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文化と疾病の狭間

― ケニアで風土病を学ぶ ―

Studying endemiology in Kenya

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プロローグ

標高 1,700 m にある首都ナイロビから南東 450 km のケニア第 2 の都市モンバサへ向かうモンバサロードは幹線道路のわりには全体が完全であったためしがなく，サバンナのなかをところどころ穴のあいた片道一車線の舗装道路がはてしなく続く．朝，ナイロビ発のバスに乗ると 2 時ごろ途中のムティトアンデイで昼飯休みをとり，6 時ごろモンバサの町に着く．著者がはじめてこの道路を通ったのは 1991 年 1 月末，前年からはじまった JICA(現：日本国際協力機構)の“ケニア感染症対策研究プロジェクト”の短期専門家としてビルハルツ住血吸虫症の健康教育評価をするためであった．このプロジェクトは実質的に第三次 5 カ年プロジェクトであり，活動はその 10 年ほど前からはじまっていた．

モンバサはアラブ人が拓いた町であり，その後ポルトガルの拠点として交易で栄えた．モンバサ島は陸橋で本土と繋げられ，イギリス植民地時代にナイロビを経てウガンダに至る東アフリカ鉄道の出発点となった．調査地があるクワレ郡はモンバサの南に位置している．貨物船と軍艦が通るモンバサ港に至る海をリコニフェリーで渡り，そこからタンザニアのタンガに向かう道を南下して途中のコッパーニから西に折れて海岸稜を登って 15 km ほど走るとクワレの町に着く．町は標高 400 m の森に囲まれた丘のうえにあり，灼熱の沿岸州のなかでは涼しく，イギリス植民地時代に郡都とされた．しかし，郡の経済的中心は沿岸道路沿いにあり，また，この地域の有力民族であるドルマ族の中心はキナンゴという 30 km ほど西に行ったところにある．したがって，クワレの町は行政の中心ではあるが，この 15 年間あまり経済的に発展しないのんびりした田舎町のたたずまいを保ってきた．

クワレの町から内陸側は暑く乾燥した低地サバンナウッドランドが広がり，その地域は沿岸州のなかでもきわめてビルハルツ住血吸虫症の感染が多い地域である．いまだに治療しても 2 年後には小学生の 9 割が感染してしまう．クワレから未舗装路を 13 km ほど行くと，著者らが長年対策と研究を実施してきたムワチンガ村がある．この村での住血吸虫症対策研究は 1981 年に長崎大学熱帯医学研究所の青木克己，嶋田雅暁などによってはじめられた．彼らはクワレ郡の 40 余の小学校で虫卵検査を実施し，感染率が高く，まったく治療がなされていないムワチンガ村を調査地とした．その後，プロジェクトはナイロビに新設された中央医学研究所(KEMRI)のカウンターパートとともに多くの寄生虫学者が派遣専門家として参加して継続され，JICA プロジェクトの後は科学研究費補助金などで研究を継続して現在に至っている．2001 年からは ESACIPAC(国際寄生虫対策東・南アフリカセンター)プロジェクトが開始され，クワレ郡でも活動を展開している．

住血吸虫症は巻貝を中間宿主とする寄生虫疾患で，人間が川を歩いて渡ったり水浴をしたりすると感染する．感染者の 8 割と重症例のほとんどはアフリカに集中している．ビルハルツ住血吸虫の成虫は膀胱壁周辺の静脈で産卵し，卵は膀胱壁を

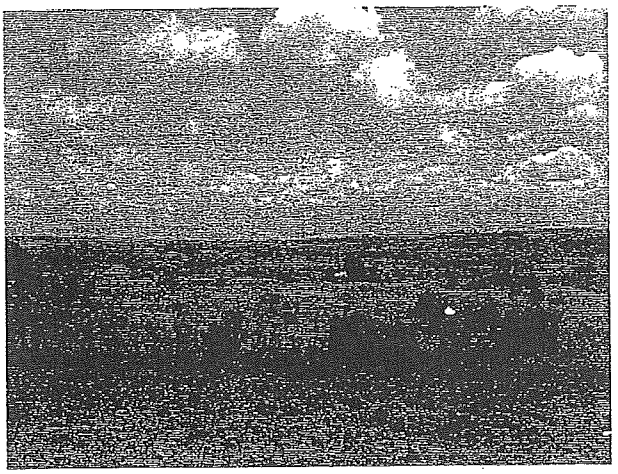

図 1　ケニア沿岸州クワレ郡ムワチンガ村周辺

図 2　川で洗濯をする親子連れ

破って尿とともに体外にでる．そのため，血尿がもっとも一般的にみられる症状である．サハラ以南アフリカで 4.4 億人が感染危険地域に住み，1.1 億人が感染し 0.7 億人に血尿症状があると報告されている[1]．基本的に田舎に住む貧しい人の疾患であり，“忘れられた疾患(neglected diseases)”のひとつである．

著者は寄生虫学者ではなく，人類生態学をバックグラウンドとして公衆衛生・国際保健分野の仕事に従事してきた者としてこのプロジェクトで多くのことを学ばせていただいた．文化の専門家でも疾病の専門家でもないが，おそらくその関係をみる者としての役割が与えられたのだと思う．はじめての訪問から 14 年，ほぼ毎年のペースで調査地を尋ね，“文化と疾病の狭間”で苦しんできた者として，このプロジェクトを通してみえてきた熱帯地での風土病について考えを整理してみたい．

ムワチンガ村

ムワチンガ村には 200 世帯 1,500 名ほどが居住している．村といっても 10 km 四方内のいくつもの小丘陵に家屋が点在しているだけで，確固たる村の中心があるわけではない(図 1)．一応，小学校がある地域(ムワチンガ)が中心だと著者らは考えている．ミジケンダ族に属するディゴ，ドルマ，ギリヤマ族の人びとが生活している．多くがイスラム教徒であるが，生活の基礎には伝統的要素が多く残っている．生活は厳しく，人びとはケニアのなかでもきわめて貧しい．土地はやせていて降雨量も少なく，トウモロコシが捨植えのように栽培される．多くの世帯は収穫を 3 カ月程度で食べつくし，何らかの方法で現金収入を得て食料を買わなければいけない．ウシ，ヤギは重要な収入源であるが，それほど数もない．

村の端をマレレ川という大きな川が流れ，村のなかの小丘陵の間にはいくつにも分かれて小さな川が存在し，それがカディンゴ川，マロノ川となってマレレ川に合流する．支流の多くは通常はほぼ干上がっていて雨が降ると水がたまり，たくさん降ると流れになるといったものである．これらの川にビルハルツ住血吸虫の媒介貝 *Blinus globosus* が棲息している．ただでさえ厳しい生活状況であるうえに，野生の象が多く棲息していて村の農作物や果樹を荒らし人命も脅かしていた．著者が 1991～1993 年に行った活動のひとつは，ケニア中央医学研究所を通してケニア野生動物保護局に電気柵を村と野生動物保護区の間につくってもらったことであった．その後，象の棲息地は整備されてムサンブウェニ象保護区となり，外国人観光客がくるようになり，小さな子どもたちは屈託なく“Give me money！”とあいさつするようになった．いまでは象は電気柵の支柱部分をうまく倒すことを学習し，ときどき村に侵入してくる．

対策，およびそれを困難にする生活要因

汚染水との接触は，水道水などの安全水の利用が多くなれば減少する．ムワチンガ村はマレレの湧き水をモンバサに運ぶ水道管が通っている．

れを利用して1984年から安全水の供給を開始した．しかし，水道水は有料で川の水は無料であり，水道蛇口までの距離が遠い家庭もある．村人は飲み水や調理水には安全水を使い，川の水との接触をある程度下げる効果を示した[2]．しかし，安全水の供給で川の水との接触をすべてなくすことは困難であった．いまでも多くの人びとは，水浴，洗濯，食器洗い，渡川，水遊びのために川の水との接触を頻繁に続けている(図2)．川での魚とりも重要な生業活動であり，また子どもたちの娯楽である．

生活が厳しく村内での就業機会が限られているために，長期，短期の出稼ぎが重要な収入源である．モンバサやクワレの町で親族などを頼って不安定な職に就き，少額の金をもって村に戻り村では半失業者的生活を繰り返す．村人が頻繁に移動を繰り返すため，人口の把握，疫学調査，対策が難しくなる．住血吸虫に感染したまま都会にでて集団治療時に不在のために治療を受けず，その後，村に帰って虫卵の供給者となる．また，帰村者はHIV/AIDSなど都市の病気を村にもちこむ．

