなじみのある疾患になり，いつか「苦手感染症」ではなくなるのではないだろうか．今が，具体的な動きを起こすときであると考えている．

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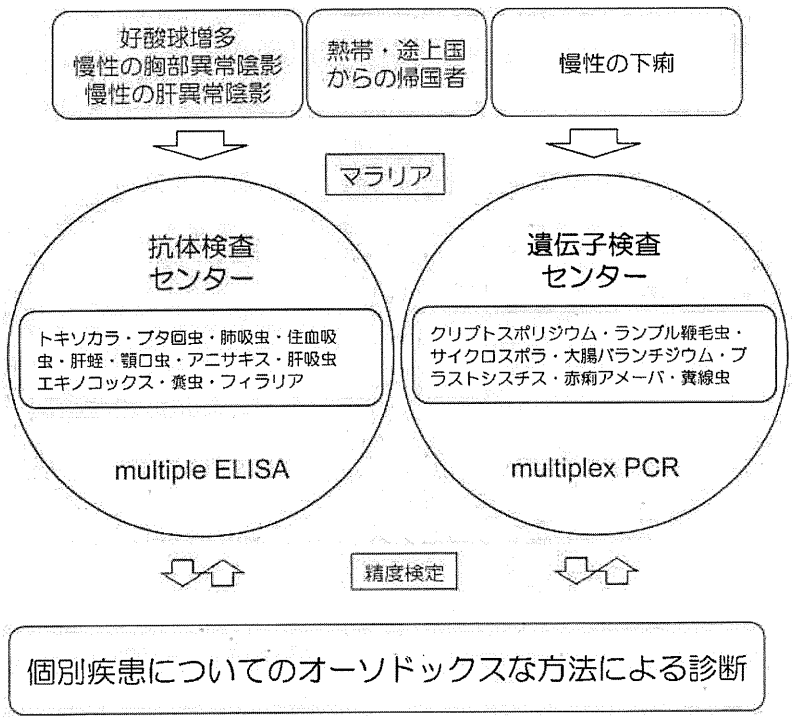

図 2　寄生虫検査体制構築に向けた多施設共同研究案

好酸球増多を伴った糞線虫症の1例

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2)長崎大学医学部　第二内科
3)宮崎大学医学部　寄生虫学
4)長崎大学医学部　第一病理

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原　耕平[1)]，泉川欣一[1)]，泉川公一[2)]
河野　茂[2)]，丸山治彦[3)]，林　洋子[4)]

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症例報告

好酸球増多を伴った糞線虫症の1例

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要　旨

症例は78歳女性．2008年6月乾性咳嗽と38℃の発熱があったため，当院を受診した．CRP値は上昇し，白血球増多と好酸球増多，胸部X線上左下肺野に網状の浸潤影をみとめ，CTでは小葉中心性の粒状影や浸潤影をみとめた．メロペネムを投与したが反応せず，プレドニゾロンの投与でも症状や胸部陰影の増悪や寛解を繰り返した．約2ヵ月後から再び好酸球の増多とIgE値の上昇をみとめた．上部消化管の内視鏡検査で十二指腸に小白粉性隆起をみとめ，病理学的に同部に寄生虫体の構造をみとめ，また糞便中にラブジチス型の幼虫が認められ，糞線虫に対する血清抗体価も上昇していた．糞線虫症と診断し，ivermectinを2週間隔で2回投与したところ，症状も所見も全く消失した．幼少時にフィリピンにて感染し，老齢化により症状が顕性化したものと考えた．

キーワード：糞線虫症，小白粉性隆起，イベルメクチン

はじめに

糞線虫（通常ヒトへの感染は*Strongyloides stecoralis*）は熱帯・亜熱帯に分布し，日本では沖縄地方に多くみられる寄生虫で，フィラリア型幼虫が経皮的に侵入して感染が成立するとされている[1)]．私達は九州・長崎において，その発症初期に慢性の好酸球性肺炎の徴候を示し，十二指腸内視鏡では小白粉性隆起を認め，血清中に糞線虫抗体の上昇と，且つ糞便中に糞線虫の虫体を認めた一例を経験したので，文献的考察を加えて報告する．

症　例

患者：78歳　女性．

既往歴，家族歴：特記すべきものはない．

生活歴：1933年フィリピン・ミンダナオ島で出生．1945年終戦で長崎県口之津に引き揚げ（13歳時），その後成人になってからは農業に従事．豚堆肥を用いた有機野菜を摂取．生肉，川魚，沢蟹は食べていない．ペットの飼育歴はなく，喫煙歴や旅行歴もない．

臨床経過：

第1回入院時：2008年5月中旬より乾性咳嗽が続き，前日より38℃台の発熱があった

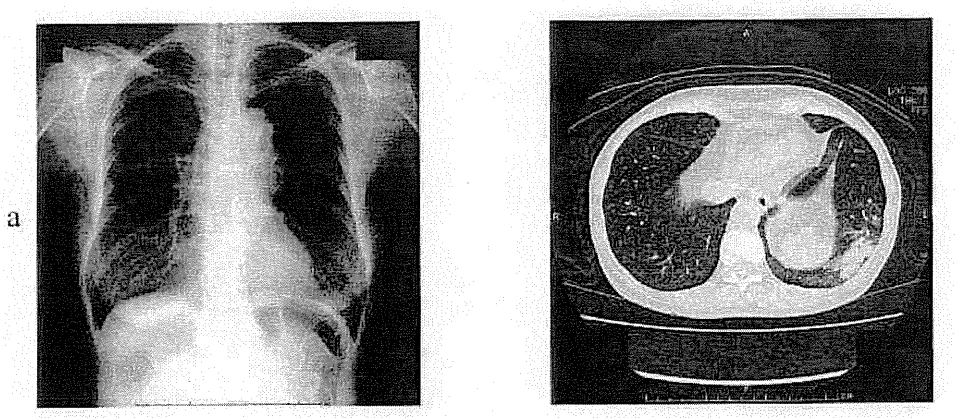

Fig. 1　Rontgenographic findings of chest

Dirofilaria immitis	+−	−	*Paragonimus westermani*
Toxocora canis	−	−	*P. miyazakii*
Ascaris suum	+−	+−	*Fasciola sp.*
Anisakis sp.	+−	−	*Clonorchis sinensis*
Gnathostoma doloresi	+−	−	*Spirometra erinacei*
Strongyloides sp.	++	−	*Cysticercus cellulosus*

Fig. 2　Multiple-dot ELISA of the patient's serum showing positive reaction against S. stercoralis.

ため，2008年6月下旬当院を受診，入院した．入院時，CRP値は上昇し，白血球増多と好中球増多，さらに好酸球増多(実数1436)をみとめ，胸部X線像では左下肺野に斑状の浸潤影をみとめ，CTでは小葉中心性の粒状影や淡い境界不明瞭な斑状の陰影を認めた(Fig 1a)．

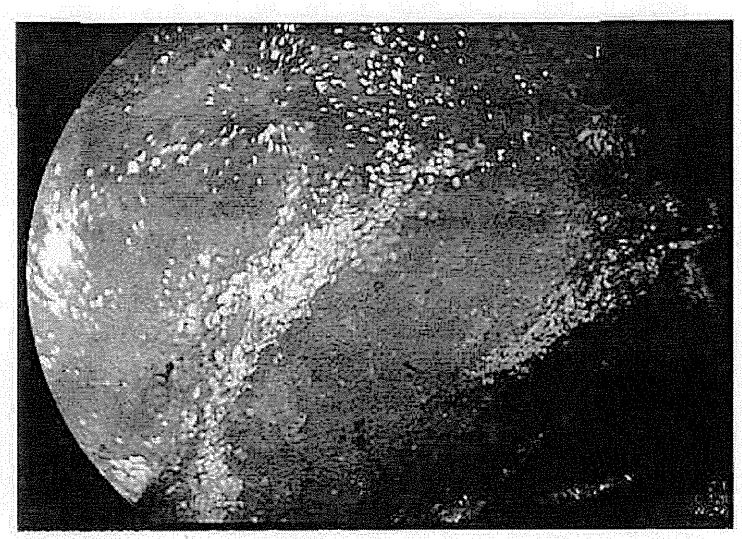

Fig. 3 Endoscopy findings in duodenum indicate a huge number of small white powder-like protrusions.

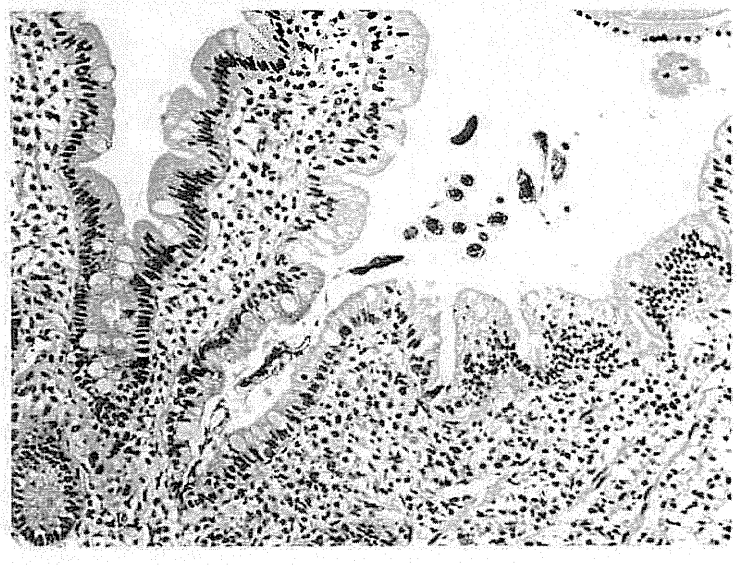

Fig. 4 In a duodenal biopsy specimen, eosinophilic inflammation of mucosa and parasites are recognized.

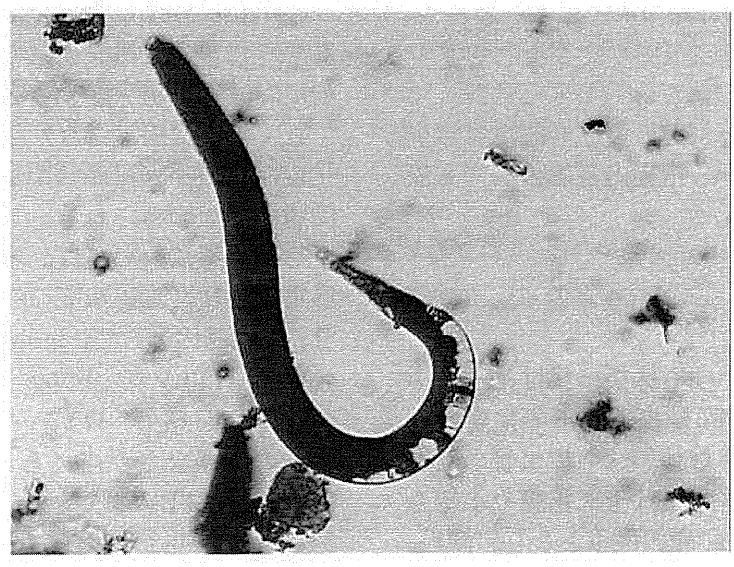

Fig. 5 Stool examination shows strongyloides larva.