伝統的な“やまいillness”の概念

病気に対する考え方はそれぞれの文化によって異なり，病気に対してどのような対処・予防行動をとるかも各地域文化ごとに異なる．この地域でドルマ族の医療人類学研究を行っている浜本満[3]によれば，ドルマ(浜本はドゥルマと表記)の病気分類は，“悪霊，祖霊，妖術のやまい”といった呪医によって治療されるべきものと，呪医による治療の必要ない“神・運命のやまい”に分類される．後者は“西洋医学でも直せる単純な疾患disease”であることが多く，マラリアや住血吸虫症などは通常はこれに分類され，単なる薬(売薬，病院の薬，単なる薬草)が効く．しかし，悪霊によってマラリアや住血吸虫症にかかることもあり，その場合は呪医によるまったく違った治療が選択されねばならず，呪医が呪文を唱えてはじめて効力をもつ呪薬が必要となる．

現在では健康教育活動などの結果，多くの人びとが「ある種の巻貝から虫がでて水中で皮膚を貫通して体内に侵入し，血尿などを起こし，放置すると病気が重篤化し，死亡することもある．巻貝から虫がでてくるのは感染者が川に排尿するからである」ということを表面上は理解している．“死亡する”と理解されねばならないのは，“死亡しない病気”は彼らの病気の範疇に入らないからである．従来，子どもが血尿を出すのはサトウキビやトウモロコシ，トウガラシを食べすぎたからだと説明された．そしてビルハルツ住血吸虫症をずっとわずらっていると大人になって淋病になるとも説明された．さらに，淋病やビルハルツ住血吸虫症に長期間かかっていると“テゴ t'ego”という病気になり，不妊になったり死亡したりすると信じられている．

テゴは成人あるいは老人の病気で，その一部はビルハルツ住血吸虫症流行地域に多いとされる腎不全，膀胱癌だと考えられる．テゴの原因は，密通相手の配偶者が呪文をかけた草木を寝室の入口におき，それに気づかず密通したからだと信じられている．住血吸虫症以外の多くのテゴが性感染症の合併症であるとすれば，この説明はわれわれにも理解しやすい．テゴは呪医による儀礼的治療を行わなければならない重篤な妖術によるやまいであり，単なる住血吸虫症とは大きく異なる．

伝統的な病因論は，一般にみられる症状がなぜある特定の個人の特定の時期に重篤化し，死をもたらすかを説明する概念である．西洋医学の説明理論とは異質であり，異質であるがゆえに両立している．住血吸虫に対する対策も死と結びつけないかぎり彼らは重要性を本心では納得しない．そして，まだその点が十分に納得されていないことが，この地域での住血吸虫症対策の難しさの根底にある．われわれは住血吸虫症が寄生虫疾患であることを当たり前のこととして治療や予防に対する協力を求めがちであり，住民が協力したり指示に従ったりすることが当然だと考えやすい．しかし，そもそもその態度そのものが疾病対策の弊害になることもあるのである．

治療の可能性——WHOの戦略の変遷

1979年に駆虫剤プラジカンテルが発明され，住

血吸虫の対策は1980年代に一変する．日本での対策が有効な薬もなく副作用も多かった時代に貝対策・環境改良対策によってなされたのとは大きな違いである．プラジカンテルは1回の投与で9割の患者が虫卵陰性となり，副作用も少なく，その出現は画期的であった．1984年にWHO専門委員会が発表した住血吸虫症対策はプラジカンテルによる集団治療を中心としたものであり，それまでの媒介貝対策を中心とした伝播対策(transmission control)に対し疾患対策(morbidity control)とよばれた．疾患対策としたのはプラジカンテルで集団治療しても再感染は確実に起こり，根絶，排除するには至らないことがはじめから明確であったからである．健康教育，安全水供給とトイレの普及による伝播対策，媒介貝対策と環境管理は集団治療をサポートするものとして言及されていた．

1991年にWHO専門員会は再度この方針を支持しつつも，伝播対策も含めたより柔軟な実施を推奨し，対策は包括的ではあるが曖昧なものとなった．これは当時まだプラジカンテルが高価であり，プラジカンテルの出現にもかかわらず対策が進まなかったからである．実際に1991年当時，成人への1回投与量は7ドル程度であり，ケニア人1人当りの保健関連の年間予算を超えていた．集団治療を中心とした対策は実施ができれば有効であったが，とても全体に広がる状況ではなく，全体としては患者数も減少しなかった．プラジカンテルは郡病院でも入手不可能であったし，必須医薬品のリストにも載っていなかった．必須医薬品とするには途上国は貧しすぎた．ケニアのクワレ地方でもプラジカンテルは郡病院になく，治療はできない状況であった．著者はこの当時，公衆衛生学的観点からプラジカンテルによる治療には多くの疑問をもった．高い薬で治療してもすぐに再感染が起こり，実際の治療の長期的効果を判定するのは困難であった．

この状況を変えるために，1990年代に具体的なプラジカンテルの使い方などが検討されるようになった．とくに当時オックスフォード大学からスタートしたPartnership for Child Development(PCD)は科学的で現実的な住血吸虫対策と土壌伝播腸管寄生虫対策のための研究を行った．ビルハルツ住血吸虫の場合，自覚的血尿の有無，あるいは自覚的な感染を学童から聞き取ることによってかなりの程度，感染がわかること，とくに学校レベルでの感染率を評価し，集団治療するかどうかを判断するのに十分使えることなどを明らかにした．

一方，超音波診断によるビルハルツ住血吸虫感染の身体影響が明確に客観化された．1993年に“病気負担を考慮した人生年(DALY)”損失の概念が導入され，住血吸虫の慢性的感染による“病気負担(disease burden)”が無視できない規模であること，さらにプラジカンテルが廉価になったことにより住血吸虫症対策が費用対効果が高いことなどが判明した．いまでは小学生の治療は1人10セント以下となった．

これらの研究をもとにWHOは1998年に非公式レポートを作成し，高流行地での対策に対する指針を発表した(Report of the WHO Informal Consultation on schistosomiasis control, Geneva 24December 1998, WHO/CDS/CPC/SIP/99.2)．指針では住血吸虫症と土壌伝播寄生虫の総合対策が提案され，公衆衛生的重要性がなくなるまで有病率を下げ，発育を改善することが目標とされた．そのための戦略として，①末端の診療所での治療体制，②学校保健プログラム，③地域介入プログラムの展開が採用された．

2000年には低流行地での実質的制圧eliminationを視野に入れた指針を発表し(Report of the WHO Informal Consultation on schistosomiasis low transmission areas：control strategies and criteria for elimination, London, 10-13, April, 2000)，①プラジカンテルと腸管寄生虫に対するアルベンゾルなどの駆虫薬が流行地の診療所などで常備使用できる，②医療機関において有症状の感染の正しい治療ができる，③5歳以下の子どもIMCI(子どものやまいの統合ケア)のなかで治療することが目標とされた．そして，2001年5月には，第54回WHO総会において“住血吸虫症と土壌伝播腸管寄生虫対策WHA54.19, Agenda item 13.3 22”が採択され，2010年までに高流行地75%の小学校で(あるいは高流行地の学齢期の子どもの75%を対象として)定期的集団治療が実施されることを目標に設定した．