細菌性肺炎の併発も考えてsulbactam/ampicillin(SBT/ABPC)で治療したが解熱せず，さらにmeropenem(MEPM)に変更したが，陰影は改善しなかった．好酸球性肺炎の診断のもとに，2008年7月上旬からpredonisolone 30mg/日より開始し，毎週5mgで漸減したが，症状も胸部の陰影も増悪や寛解を操り返した．さらに精査を進めようとしたが，同意が得られなかったので，外来で経過を観察することとした．その後も，慢性の咳嗽は持続したが，胸部の陰影は次第に消失した(Fig 1b)．2009年7月にPredonisoloneを中止したところ，同年9月頃から徐々に好酸球数の増加をみとめた．

第2回入院時：2010年2月上旬，咳嗽も続くため，精査のため第2回目の入院となった．入院時に貧血や黄疸はなく，口腔内は正常で，頚部や表在リンパ節は触知せず，心音は純，呼吸音も正常，腹部にも異常を認めなかった．検査では好酸球増多(実数1330)とIgEの上昇(1865，7 IU/ml)を認め，HTLV-1は陰性で，気管支肺胞洗浄液では，細胞数1130/μl，CD4/8 1.1，好中球42%，好酸球11%，リンパ球33%，肺胞マクロアージ14%であった．血清の寄生虫検査ではmultiple-dot ELISAにて糞線虫が陽性(Fig 2)で，十二指腸の内視鏡検査では小白粉性隆起(small white powder-like protrusion)を認め(Fig 3)，この部の生検では病理学的に十二指腸の腺窩に寄生虫(糞線虫に矛盾しない)と考えられる構造を認めた(Fig 4)．さらに糞便検査ではラブジチス型幼虫が確認された(Fig 5)．

糞線虫症と診断し，2010年3月上旬よりivermectin 6 mg(0.2mg/kg)/日を2週間隔で2回に分けて投与したところ，好酸球は減少し，咳嗽も消失，十二指腸内視鏡検査でも表層粘膜は正常となった．

考　　察

糞線虫のヒトにおける感染形式は，土の中のフィラリア型(F型)幼虫がヒトの皮膚から侵入し，リンパ系から肺，さらに気管・食道などを移動し，最終的には十二指腸や小腸上部に寄生する．そこで雌の成虫となり産卵し，孵化したラブジチス型幼虫は便と共に体外に排出されるが，一部の幼虫は排出される前に腸管内でフィラリア型幼虫に発育し，腸粘膜や肛門周囲の皮膚から再び体内に入ることがある．これを自家感染といい，糞線虫が長期に亘り持続感染する原因となっている[2]．

本患者においては，長期に亘って好酸球増多が認められ，肺の浸潤影と共に，気管支肺胞洗浄液中にも好酸球増多が認められた．糞線虫症による肺病変として，斉藤は虫体の直接的な機械的肺障害(direct destruction of vessels)すなわち肺内に寄生したラブジチス型幼虫が肺内毛細血管から遊出して血管の破綻をきたすものと，生体反応によるallergic reaction，および二次的な細菌感染によるaccompanied lesionの3つの型に分けている[3]．本症例では肺の組織学的所見が得られていないので明らかでないが，抗菌薬の効果が得られなかったことからaccompanied lesionは考え難く，前二者のいずれかであろうと考えられた．

消化器症状は本症にみられる一般的な症状で，腹痛・腹鳴，腹満感，軟便など，その程度は多様とされる．このような症例では，十二指腸や小腸上部の粘膜にカタル性ないしは浮腫性変化が強く認められ(small white powder-like protrusion)，潰瘍形成を伴うこともあるとされている[2]．糞線虫症の病理解剖学的検討を行った所見としては，十二指腸から回腸にかけて高度の浮腫状肥厚があり，組織的に粘膜に多数の虫や卵を認めたとしている[4]．私達の症例では，消化器症状は認められなかったが，これを裏付ける内視鏡所見と生検における病理所見を得ることができた．

とくに基礎疾患がない場合には，軽度の消化器症状や呼吸器症状を示す程度で，臨床上問題は少ないとされる．しかし，免疫能が低下した状態においては，本病原体による過剰感染や播種性糞線虫症の病態となることがあり，その誘因として，human T-lymphotropic virus 1(HTLV-1)，human immunodeficiency virus(HIV)感染のほか，免疫抑制剤やステロイドの投与などが考えられている[5),6]．本症例では，HTLV-1陰性で，とくに免疫不全を思わせる病態は考えられなかった．

本症の治療薬剤について，Zahaらはmebendazoleは高頻度の肝機能障害があり，albendazoleと共に，その駆虫効果は十分でなかったが，ivermectinは強い駆虫効果が認められ，毒性も少なかったことから，本症の駆虫における最も有用な薬剤として推奨している[1]．本症例においても，ivermectin 6 mg/日の2週間隔2回の投与によって，好酸球数の低下と共に諸症状の改善をみとめた．

最後に本症例の感染であるが，おそらく幼少時のミンダナオ島での生活で起こったと考えるのが妥当であろう．その後，誘因は明らかでないが，高齢化などの何らかの免疫能の低下がおこり，症状や所見が明らかになったものと推察された．しかし，長年農業に従事してきたことや，有機野菜を摂取してきたことなど，長崎県口之津での感染も全く否定はできない．日本においては最近になって，沖縄以外に宮崎[7]や福岡[8]，さらに長崎においても本症の報告がみられるようになってきた[9),10),11]．いずれにしろ，好酸球増多を伴って呼吸器系や消化器系の症状を示す感染性疾患においては，糞線虫症も念頭に入れるべきと思われた．

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EFFICACY OF SODIUM METAPERIODATE (SMP)-ELISA FOR THE SERODIAGNOSIS OF SCHISTOSOMIASIS MEKONGI

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Abstract. Schistosomiasis mekongi is an important public health issue in endemic countries. In this study, we evaluated an indirect immunodiagnostic ELISA method using *Schistosoma mekongi* soluble egg antigen. Sodium metaperiodate (SMP)-ELISA was utilized in order to remove the glycosylated epitopes responsible for false positive reactions and the results using this method were compared with those using conventional ELISA (conv-ELISA). Forty-two serum samples from schistosomiasis mekongi egg-positive patients and 100 serum samples from schistosomiasis-negative Cambodian subjects were tested using both ELISA methods. The ranges of ELISA values for positive and negative sera were distinct on SMP-ELISA, but the ranges of the two groups of sera overlapped on conv-ELISA. Therefore, diagnostic criteria may be established based on the highest ELISA value on negative sera and the lowest ELISA value on positive sera. In the present study, both the sensitivity and specificity of SMP-ELISA reached 100% using the criteria in which an ELISA value ≥ 0.2 was positive.

Keywords: SMP-ELISA, *Schistosoma mekongi*, diagnosis

INTRODUCTION

Schistosomiasis mekongi is a significant public health problem in Lao PDR and Cambodia. A mass drug administration campaign by the National Center for Parasitology, Entomology and Malaria Control (CNM), Ministry of Health, Cambodia, with international cooperation was initiated in 1994 using praziquantel in Kratie Province. At the beginning of the control program, the disease prevalence in primary schools in the Kratie Province, as determined by stool examination using the Kato-Katz method, was 72.9% (Stich *et al*, 1999). As a result of the control program, the prevalence of schistosomiasis decreased dramatically. In 2004, when stool

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surveys were conducted at five sentinel villages, no schistosome eggs were detected and only three cases of schistosomiasis were reported (Sinuon *et al*, 2007).

Despite the success of the control program, the limited sensitivity of the Kato-Katz method is a matter of concern. The detection of eggs from patient stool has become difficult because the intensity and prevalence of the disease have decreased. Therefore, a more sensitive diagnostic technique is required to determine progress of the schistosomiasis control program (Urbani *et al*, 2002; Fenwick *et al*, 2006). Bergquist *et al* (2009) stated that diagnostic methods need to be continually adapted based on the stage of control. Serological diagnosis, such as using an enzyme-linked immunosorbent assay (ELISA), is a potential alternative diagnostic method for the schistosomiasis mekongi control program (Ohmae *et al*, 2004).

We previously conducted a seroepidemiological survey of schistosomiasis japonica using circum oval precipitin (COP) tests (Tanaka *et al*, 1987) and ELISA (Matsumoto *et al*, 1999; Chigusa *et al*, 2006) and have studied *Schistosoma mekongi* control in Cambodia (Ohmae *et al*, 2004).

Immunodiagnosis of schistosomaisis mekongi using antigens of *Schistosoma japonicum* (Zhu *et al*, 2005) and keyhole limpet hemocyanin (Ittiprasert *et al*, 2000) have been carried out, but immunological diagnosis using *S. mekongi* antigen has not previously been reported.

In this study, we performed an ELISA using the soluble egg antigen (SEA) of *S. mekongi* and utilized sodium metaperiodate (SMP)-ELISA. The method of Alarcón de Noya *et al* (2000) was used in order to remove the glycosylated epitopes responsible for false positive reactions occurring with immunodiagnosis of *S. mansoni* infection. In this study, a comparison between SMP-ELISA and conventional ELISA was performed, and we discus suitable criteria for the methods.

MATERIALS AND METHODS

Serum samples

A mass drug administration campaign was conducted in Cambodia: individuals in the target population were diagnosed by stool examination and serological testing as part of the National Schistosomiasis Control Program for Cambodia. The 42 positive control sera (Group A) used in this study were collected from schistosome egg-positive residents in 2003 (n = 34) and 2009 (n = 8) in Kratie Province, Cambodia. The 34 sera collected from *S. mekongi* infected patients in 2003 were pooled and used as the positive reference serum, which was distributed into sample tubes and stored at -40°C until use.

Negative control sera were collected from volunteers without risk for schistosomiasis mekongi infection: 100 Cambodians (Group B) in 2002 and 25 Japanese (Group C) in 2003. Group B was comprised of schoolchildren in Phnom Penh, located more than 120 km away from the endemic area. Furthermore, these subjects had no history of visiting the endemic area. Serum samples were collected by CNM as a part of the parasitic diseases control program.

Individuals in Group C were healthy Japanese volunteers without a history of traveling abroad. The purpose of the study was explained and written informed consent was obtained from all volunteers.

Antigen

Schistosoma mekongi (Laotian strain) was maintained in *Neotricula aperta* snails and mice (ICR strain) at the Laboratory of Tropical Medicine and Parasitology,

Dokkyo Medical University, Japan. SEA was prepared using the method described by Matsuda *et al* (1984). Eggs of *S. mekongi* were isolated from infected mice intestines by digestion method using actinase E (No. 122, Kaken Pharmaceutical, Tokyo, Japan) and collagenase (C6885, Sigma-Aldrich, St. Louis, MO), and the collected eggs were subsequently lyophilized. SEA was extracted from lyophilized eggs with carbonate buffer (0.05 M, pH 9.6). After protein content measurement using Bradford reagent (#500-0006, Bio-Rad Laboratories, Hercules, CA), 1 ml aliquots of SEA solution were placed in small tubes and stored at -80°C until use.

ELISA

The ELISA was performed as described by Matsuda *et al* (1984) and Hirose *et al* (2005) with some modifications. In this study, the standard ELISA technique used in our laboratory was designated as conventional-ELISA (conv-ELISA). *S. mekongi* SEA was dissolved in carbonate buffer at a concentration of 2 μg protein/ml just before sensitization of the ELISA plate (No.762070, Greiner Bio-One, Frickenhausen, Germany). Each well of the ELISA plate was sensitized overnight with 0.1 ml of *S. mekongi* SEA diluent. After washing the wells with T-PBS (0.15 M phosphate buffered-saline containing 0.05% Tween 20), the inner surfaces of the wells were blocked with 0.12 ml of BSA/T-PBS solution (T-PBS containing 1% bovine serum albumin) for 10 minutes.