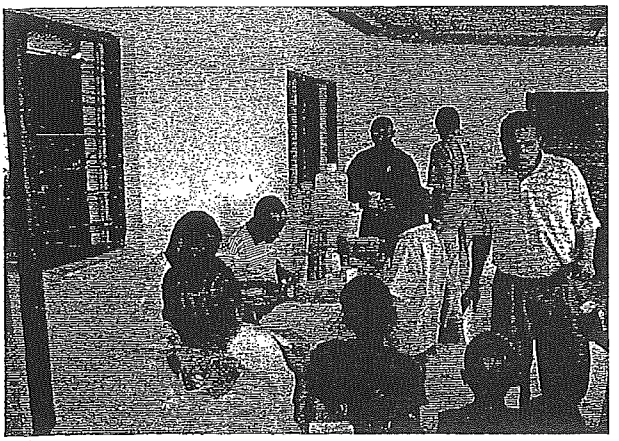

図 3　診療所での駆虫剤の集団投薬

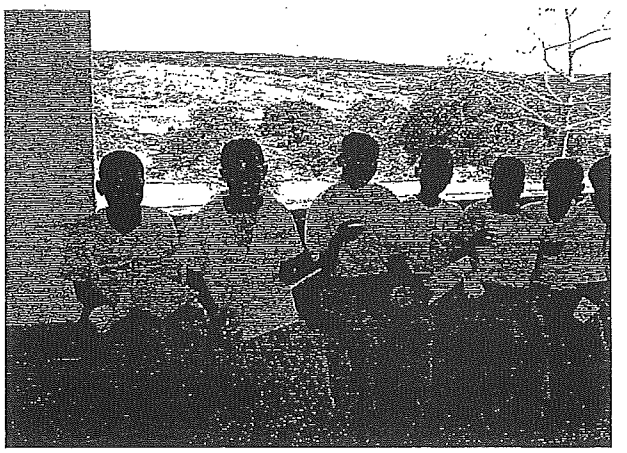

図 4　検尿をまつ小学生たち

しかし，クワレ地域では 25％以下の小学校でしか集団治療は実施されていないのが現状であり，2010 年の目標達成には多大な努力が必要である．

対策を成功させるために

プラジカンテルは有効な薬であるが，薬剤耐性が発生する危険性が指摘されており，プラジカンテルに頼るいまの対策に対する批判がなされている[4]．治療しても再感染に対する対策が不十分であり，2〜3 年で虫卵陽性率はほぼもとの値に戻ってしまうからである．しかし一方，高流行地であるにもかかわらず，一度も治療の恩恵を受けたことのない子どもも多い．したがって，短期的・中期的・長期的な視点で対策を整理しなければならない．

短期的にはプラジカンテルによる合理的な治療である．JICA が実施したジンバブエでの事例では末端の保健センターの環境技師と看護師が小学校を訪問して治療をするシステムが確立し，プロジェクト終了後も維持されている．すくなくとも住民のことをよく考える末端の医療機関がなくてはいけない．プライマリヘルスケア(PHC)のなかに住血吸虫症対策が組み込まれることが大切である．しかし，それさえ僻地では難しい情況である．中期的には，丸木橋をつくったり水場を安全にしたりすること，貝対策を実施するために草刈りを実施したり簡単な環境改変を行うこと，ときには有効に殺貝剤を使うことなどであり，そのための教育を実施することである．長期的には，人びとの生活水準，経済水準，環境衛生がよくなることであり，安全な水道水が使えること，トイレが完備されること，水道代や薬代が出せるようになること，そして健康で長生きすることの価値が現実性をもって理解される必要がある．

エピローグ

著者らのケニアのプロジェクトも，小さな診療所と診療所のスタッフハウス(研究者が滞在できる部屋がある)を村人の協力で完成させ，さまざまな対策を実施している(図 3，4)．しかしである．病気はいまだになくなる気配がなく，生活の一部とすらなっており，まさに住血吸虫症が風土病であることを思い知るのである．一次の流行(epidemic)ならば対策は可能かもしれない．疫学研究(epidemiology)が原因を推論し，その除去が試され，成功するであろう．しかし，原因がわかってもそれが除去できず，生活のなかに根づいているのが風土病であり，それは生活が変わらないとなくならない．熱帯医学は風土病を扱うことが多く，風土病をなくすには人びとの生活を十分に理解したうえで疾病対策に取り組む風土病学(endemiology)を確立していかなければならない．

近代医学の最先端を適用することは重要である．しかし同時に，人びとの生活と疾病との関係を十分に理解し，その対策に向けた健康転換・健康開発を促進していくこともまたきわめて重要な課題であり，そのためにも近代の叡知が使われなければならないと考える．

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Expression of mRNAs and proteins for peroxiredoxins in *Plasmodium falciparum* erythrocytic stage

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Abstract

mRNA and protein expression profiles for three peroxiredoxins (PfTPx-1, PfTPx-2 and Pf1-Cys-Prx) and a thioredoxin (PfTrx-1) of *Plasmodium falciparum* during the erythrocytic stage were examined by real-time quantitative reverse transcription-PCR (RT-PCR), Western blotting and confocal laser scanning microscopy. PfTPx-1 was expressed constitutively in the parasite cytoplasm throughout the erythrocytic stage, suggesting a housekeeping role of this enzyme for control of intercellular reactive oxygen species (ROS) in the parasite. Pf1-Cys-Prx showed elevated expression during the trophozoite and early schizont stages in the parasite cytoplasm, and this profile suggested that this peroxiredoxin (Prx) detoxifies metabolism-derived ROS such as those released from heme iron. The other 2-Cys Prx, PfTPx-2, was detected in mitochondria and was expressed in both the trophozoite and schizont stages. Detection of the Prx in mitochondria is consistent with recent reports of the existence of a respiratory chain, which produces ROS, in the mitochondria of *P. falciparum*. PfTrx-1 showed elevated expression during the trophozoite and schizont stages in the parasite cytoplasm. Finally, expression of these antioxidant protein genes is most likely regulated at the transcriptional level because their mRNA and protein expression profiles overlapped.

Keywords: Antioxidant; Malaria; Peroxiredoxin; *Plasmodium falciparum*; Thioredoxin

1. Introduction

Plasmodium falciparum is responsible for 1–2 million malaria deaths each year. The parasite actively proliferates in human erythrocytes, and is subject to the toxic effects of reactive oxygen species (ROS) during the period of asexual development [1]. Because *P. falciparum* is sensitive to oxidative stresses [2], its antioxidant defenses are considered to play essential roles in its survival and thus are promising targets for new chemotherapeutic strategies [3,4]. *P. falciparum* has two major functional antioxidant defense systems: a thioredoxin system and a glutathione system [2,5].

The thioredoxin system is based on NADPH-dependent reduction of thioredoxin (Trx) by Trx reductase (TrxR). Trx supplies reducing equivalents to enzymes such as thioredoxin peroxidases (TPxs) [6] and the system is thought to be important for *P. falciparum* detoxification of hydroperoxides because the parasite lacks catalase and a true glutathione peroxidase [3]. The importance of the TPx [7] and TrxR [8] in the development of *P. falciparum* in the host erythrocytes has been shown in vitro.

Abbreviations: Prx, peroxiredoxin; TPx, thioredoxin peroxidase; Trx, thioredoxin; TrxR, thioredoxin reductase.

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Proteins that are homologous to the TPx (thiol-specific antioxidant; TSA) of yeast [6] have been identified in organisms from bacteria to humans and are called peroxiredoxins (Prxs) [9]. Three Prx genes have been deposited in the *P. falciparum* genome database (PlasmoDB). These genes include two 2-Cys Prx (PfTPx-1 and PfTPx-2) and Pf1-Cys-Prx [10]. PfTPx-1 showed Trx (PfTrx-1)-dependent peroxidase activity, whereas PfTPx-2 showed only slight activity with the Trx [10]. The physiological electron donor used by 1-Cys-Prx is controversial in general [11–13] and on *P. falciparum* 1-Cys-Prx in particular [14,15]. High levels of expression of PfTPx-1 and Pf1-Cys-Prx in trophozoite cells have been shown by Western blot experiments [14–16]. PfTPx-2 has a putative mitochondrial targeting sequence at the N-terminal region [17]. However, the subcellular localization and expression profile of PfTPx-2 has not been characterized.

In the present study, we examined mRNA and protein expression profiles for the Prxs and Trx of erythrocytic-stage *P. falciparum* by real-time quantitative reverse transcription-PCR (RT-PCR), Western blotting and confocal laser scanning microscopy, respectively, as a first step to clarify how the parasite regulates expression of these proteins for management of intracellular ROS.

2. Materials and methods

2.1. Parasite culture

The FCR-3 strain of *P. falciparum* was cultured according to the modified method of Trager and Jensen [18]. For tight synchronization, the parasite culture was initially synchronized in the late trophozoite and the schizont stages by 60% Percoll (Amersham Biosciences, USA) density centrifugation at 1870×*g* for 20 min. After 4 h of first synchronization, a second synchronization was done by treating the culture with 5% D-sorbitol [19], yielding parasites tightly synchronized in the early phase of the ring stage (0–4 h after parasite invasion of the erythrocyte). The developmental stages of the cultured parasite were discriminated as follows; a non-pigmented parasite cell with a single nucleus was designated as a ring stage parasite; a pigmented parasite cell with a single nucleus was designated as a trophozoite; and a pigmented parasite cell with more than two nuclei was designated as a schizont.