We used horseradish peroxidase (HRP)-conjugated anti-human IgG goat serum (55252, MP Biochemicals, LLC-Cappel Products, Soton, OH) for the enzyme-conjugated antibody and 2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) di-ammonium salt (ABTS)(A-1888, Sigma-Aldrich, St. Louis, MO) as a substrate for HRP. The optimal concentrations of antigen, test sera, and enzyme conjugate were determined using checkerboard titration. The test serum was diluted to 1:200 with BSA/T-PBS, and 0.1 ml of the dilution was put in each well. After incubation of the plate at 37°C for 45 minutes, the wells were washed three times with T-PBS. Zero point one milliliter of HRP conjugate diluted to 1:1,200 was then added, and the plate was incubated at 37°C for 60 minutes. After washing, 0.2 ml of ABTS solution (0.03% ABTS, 0.25 M citric acid, 0.25 M sodium dihydrogen phosphate, 0.003% H_2O_2) was put in each well and the plate was kept at room temperature for 1 hour to allow enzyme reactions. The optical density (OD) of each well was read by a microplate reader (MTP-120, CORONA ELECTRIC, Ibaraki, Japan) at 415 nm.

For SMP-ELISA, SMP treatment of antigen was performed as described by Alarcón de Noya *et al* (2000) except for the concentration of SMP solution. The *S. mekongi* SEA-sensitized plate was prepared using the same method as conv-ELISA and was washed with 50 mM sodium acetate buffer (pH 4.5). Each well was treated with 0.1 ml of 0.5 mM SMP solution (0.5 mM SMP in sodium acetate buffer) at room temperature for 1 hour in the dark. After briefly washing the treated plate with PBS, 0.12 ml of 50 mM sodium borohydride in PBS was added to the wells and the plate was incubated for 30 minutes at room temperature. After washing three times with T-PBS, the plate was subjected to ELISA in a similar manner as conv-ELISA, except the dilution of HRP conjugate was 1:6,000. Optimal concentrations of SMP solution and enzyme conjugate were determined using checkerboard titration to make the positive reference serum produce an OD value comparable with that of the conv-ELISA.

Evaluation of cross-reaction with other parasites

A parasitological survey was conducted in Kratie Province, Cambodia in May 2005 as part of the National Schistosomiasis Control Program. In this survey, eggs from other helminths were found at positivity rates of 14.6-25.8% by stool examination in a village, Talous, located 5 km south of the city of Kratie. According to a previous survey, Talous was known as an area with low transmission of *S. mekongi*. Therefore, we compared the results of stool examination with those of the two ELISA methods in samples obtained from Talous to evaluate cross-reaction with other parasite infections by ELISA. Blood and stool were collected from 151 residents in Talous. Blood samples were collected on blood sampling filter papers (Advantec Toyo Kaisha, Tokyo, Japan) after finger pricking. The blood samples on filter paper were dried and transported to the laboratory in Japan. Discs (3 mm in diameter) were cut out from the blood sampling filter paper and placed individually into wells of deep-well microplates. Each disc with blood was immersed in 400 µl of BSA/T-PBS containing 0.5% skimmed milk, shaken vigorously, and incubated overnight at 4°C after vigorous shaking. The resulting extract was estimated to have a 1:200 dilution of the plasma specimen and was tested by the ELISA methods as described above. For reasons described in the Discussion section, we used the criterion that ELISA values ≥ 0.2 were positive. Stool examination was conducted using a formalin-detergent technique (Waikagul *et al*, 1997). In brief, 0.5 ml of each stool sample was suspended in a formalin-detergent solution (10% liquid dish-washing detergent, 2% formalin in water) and incubated for 30 minutes at room temperature. The suspension was filtered through gauze into another tube and then shaken vigorously. The suspension was allowed to settle for 3 hours, after which the supernatant was discarded. The remaining pellet was dissolved in 10% formalin to give a volume of 1 ml. The resulting specimen was mixed well, then 0.04 ml of the specimen was examined under the microscope. Each sample was examined twice.

Statistical analysis

The software program Microsoft Excel (Microsoft Office Excel 2003, Microsoft, Washington, WA) was used to calculate the correlation coefficient (r).

The two-sided probability (P) for the Fisher's exact probability test was calculated by standard statistical software (Dr. SPSS 2 for Windows, Version 11.0.1J, SPSS, Chicago, IL). P-value < 0.05 was considered significant.

RESULTS

The results of the two ELISA methods are shown in Fig 1 and Table 1. The range of ELISA values in Group A was wider with the SMP-ELISA on both the higher and lower sides. The highest ELISA value of Group B with the conv-ELISA (0.578) was markedly lower with the SMP-ELISA (0.198). The range of ELISA values from Group C was very narrow in comparison with the other groups, and there was little difference between the two ELISA methods. With conv-ELISA, the lowest value in Group A (0.330) was lower than the highest value in Group B. Therefore, 10 individuals (23.8%) from Group A and 6 individuals (6%) from Group B had ELISA values ranging from 0.330 to 0.578. In contrast, the distribution of the ELISA values from Group A was different than those from Groups B and C with SMP-ELISA.

The correlation between conv-ELISA and SMP-ELISA, from Groups A and B is

Table 1
A comparison of the ELISA methods for Schistosomiasis mekongi.

	conv-ELISA			SMP-ELISA		
	Group A	Group B	Group C	Group A	Group B	Group C
No. examined	42	100	25	42	100	25
Minimum	0.330	0.006	0.004	0.233	0.015	0.010
Maximum	1.232	0.578	0.054	1.526	0.198	0.058
Mean	0.755	0.089	0.018	0.694	0.058	0.029
SD	0.211	0.107	0.014	0.314	0.041	0.011
Mean + 3SD		0.410	0.059		0.180	0.061

Table 2
Relationship between the ELISA results and other parasitic infections detected by stool examination (n = 151).

			Stool examination [a]					
			A. lumbricoides		Hookworms		*Echinostoma* sp	
			Positive	Negative	Positive	Negative	Positive	Negative
			22	129	35	116	39	112
conv-ELISA [b]	Positive	25	3	22	7	18	9	16
	Negative	126	19	107	28	98	30	96
	Fisher's exact probability test (P)		1.000		0.605		0.217	
SMP-ELISA [b]	Positive	8	2	6	3	5	3	5
	Negative	143	20	123	32	111	36	107
	Fisher's exact probability test (P)		0.329		0.388		0.426	

[a]Stool examination was conducted using the formalin-detergent method.
[b]ELISA values ≥ 0.2 were considered positive.

shown in Fig 2. There were significant correlations between the two ELISA methods in both groups. The correlation coefficient of Group A was higher (r = 0.951, <0.001) than the correlation coefficient of Group B (r = 0.744, <0.001).

Stool examination and ELISA were conducted to determine cross-reactivity with other parasites (Table 2). Of 151 subjects, 25 (16.6%) and 8 (5.3%) had positive ELISA results with the conv-ELISA and the SMP-ELISA, respectively. No *S. mekongi* eggs were detected. Eggs from *Ascaris lumbricoides*, hookworms, and *Echinostoma* sp were detected in samples from 22, 35, and 39 individuals, respectively. Most individuals who had eggs of other parasites had negative ELISA results on both ELISAs, but some individuals had positive ELISA results. The ELISA positivity rates

Table 3
False positive and false negative in each criteria.

	conv-ELISA			SMP-ELISA		
	Group A	Group B	Group C	Group A	Group B	Group C
≥0.2	42 (100%)	9 (9%)	0 (0%)	42 (100%)	0 (0%)	0 (0%)
<0.2	0 (0%)	91 (91%)	25 (100%)	0 (0%)	100 (100%)	25 (100%)
≥X (B)[a]	41 (98%)	2 (2%)	0 (0%)	42 (100%)	2 (2%)	0 (0%)
<X (B)[a]	1 (2%)	98 (98%)	25 (100%)	0	98 (98%)	25 (100%)
≥X (C)[b]	42 (100%)	45 (45%)	0 (0%)	42 (100%)	33 (33%)	0 (0%)
<X (C)[b]	0	55 (55%)	25 (100%)	0 (0%)	67 (67%)	25 (100%)

[a]X (B) Mean + 3SD of Group B in each the method. The values are shown in Table 1.
[b]X (C) Mean + 3SD of Group C in each the method. The values are shown in Tabele 1.

among egg-positive patients were 13.6-23.1% with conv-ELISA and 7.7-9.1% with SMP-ELISA. The Fisher's exact probability test did not show bias with the ELISA for intestinal parasites.

DISCUSSION

SMP treatment of SEA was performed in order to destroy the glycosylated epitopes responsible for false-positive results (Alarcón de Noya *et al*, 2000). Because glycosylated epitopes can be recognized by antibodies in *Schistosoma mekongi* infected patients, the OD values of the patients decreased with the SMP-ELISA when we used enzyme conjugate at the same dilution rate as that used for conv-ELISA (data not shown). In this experiment, we used a higher concentration of enzyme conjugate with the SMP-ELISA. SMP treatment can increase or decrease ELISA values based on serum type and result in expansion of the range of ELISA values for Group A with SMP-ELISA. Differences in the effect of SMP treatment on ELISA values appear to depend on the composition of target epitopes in each serum sample. Most antibodies in sera with high ELISA values in Group B, which consisted of subjects from a non-endemic area in Cambodia, recognized the glycosylated epitopes in SEA.

Diagnostic criteria are established based on the protocol and/or purpose of the study. Many researchers have used mean + 3SD for the OD values of negative control sera as a cut-off limit (Alarcón de Noya *et al*, 2000). Some authors used criteria that depended on the distribution of ELISA values for positive and negative controls (Hirose *et al*, 2005). In this study, Cambodian (Group B) and Japanese (Group C) individuals were examined as negative controls. Using the mean + 3SD for the negative control for Group B as a cut-off value (Table 3), the sensitivity and specificity of conv-ELISA were both 98%, and the sensitivity and specificity with SMP-ELISA were 100% and 98%, respectively. Using the same criteria for Group C (Table 3) results in false positives with conv-ELISA of 45% and with SMP-ELISA of 33%.

The range of ELISA values for positive sera was distinct from that for negative sera with SMP-ELISA. Therefore,

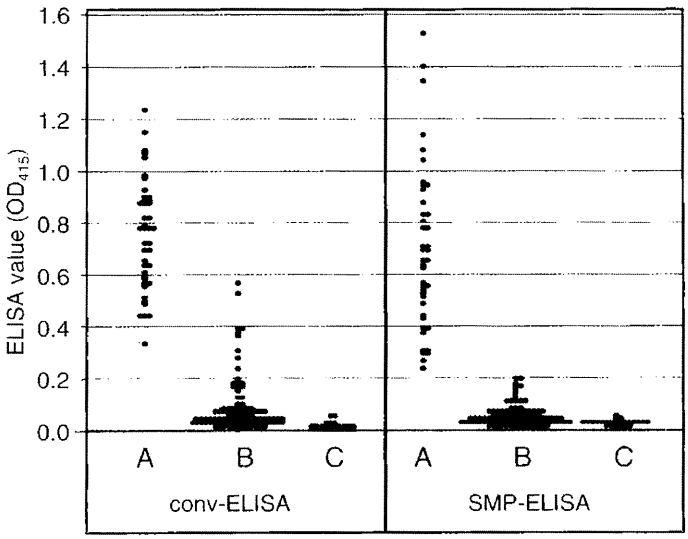

Fig 1–Distribution of ELISA values for sera from *S. mekongi* infected patients and from subjects in non-endemic areas by the two ELISA methods. A: *S. mekongi* infected patients (n = 42); B: subjects in a non-endemic area in Cambodia (n = 100); C: subjects in Japan (n = 25).