2.2. Real-time quantitative RT-PCR

Cultures of parasite-infected erythrocytes that were tightly synchronized in the early phase of ring stage were harvested every 4 h from 36–76 h after the final synchronization. This time frame corresponds to the parasite's development from the late schizont to the same stage in the next cycle (see Fig. 1E). Total RNA was extracted from erythrocyte samples with TRIzol reagent (Invitrogen, USA). Total RNA was reverse transcribed with Ready-To-Go You-Prime First-Strand Beads and pd $(N)_6$ random hexamers (Amersham Biosciences). PCR was performed with the resulting cDNA as template and specific oligonucleotide primers. Primers used for the sequence-specific PCR were PfTPx-1, 5′-TCT GAA ATG GCA TCA TAT GTA GGA-3′ and 5′-CGG AAT TCT TAC AAC TTT GAT AAA TAT T-3′; Pf1-Cys-Prx, 5′-TCG TGA GAT TCT TTG GAG TT-3′ and 5′-ATG GCT TAC CAT TTA GGA GCT AC-3′; PfTPx-2, 5′-TCG TGA GAT TCT TTG GAG TT-3′ and 5′-ATG GCT TAC CAT TTA GGA GCT AC-3′; and PfTrx-1, 5′-TCG TGA GAT TCT TTG GAG TT-3′ and 5′-ATG GCT TAC CAT TTA GGA GCT AC-3′. Reaction mixtures were amplified with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems Japan, Japan). After initial incubation for 10 min at 95 °C, the cDNA was subjected to 40 cycles of amplification. The program used was as follows; denaturation at 95 °C for 30 s; reannealing at 50 °C for 30 s; and amplification at 65 °C for 1 min. Continuous fluorescence observation of amplifying DNA was done with SYBR Green PCR Master Mix Kit (Applied Biosystems Japan). After amplification, the PCR end-products were checked by electrophoresis on 1.5% agarose gels to confirm the specificity of each amplification. To compare the relative amounts of PCR products, the fluorescence intensity was recorded by cycles for each amplification and analyzed with the ABI PRISM Sequence Detection System software Version 1.7 (Applied Biosystems Japan). Amplification of the 18S rRNA sequence served as an internal control. The primers used for the amplification of 18S rRNA were 5′-CAA GGA AGT TTA AGG CAA CAA CAG G-3′ and 5′-CAT ATC TTT CAA TCG GTA GGA GCG-3′.

2.3. Antisera and antibodies

Rabbit antiserum to rPfTPx-1 [16] and rabbit antiserum to rPf1-Cys-Prx [20] were used. Antiserum to rPfTPx-2 was prepared by immunizing rabbits with recombinant PfTPx-2, which had been expressed with pTrcHisB (Invitrogen) as a fusion protein with the N-terminal 6-His tag. The protein was purified over Ni columns (ProBond™ Resin, Invitrogen) under non-denaturing conditions. Antiserum to rPfTrx-1 was prepared by immunizing BALBc mice with recombinant PfTrx-1 that had been expressed with pGEX-6P-1 (Amersham Biosciences) as a glutathione *S*-transferase (GST) fusion protein. The GST-tag on the fusion protein was removed by PreScission™ protease treatment and GST-glutathione affinity chromatography (Amersham Biosciences). The IgG fractions of the antisera were prepared using the HiTrap™ rProteinA affinity columns (Amersham Biosciences) according to the method of Miller and Stone [21]. The animal experiments in this study were carried out either at the International Medical Center of Japan or at Genenet (Fukuoka, Japan) in compliance with the Guide for Animal Experimentation.

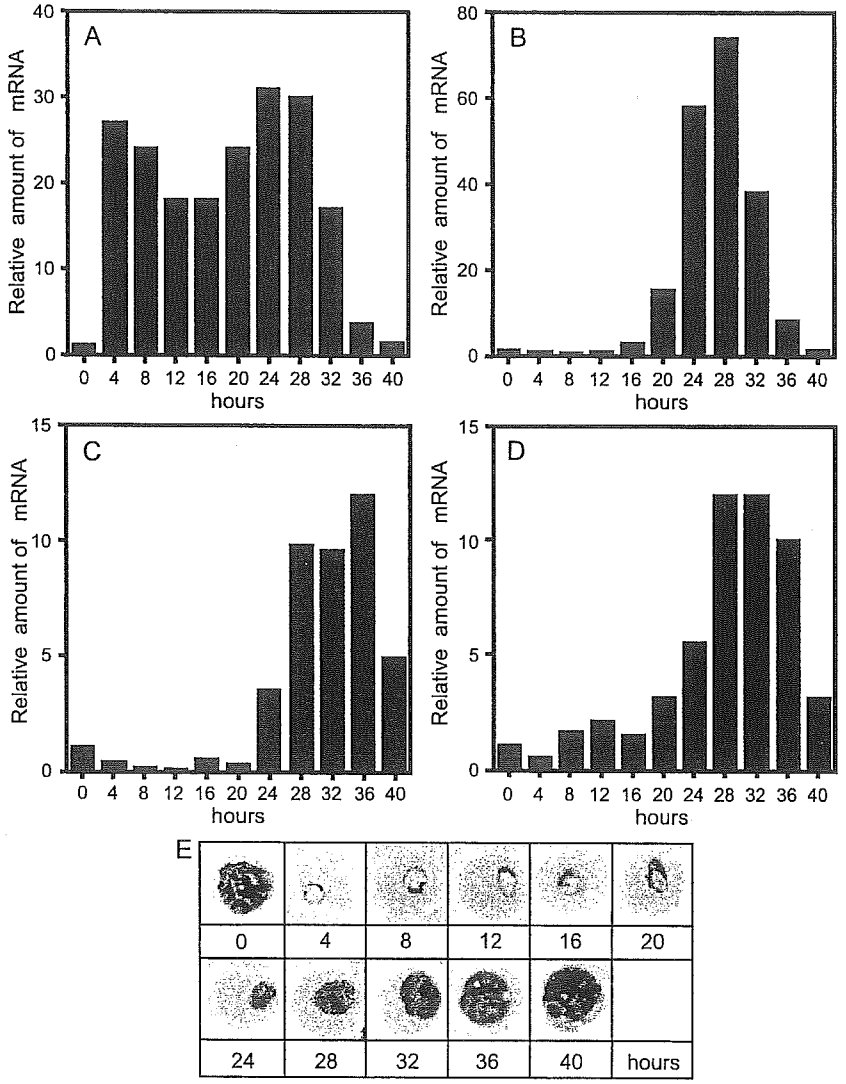

Fig. 1. Patterns of expression of PfTPx-1 (A), Pf1-Cys-Prx (B), PfTPx-2 (C) and PfTrx-1 (D) mRNAs in *P. falciparum* cells during the erythrocytic stage. Parasite cultures were tightly synchronized, and parasite-infected erythrocytes were harvested at the indicated times for total RNA extraction and cDNA synthesis. Images of various stages of parasite growth at indicated times were shown in panel E. The time 0 in this panel corresponds to the parasite invasion of the erythrocyte. The real-time quantitative RT-PCR was performed with the cDNA templates, and the values recorded were normalized to the amount of 18S rRNA in each sample measured in the same RT-PCR run. The figures show representative results of two independent assays, including the parasite culture, RNA sampling, cDNA synthesis, and RT-PCR.

2.4. Western blot analysis

Cultured unsynchronized parasites were treated with 5% D-sorbitol and the selected parasite population was used as ring stage (≤20 h after parasite invasion of the erythrocyte). A part of the ring stage parasites was cultured for 20 h and the yielded parasite population was used as trophozoite/schizont stage(s) (20–40 h after parasite invasion of the erythrocyte). The parasite-infected erythrocytes were lysed with phosphate-buffered saline (PBS) containing 0.05% saponin. The parasite pellets were washed several times with PBS and solubilized in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer [22]. After separation by the SDS-PAGE, the proteins were electrophoretically transferred to polyvinylidene difluoride sheets (Immobilon transfer membranes; Millipore, USA), and they were reacted with either the antisera or the antibodies. The IgG fractions of rabbit antisera to rPfTPx-1, rPf1-Cys-Prx and rPfTPx-2 were used at 25, 25 and 50 μg/ml, respectively. Mouse antiserum to rPfTrx-1 was used at 1:100. The blot was developed using horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibody (Cappel) at 1:1250 dilution.

2.5. Indirect immunofluorescence microscopy

The parasite culture was washed twice with PBS by centrifugation at 800×*g* for 5 min. The pellet of parasite-infected erythrocytes was suspended in ice-cold PBS containing 3% (w/v) paraformaldehyde and fixed on ice for 20 min. For the staining experiments with Mito Tracker®, the parasite-infected erythrocytes were fixed on

ice for 20 min in PBS containing 3% (w/v) paraformaldehyde and 0.1% (w/v) glutaraldehyde. The fixed erythrocytes were collected by centrifugation at 1870×*g* for 10 min and were suspended in PBS containing 3% bovine serum albumin. Three to four microliters of the suspension was spotted onto immunofluorescence microscopy-grade micro slide glass and dried at room temperature (RT). The slide was then treated with PBS containing 0.1% (v/v) Triton X-100 for 60 min at RT and rinsed three times with PBS for 5 min. Blocking was done with PBS containing 5% nonfat dry milk for 60 min at RT, and the slide was rinsed three times with PBS for 5 min before staining. Rabbit antiserum to rPfTPx-1 and rabbit antiserum to rPf1-Cys-Prx were both used at a 1:100 dilution. Rabbit antiserum to rPfTPx-2 was used at a 1:100 dilution. Mouse antiserum to rPfTrx-1 was used at 1:100. The slides were incubated for 60 min at 37 °C and rinsed three times with PBS for 5 min. After incubation with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG antibody (1:50, Cappel, USA), Alexa Fluor 568-conjugated anti-rabbit or anti-mouse IgG antibody (1:100, Molecular Probes, USA) and TO-PRO-3 iodide (1:1000, Molecular Probes) for 60 min at 37 °C, slides were rinsed five times with PBS for 5 min. Mito Tracker® Red CM-H_2XROS (Molecular Probes) was applied to the parasite culture at 200 nM and incubated at 37 °C for 30 min before fixation. The slides were mounted under cover glasses in Prolong Antifade solution (Molecular Probes) and observed with a confocal laser scanning microscope (Model LSM510, Carl Zeiss, Germany).

3. Results

3.1. Prx and Trx expression

To examine patterns of expression of the Prx and Trx genes in the erythrocytic stage of *P. falciparum*, we synthesized cDNAs from total RNA extracted from tightly synchronized parasite cultures and performed real-time quantitative RT-PCR. We calculated the relative amount of Prx and Trx mRNAs in each stage using 18S rRNA as an internal standard. We found that expression of PfTPx-1 mRNA was elevated immediately after parasite invasion of erythrocytes, remained elevated until the early schizont stage, and rapidly decreased in the late schizont stage. Therefore, the PfTPx-1 gene showed constitutive expression through the erythrocytic stage (Fig. 1A). In contrast, expression of Pf1-Cys-Prx mRNA began increase when the parasite had developed to the trophozoite stage and decreased in the late schizont stage. Therefore, the Pf1-Cys-Prx gene showed limited expression in the trophozoite and early schizont stages (Fig. 1B). Expression of the PfTPx-2 mRNA was initiated during the trophozoite stage and was sustained during the schizont stage (Fig. 1C). The PfTrx-1 gene was expressed at a low level during the ring stage and showed highest expression during the trophozoite and schizont stages (Fig. 1D). The mRNA expression levels at 0 h and 40 h were different in PfTPx-2 and PfTrx-1 (Fig. 1C and D). This may be due to the difference in the parasite development in 0 and 40 h samples; the former contained more ring stage parasites than the latter.

The mRNA expression profiles observed for these three Prxs and the Trx genes by RT-PCR were all well consistent with their similar profiles that had already been published in the PlasmoDB by the microarray analyses [23,24]. This finding suggests that the two data sets can complement each other in order to prove their accuracy and applicability. For instance, it proves that these profiles are common among different *P. falciparum* strains, because our analysis was made in the FCR-3 strain of the parasite, while the data in PlasmoDB were compiled with the 3D7 and the HB3 strains.

3.2. Expression of Prx and Trx proteins during the erythrocytic stage

In Western blotting, each antiserum or antibody specifically recognized the parasite proteins with a molecular mass of 25 kDa (Pf1-Cys-Prx), 22 kDa (PfTPx-1), 22 kDa (PfTPx-2) and 12 kDa (PfTrx-1), respectively (Fig. 2). These molecular masses all agree with the sizes predicted from their amino acid sequences [10]. Another blotting of the parasite extracts in the same system with the pre-immune sera did not show any bands (data not shown). The Pf1-Cys-Prx was detected in the ring stage and the trophozoite/schizont stage(s) (see Section 2.4 for the sample designation). However, the band observed in the ring stage was faint. The band intensity observed in trophozoite/schizont stage(s) was at least 10 times stronger than that in the ring stage. The PfTPx-1 was also detected in the ring stage and the trophozoite/schizont stage(s). The band intensity observed in the ring stage was, however, almost as strong as that in the trophozoite/schizont stage(s). The comparable expression of PfTPx-1 protein in the blot may

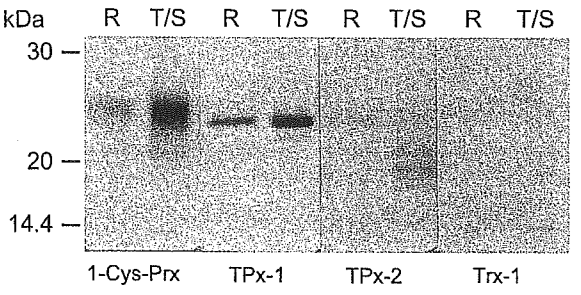

Fig. 2. Western blot analyses of Pf1-Cys-Prx, PfTPx-1, PfTPx-2 and PfTrx-1 proteins in *P. falciparum* cells. Extracts prepared from the erythrocytic-stage parasites were subjected to 12.5% (w/v) SDS-PAGE under reducing conditions. Twenty micrograms of extract from ring stage parasites (R) and trophozoites/schizonts (T/S) were separated by electrophoresis. Proteins were transferred to Immobilon™ membranes and reacted with rabbit anti-rPf1-Cys-Prx IgG (1-Cys-Prx), anti-rPfTPx-1 IgG (TPx-1), anti-rPfTPx-2 IgG (TPx-2) and mouse anti-rPfTrx-1 serum (Trx-1). Molecular weight markers in kDa are indicated on the left.

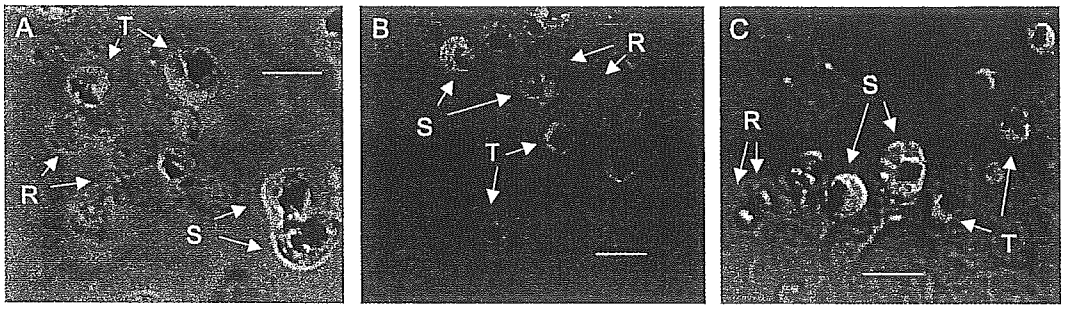

Fig. 3. The expression of PfTPx-1 (A), Pf1-Cys-Prx (B), and PfTrx-1 (C) proteins in *P. falciparum* cells during the erythrocytic stage. Parasite cells in panels (A–C) are stained with antiserum specific for each antioxidant protein (Alexa Fluor 568, red) and TO-PRO-3 iodide (blue; nucleolus staining). Letters with arrows are ring stage (R), schizont (S), and late trophozoite (T). See Section 2.1 for designation of the parasite developmental stages. Bars 2 μm.

prove equal loading between the samples. The PfTPx-2 was detected in the two developmental stages, but the bands observed were faint. This may be due to its mitochondrial localization. The additional bands with molecular masses of less than 22 kDa, the size without the putative signal sequence [17], in the trophozoite/schizont stage(s) may represent the protein degradation following its elevated expression. The PfTrx-1 was also detected in the two developmental stages, and the band intensity observed in the trophozoite/schizont stage(s) was stronger than that in the ring stage. Lower intensity of the bands seen in the blot of PfTrx-1 as compared with that in other proteins may be due to the difference in the probe; the blot was reacted with antiserum but not with its IgG fraction.