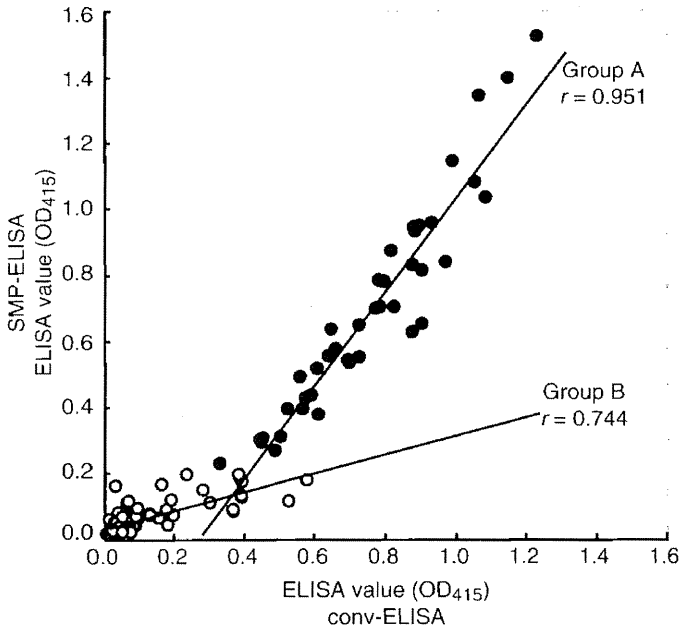

Fig 2–Plot of OD values comparing conv-ELISA to SMP-ELISA to evaluate the correlation between the two ELISA methods. The OD values of sera from *S. mekongi* infected patients (solid circle) and Cambodians living in a non-endemic area (open circle) are shown. Group A: *Schistosoma mekongi* infected patients (n = 42); Group B: persons in a non-endemic area in Cambodia (n = 100).

diagnostic criteria can be established based on the highest ELISA value for negative sera and the lowest ELISA value for positive sera. Using the criterion in which an ELISA value ≥0.2 was positive, both the sensitivity and specificity of SMP-ELISA reached 100% (Table 3).

Concerning negative control sera, the range of ELISA values for Group B (Cambodian) was higher than for Group C (Japanese). This is possibly due to a difference in antigens to which each group were exposed, both qualitatively and quantitatively. Our results suggest SMP-ELISA can reduce the influence of cross reactive antigens.

A positive ELISA reaction without schistosome eggs indicates one of three scenarios: 1) active infection with *S. mekongi* but no eggs were detected, 2) *S. mekongi* has already been treated but residual antibodies still existed, and (3) non-specific cross-reaction was detected. The lower sensitivity of the stool examination compared to the ELISA may explain why scenario 1) might occur. In low transmission areas, such as in Talous, it is difficult to detect eggs in stool samples because of lower disease intensity (Urbani *et al*, 2002; Fenwick *et al*, 2006). Scenario 2) is a characteristic feature of diagnostic methods detecting specific antibodies. This reaction indicates the subject had a risk for infection. In

general, residual antibodies tend to diminish progressively, and withdrawal periods vary between individuals (Hayashi *et al*, 2000). In scenario 3), a non-specific cross-reaction, might be one of the causalities, at least with the conv-ELISA (Fig 1). Most patients with intestinal parasite eggs had negative ELISA values, although some had positive values. The present data suggest infection with one of these three intestinal parasites should not result in a significant cross-reaction. Given the results of Table 3, it can be seen that the positive reactions on SMP-ELISA are most likely caused by scenarios 1) or 2). Assuming that all positive ELISA tests were due to cross-reaction with other parasites, the maximum rate of false positives with SMP-ELISA would be 9.1% (2/22). In another survey in a *Schistosoma mekongi* endemic area in Champasack Province, Lao PDR in 2006, we detected *Opisthorchis viverrini* eggs without *S. mekongi* eggs in 13 of 41 individuals who took the survey (Nakamura *et al*, 2006; unpublished data). Three of 13 opisthorchiasis patients had positive ELISA values, and 14 out of 41 had positive ELISA values on SMP-ELISA. The ELISA positivity rate for *O. viverrini* egg-positive persons (23%) was lower than the positivity rate of the targeted 41 persons (34%). These data suggest cross-reaction should not be significant with these helminths, although further studies are required for validation.

We conclude the use of SMP-ELISA improves diagnostic specificity and sensitivity for schistosomiasis mekongi. This method should become a powerful tool for diagnosing infection.

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ORIGINAL PAPER

Compartmentalization of a Glycolytic Enzyme in *Diplonema*, a Non-kinetoplastid Euglenozoan

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Glycosomes are peroxisome-related organelles containing glycolytic enzymes that have been found only in kinetoplastids. We show here that a glycolytic enzyme is compartmentalized in diplonemids, the sister group of kinetoplastids. We found that, similar to kinetoplastid aldolases, the fructose 1,6-bisphosphate aldolase of *Diplonema papillatum* possesses a type 2-peroxisomal targeting signal. Western blotting showed that this aldolase was present predominantly in the membrane/organellar fraction. Immunofluorescence analysis showed that this aldolase had a scattered distribution in the cytosol, suggesting its compartmentalization. In contrast, orotidine-5'-monophosphate decarboxylase, a non-glycolytic glycosomal enzyme in kinetoplastids, was shown to be a cytosolic enzyme in *D. papillatum*. Since euglenoids, the earliest diverging branch of Euglenozoa, do not possess glycolytic compartments, these findings suggest that the routing of glycolytic enzymes into peroxisomes may have occurred in a common ancestor of diplonemids and kinetoplastids, followed by diversification of these newly established organelles in each of these euglenozoan lineages.