Immunofluorescence microscopy of *P. falciparum* erythrocytic-stage parasites revealed that PfTPx-1 was expressed in the parasite cytoplasm during the ring, trophozoite and schizont stages. PfTPx-1 thus showed constitutive expression through the erythrocytic stage (Fig. 3A). In contrast, significant staining of Pf1-Cys-Prx was detected in the trophozoites in the cytoplasm of the parasite. Expression of Pf1-Cys-Prx in late schizonts was weak in comparison to that of PfTPx-1 in parasites of the same stage, and it was not detected in the ring stage. Pf1-Cys-Prx thus showed limited expression during the trophozoite and early schizont stages (Fig. 3B). Significant expression of PfTrx-1 was observed after the trophozoite stage, but expression was weak during the ring stage (Fig. 3C). Double staining of parasite cells with anti-PfTPx-2 serum and Mito Tracker® revealed that PfTPx-2 was present in mitochondria (Fig. 4A–F). The mitochondrial localization of PfTPx-2 was also confirmed by double staining of parasite cells with anti-serum to *P. falciparum* complex II Ip subunit [25], subunit of a marker enzyme for mitochondria, and anti-PfTPx-2 serum (data not shown). PfTPx-2 was expressed in the trophozoite and schizont stages (Fig. 4G). Staining of parasite cells with specific pre-immune serum yielded no specific fluorescence (data not shown).

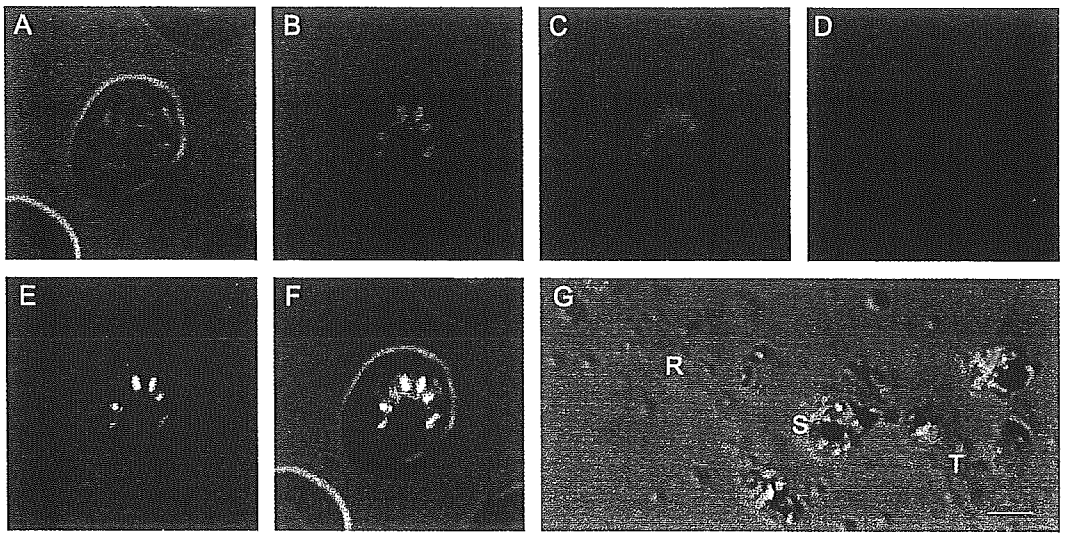

Fig. 4. Mitochondrial localization (A–F) and expression (G) of PfTPx-2 protein in *P. falciparum* cells during the erythrocytic stage. Parasite-infected erythrocytes (A–F) were fixed with ice-cold PBS containing 3% (w/v) paraformaldehyde and 0.1% (w/v) glutarlaldehyde before they were spotted onto glass slides (see ""Materials and methods""). Panel (A) shows nonconfocal transmitted image. Parasite cells in panels (B–G) are stained with antiserum to PfTPx-2 (FITC, green; B and G), Mito Tracker® (red; C) and TO-PRO-3 iodide (blue; D and G). Panels E and F show merged images of B, C, and D, and of A and E, respectively. Letters with arrows in panel G are ring stage (R), schizont (S), and late trophozoite (T). See Section 2.1 for designation of the parasite developmental stages. Bars 2 μm.

The expression profiles observed for these four antioxidant proteins by two methods, namely, Western blotting and immunofluorescence microscopy are well comparable. These two data could thus complement each other. The results in Western blotting proved the protein specific recognitions of the antibodies; thus they were not cross-reacting in immunofluorescence microscopy. The observations by immunofluorescence microscopy supported the expression profiles in Western blotting and determined subcellular localization of each protein.

4. Discussion

PfTPx-1 is expressed in the parasite cytoplasm throughout the erythrocytic stage; thus, it likely plays a housekeeping role in intracellular redox homeostasis of *P. falciparum*, which may be affected by the intraerythrocytic environment and parasite metabolism. Such a role for PfTPx-1 was also suggested by the fact that disruption of this gene renders the parasite hypersensitive to reactive oxygen and nitrogen species [7]. PfTPx-1 was shown to reduce hydrogen peroxides and organic hydroperoxides via the PfTrx-1-dependent catalytic reaction in vitro [17]; however, the expression profile of this enzyme in vivo does not completely overlap that of PfTrx-1, which showed lower level expression in the ring stage. Although actual amounts of these two proteins should be compared, this finding suggests that PfTPx-1 may use other members of the Trx subfamily instead of PfTrx-1 as an electron donor or that this enzyme has biological activity in addition to Trx-peroxidase during the ring stage. There are at least three entries for Trx-like proteins in PlasmoDB: PF14_0545, PF1250w and MAL13P1.225. PF14_0545 corresponds to PfTrx-1 [5]. However, none of these proteins was significantly expressed during the ring stage at the mRNA (data not shown) or protein level [5]. DNA microarray and proteomic analyses of PfTPx-1-null parasite cells [7] during the ring stage would be one possible approach to examining the novel function of this enzyme in *P. falciparum*.

In contrast to PfTPx-1, Pf1-Cys-Prx shows elevated expression in cytoplasm of trophozoites, the active growth stage. Trophozoites digest large quantities of host hemoglobin for nutrition, and this process increases the parasite's intracellular oxidative stress due to discharge of pro-oxidative heme iron [26]. Malaria parasites are known to be highly sensitive to the toxic effects of this heme, and this is the basis of the antimalarial action of chloroquine [26]. Further biochemical and biophysical studies of this Prx protein may provide insights into its involvement in heme metabolism, and such experiments are currently underway in our laboratory.

PfTPx-2 was expressed in mitochondria during the trophozoite and schizont stages. This Prx was identified as a member of the 2-Cys Prx family with a putative mitochondrial targeting sequence at its N-terminal region [17]; however, its subcellular localization and expression profile were unknown until the present study. None of the Trx-like proteins has been annotated for mitochondrial localization in PlasmoDB, although one of them (MAL13P1.225) has a putative N-terminal leader peptide. Although this sequence is not a typical mitochondrial targeting signal, detection of this protein in the mitochondria would support the presence of the thioredoxin system in malaria parasite as has been shown in mammalian cells [27]. One subtype of superoxide dismutase (SOD) that lacks a classical mitochondrial targeting signal (TgSOD3) is localized to the mitochondria in *Toxoplasma gondii* [28]. However, the fact that recombinant PfTPx-2 could not efficiently use PfTrx-1 as its electron donor [17] could indicate that this protein has function other than as a Trx peroxidase. The reason for degradation of PfTPx-2 protein observed in Western blotting in the trophozoite/schizont stage(s) is unknown, but may relate to its function. Regulation of TPx activity by its C-terminal truncation, which has been reported in *Schizosaccharomyces pombe* [29], will be interesting to be investigated for PfTPx-2. The presence of a peroxidase such as PfTPx-2 in mitochondria, together with the entry into PlasmoDB of an SOD (MAL6P1.194) with potential mitochondrial localization, is consistent with a recent report of a respiratory chain, which produces ROS, in the mitochondria of *P. falciparum* [30]. Further efforts to clarify biological roles of PfTPx-2 by reverse genetics approaches may offer new insights into the physiology of the mitochondria of *P. falciparum*.

Because the expression profiles of the mRNAs and proteins for all three Prx and Trx overlap to the same degree, expression of these genes is likely regulated at the transcriptional level. Transcriptional activation of genes encoding antioxidant proteins through transcriptional activator proteins Yap1p/Skn7p occurs in budding yeast [31]. Sequences homologous to these regulatory proteins and those in other organisms are not present in the genome of *P. falciparum*. In addition, homology searches for known transcriptional elements in other organisms were also unsuccessful, possibly due to the extremely high AT content of the non-coding sequences, and thus there is little information regarding regulatory sequences in the genome of *P. falciparum* [32]. The mechanism by which the malaria parasite regulates transcription of antioxidant genes may be unique, so identification of the regulatory sequences and proteins involved would be of great interest.

In summary, the results of the present study indicate that *P. falciparum* uses three Prx proteins in different organelles for management of intracellular oxidative stresses during intraerythrocytic development. How the parasite can manage proper expression of antioxidant proteins under different physiological situations is a matter of great interest. Further studies to clarify the role of each Prx in adaptation and development of *P. falciparum* in the intraerythrocytic environment and to understand the mechanisms that underlie transcriptional regulations of these antioxidant

proteins in parasite cell should provide further insights into the response of *P. falciparum* to ROS at the cellular level. It will also be a great interest if the thioredoxin reductase, which is another key player in the thioredoxin redox system, is to be analyzed together. In addition, these studies may provide potential targets for development of novel malaria chemotherapies.

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LIMITED ALLELIC DIVERSITY OF *PLASMODIUM FALCIPARUM* MEROZOITE SURFACE PROTEIN 1 GENE FROM POPULATIONS IN THE SOLOMON ISLANDS

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Abstract. Meiotic recombination generates allelic diversity in the *Plasmodium falciparum* merozoite surface protein 1 (*msp1*) gene. In this study, we monitored recombination-based diversity of *msp1* in Guadalcanal, the Solomon Islands, where malaria transmission is high. We identified 5′ recombinant types, 3′ sequence types, and *msp1* haplotypes (unique associations of 5′ recombinant types and 3′ sequence types), and compared them with those from areas of low transmission in Thailand and Vanuatu. The mean number of 5′ recombinant types per person (multiplicity) was lower in Guadalcanal than in Thailand. Guadalcanal populations had 6–8 *msp1* haplotypes; the numbers are comparable to Vanuatu but much lower than in Thailand. There were marked geographic differences in distribution of *msp1* haplotypes. Linkage disequilibrium in *msp1* was stronger in Guadalcanal than in Thailand, suggesting limited recombination events in the Solomon Islands. We suggest that the frequency of recombination events in *msp1* is determined not only by transmission intensity but by the number of *msp1* alleles prevalent and multiplicity of infections.

INTRODUCTION

One of the obstacles to development of effective vaccines against the human malaria parasite *Plasmodium falciparum* is the diversity of vaccine candidate antigens in natural parasite populations. Evidence indicates that antigen diversity limits the efficacy of acquired immunity based on strain-specific anti-parasite immunity.[1–3] The diversity of antigen genes differs greatly among different areas endemic for malaria.[4–6] Therefore, it is important to investigate geographic differences in the genetic diversity of *P. falciparum*. The merozoite surface protein 1 (MSP-1) of *P. falciparum* is a major blood-stage surface antigen.[7] The gene (*msp1*) consists of 17 blocks according to the degree of sequence conservation among alleles.[8,9] Sequence variation in *msp1* is mainly dimorphic (two major allelic forms) in all variable blocks other than block 2, which is trimorphic (three allelic forms) (Figure 1). The block is grouped according to the presence or absence of nine-basepair repeats and the type of repeating sequences. MSP-1 contains at least two regions of protective immune target: block 2 near the N-terminus and block 17 at the C-terminus. In an area of Africa highly endemic for malaria, human antibodies against block 2 are associated with clinical immunity in an allelic-type-specific manner.[10] Block 17 encodes a C-terminal 19-kD polypeptide fragment that is produced by processing of MSP-1 at the merozoite surface. Antibodies against this 19-kD fragment inhibit merozoite invasion into red blood cells,[11,12] and immunization with the 19-kD fragment confers protection against challenging infections in animals.[13] Invasion-inhibiting antibodies against the 19-kD fragment have been detected in sera from individuals living in areas highly endemic for malaria.[14,15]

Meiotic recombination is a major genetic mechanism in the generation of allelic diversity in *msp1*.[8,16] The frequency of recombination events in *P. falciparum* depends primarily on the frequency of its transmission because *P. falciparum* undergoes meiotic recombination at the mosquito stage. We have been using recombination-based haplotypes of *msp1* as a polymorphic genotype marker to study allelic diversity of *msp1* in parasite populations.[17–19] The *msp1* haplotypes are defined as unique associations of 5′ recombinant types (blocks 2–6) and 3′ sequence types (block 17). In analysis of linkage disequilibirum, the prevalence of *msp1* haplotypes acts as an indicator of the frequency of recombination events in parasite populations. In a high-transmission area, there is little linkage disequilibrium in the *msp1* sequence,[20] whereas there are moderate levels of linkage disequilibrium in mesoendemic areas such as Thailand and Vietnam.[21] In contrast, there are very high levels of linkage disequilibrium in Brazil, a hypoendemic area.[22] However, nearly complete linkage disequilibrium occurs in Vanuatu in the southwestern Pacific,[19] where the level of malaria transmission is comparable to that of Southeast Asia, suggesting that the frequency of transmission is not the sole factor determining frequency of recombination events in *msp1*.

In the present study, we examined *P. falciparum* populations from Guadalcanal, the Solomon Islands, which are located between Papua New Guinea and Vanuatu. Malaria is highly endemic in the Solomon Islands, and malaria transmission is more intense than in Vanuatu. *Plasmodium falciparum* populations in the Solomon Islands are the most likely source of the Vanuatu populations because Vanuatu is located at the southern margin of the malarious band extending from Papua New Guinea through the Solomon Islands, and the Solomon Islands are the only neighboring country where malaria is prevalent. Therefore, it would be highly instructive to investigate the diversity of *msp1* haplotypes and linkage disequilibrium in *msp1* in populations from the Solomon Islands, and compare them with those of populations from Vanuatu and other geographic areas. The present results show that the diversity of *msp1* haplotypes is greater in Guadalcanal than in Vanuatu, as expected. In contrast, despite high endemicity, the diversity in the Solomon Islands was relatively limited, compared with populations from Thailand, and linkage disequilibrium in *msp1* was greater in the Solomon Islands than

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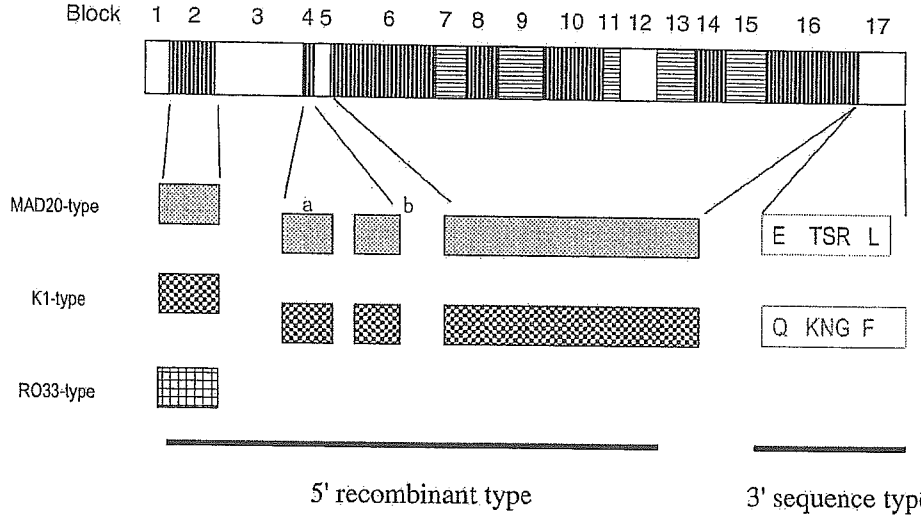

FIGURE 1. Structure of the *Plasmodium falciparum* merozoite surface protein 1 gene and the strategy for determination of 5′ recombinant type and 3′ sequence type. Inter-allele conserved, semi-conserved, and variable blocks are indicated by open, horizontally hatched, and vertically hatched columns, respectively. For variable blocks, MAD20-type, K1-type, and RO33-type sequences are represented by half-tone, checkerboard, and cross-striped bars, respectively. The 5′ recombinant type was determined by polymerase chain reaction (PCR) amplification of blocks 2–6 using allelic type-specific primers of blocks 2 and 6, followed by a nested PCR for blocks 4a and 4b using allelic specific primers of blocks 4a and 4b. The five amino acid substitutions in block 17, indicated by the one-letter codes, were determined by sequencing. The 3′ sequence type is the combination of those residues.

in Thailand. Also, all of our Solomon Islands isolates had the Papua New Guinea-type *P. falciparum* chloroquine resistance transporter (pfcrt),[23] the locus that determines chloroquine resistance in *P. falciparum*.