Key words: Euglenozoa; evolution; glycosome; kinetoplastids; peroxisome; trypanosomatids.

Introduction

Peroxisomes are cellular compartments surrounded by a single membrane and present in almost all eukaryotes. These specialized organelles are involved in the oxidation of various organic substrates using molecular oxygen. This process results in the generation of toxic hydrogen peroxide, which must be detoxified by catalase. Peroxisomes participate in important metabolic pathways, including the β-oxidation of fatty acids, free radical detoxification, and purine/pyrimidine degradation (Gabaldón et al. 2006; Parsons et al. 2001; Schlüter et al. 2006).

The physiological functions and enzymatic contents of peroxisomes vary across species and often among growth conditions (Michels et al. 2005; Parsons et al. 2001). For example, glyoxysomes, the specialized form of peroxisomes found in germinating plant seeds, contain enzymes of the glyoxylate cycle, whereas peroxisomes of leaves

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contain enzymes of the glycolate pathway, a difference regarded as a typical example of organellar diversification (Gabaldón 2010; Hayashi et al. 2000; Tolbert et al. 1968).

The protistan group Euglenozoa includes three major lineages, euglenoids, diplonemids, and kinetoplastids, with euglenoids constituting the earliest branch followed by the separation of the diplonemid and kinetoplastid lineages (Cavalier-Smith 1981; Simpson et al. 2002; Simpson and Roger 2004). Kinetoplastids consist of bodonids and trypanosomatids, the latter of which include medically important pathogens, such as *Trypanosoma brucei*, which causes sleeping sickness (African Trypanosomiasis); *T. cruzi*, which causes Chagas disease (American Trypanosomiasis) and *Leishmania* spp., which cause leishmaniasis.

The presence of peroxisomes in euglenoids is not fully understood. *Euglena* possesses peroxisome-like particles but lacks catalase activity (Collins and Merrett 1975; Graves et al. 1971; Shigeoka et al. 1980). In contrast, kinetoplastids possess unique peroxisome like-organelles, called glycosomes, characterized by compartmentalization of most of the glycolytic pathway (Michels et al. 2006; Opperdoes and Borst 1977; Opperdoes et al. 1988). In addition, glycosomes contain other metabolic enzymes, such as most of the enzymes of the purine salvage pathway and the last two enzymes of the de novo pyrimidine biosynthetic pathway, orotate phosphoribosyltransferase (OPRT) and orotidine-5′-monophosphate decarboxylase (OMPDC) (Hammond and Gutteridge 1982; Michels et al. 2000). These findings highlight the unique evolution of peroxisomes in the kinetoplastid lineage.

The evolutionary trail by which peroxisomes gave rise to glycosomes in kinetoplastids, however, remains unclear, due largely to the lack of biochemical and genetic information regarding the nature of peroxisomes and peroxisome-related organelles in diplonemids, the closest relatives of kinetoplastids. We previously reported that the structure of the *OPRT* and *OMPDC* genes varies among euglenozoan lineages (Makiuchi et al. 2007, 2008). Euglenoids possess a *OPRT-OMPDC* fusion gene, with the OMPDC activity present in the cytosol (Walther et al. 1980). In contrast, kinetoplastids possess an inversely fused *OMPDC-OPRT* gene, with the OMPDC-OPRT fusion protein having a peroxisome-targeting signal (C-terminal PTS1), suggesting that it is localized in glycosomes. In the diplonemid, *Diplonema papillatum*, the subcellular localization of OMPDC (and putative OPRT) has not yet been determined. We found that *D. papillatum OMPDC* is a stand-alone gene, lacking the consensus sequence of a PTS, suggesting that the protein is localized in the cytosol (Makiuchi et al. 2008).

In the present study, we examined whether glycosome-associated enzymes are compartmentalized in diplonemids. We characterized two enzymes, the glycolytic fructose 1,6-bisphosphate aldolase (ALD) and OMPDC in *D. papillatum*. We demonstrate that a consensus sequence of an N-terminal PTS2 is present in ALD of *D. papillatum*, suggesting that this enzyme is localized in peroxisomes, as is that of trypanosomatids. Phylogenetic analysis showed monophyly of trypanosomatid and diplonemid ALDs. Biochemical analysis showed the different distributions of ALD and OMPDC, predominantly in the membrane-rich and cytosolic fractions, respectively. Immunofluorescence analysis showed that *D. papillatum* ALD has a scattered pattern of distribution, whereas OMPDC has a uniform pattern of distribution. Our findings may provide insights into the distinct evolution of peroxisome like-organelles in the different euglenozoan lineages.

Results

Presence of a Peroxisomal Targeting Signal in *D. papillatum* ALD

ALD is a glycosomal enzyme in trypanosomatids (Hart et al. 1984; Taylor and Gutteridge 1987). Full-length ALD cDNA of *D. papillatum* (GenBank™ AB550707) was cloned based on its partial sequence registered in the TBest database (http://tbestdb.bcm.umontreal.ca/searches/login.php). In general, ALDs can be divided into two classes, Class I and Class II; Class I aldolases are present in vertebrates, plants and a number of protists and prokaryotes (see Fig. 2), whereas Class II aldolases are present in fungi, several protists and many prokaryotes (Rutter 1964). We found that the amino acid sequence of *D. papillatum* ALD showed identities of 63%, 45%, 48%, and 50% with the Class I ALDs of trypanosomatids, *Euglena gracilis* (euglenoids), *Arabidopsis thaliana* (plants), and humans (animals), respectively.

D. papillatum ALD is comprised of 374 amino acids and has an estimated molecular weight of 41 kD. The amino acid residues constituting the active site of class-I ALD are fully conserved in *D. papillatum* ALD (St-Jean et al. 2005). Indeed, recombinant *D. papillatum* ALD expressed in *Escherichia coli* was able to catalyze