MATERIALS AND METHODS

Study area and sample collection. *Plasmodium falciparum* field isolates were collected from northern Guadalcanal, the Solomon Islands, in the southwestern Pacific, from July 1995 to March 1996. Guadalcanal is the largest island in the Solomon Islands with a population of 409,000 (1999 census), and has an area of 5,336 km^2, a length of 160 km, and a width of 48 km (Figure 2). Malaria in Guadalcanal is hyperendemic, with parasite-positive rates of 45% and 39% in 1994 and 1995, respectively.[24] Transmission of malaria is perennial, with a peak during the rainy season from December to May. Both *P. falciparum* and *P. vivax* are prevalent, and approximately two-thirds of the parasites are *P. falciparum*.

Ninety isolates of *P. falciparum* were collected from three areas in northern Guadalcanal (Figure 2): 40 clinical isolates from outpatients of a hospital in Honiara City (area A), the capital of the Solomon Islands; 26 isolates from 3 villages (Kaotave, Tadhimboko, and Nugalitav) in area B; and 24 isolates from 1 village (Ruavatu) in area C. Area C is directly connected to area A by a road, whereas the three villages in area B are located several kilometers from the road. In rural areas B and C, samples were collected from participants of malariometric surveys, and most of the donors were primary school children 8–15 years of age with a mean age of 13.2 years in area B (range = 6–59) and a mean age of 14.9 years in area C (range = 11–18). Parasite-positive individuals were asymptomatic in most cases in areas B and C. Approximately half of the clinical samples from Honiara were collected from subjects more than 15 years of age, with a mean age of 18.3 years (range = 4–60). Giemsa-stained thick blood smears were examined microscopically, and *P. falciparum*-positive blood was withdrawn using a syringe containing EDTA and stored at −60°C. Parasite genomic DNA was extracted from blood using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.[25] Informed consent was obtained from the donors and the school principals. This study was reviewed and approved by the Ethical Committee of the Solomon Islands for Medical Research.

Samples collected in January 1994 (n = 92)[26] were also included in this study. These samples were from area B and included three villages (Talaura, Tadhimboko, and Kolona). The mean age of the donors was 21.6 years (range = 5–89). In addition, we used clinical isolates (n = 111) from patients who attended a malaria clinic in Mae Sot in northwestern Thailand in 1995.[18] The mean age of the donors in Thailand was 24.6 years (range = 13–48). Isolates from Vanuatu (n = 164) were collected during malariometric surveys in rural villages on four islands[19]: Namasari and Ontar in eastern and western Gaua island, respectively, in 1997, Big Bay in northern Santo island in 1997, Baie Barier and Pangi in eastern and western Pentecost island, respectively, in 1998, and Lingarak and Brenwe/Leviamp in eastern and northwestern Malakula island, respectively, in 1996 and 1998. Subjects were primarily school children in each village with a mean age of 6.9 years (range = 0–25), 12.6 years (range = 2–47), 7.9 years (range = 2–30), 10.6 years (range = 3–36), and 9.8 years (range = 1–28) in Gauaisland, Santo island, Pentecost island, Malakula island in 1996, and Malakula island, respectively, in 1998. The overall parasite-positive rate was 14%. *Plasmodium falciparum* infections detected during surveys were asymptomatic in most cases.

Determination of *msp1* haplotypes. The *msp1* haplotypes are defined as unique associations of 5′ recombinant types

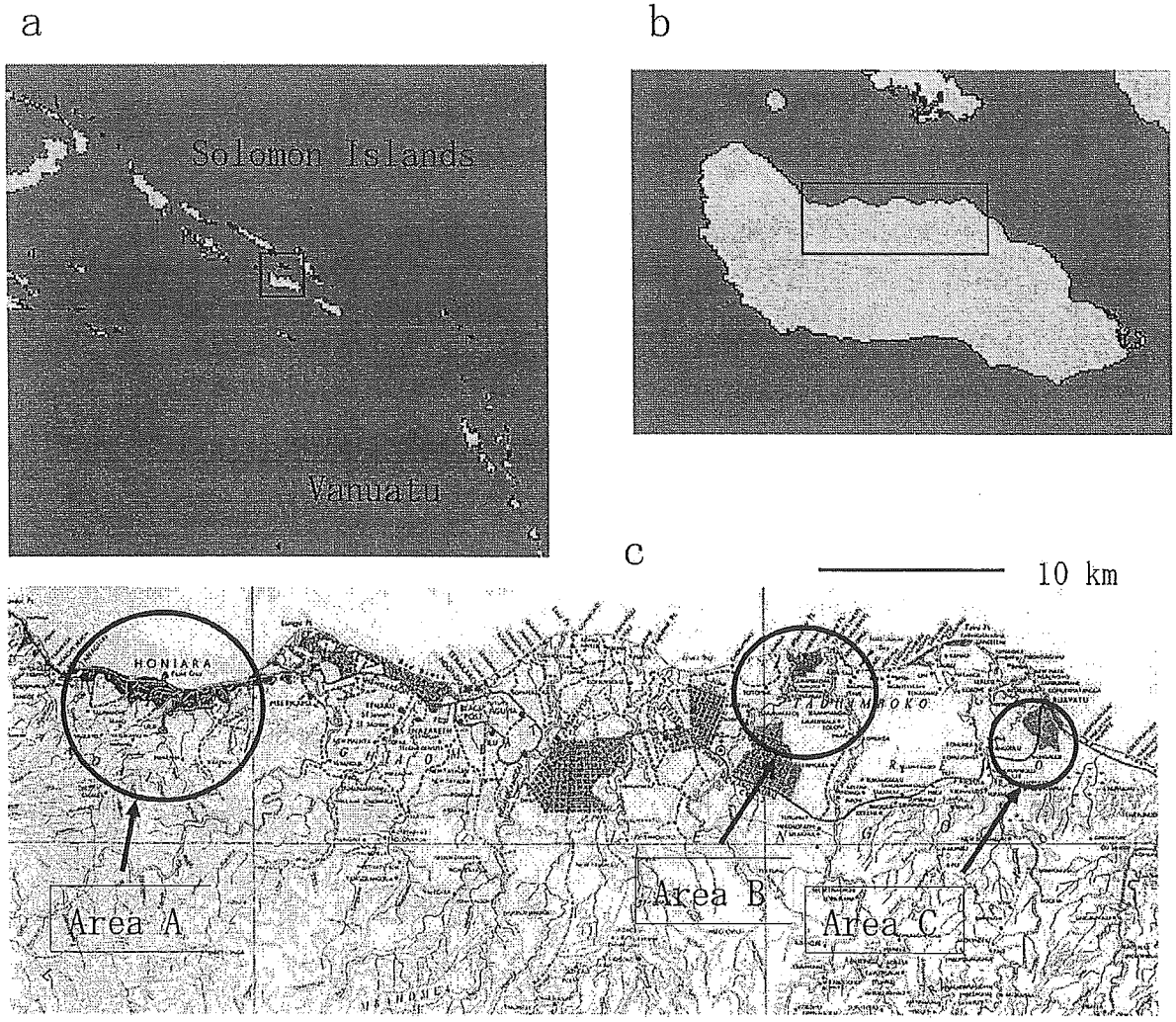

FIGURE 2. Location of study area. **a**, Solomon Islands and Vanuatu. The box shows Guadalcanal. **b**, Guadalcanal. The box shows the study area in northern Guadalcanal. **c**, The three study areas A, B and C are shown by the circles.

and 3′ sequence types (Figure 1). The methods used to determine 5′ recombinant types and 3′ sequence types were as follows.

The 5′ recombinant types (block 2–6) of *P. falciparum msp1* (a 5-kb gene) consist of 17 distinct sequence blocks (Figure 1). Sequences of all variable blocks other than block 2 are dimorphic, either one or the other of two major allelic types: K1 and MAD20. Block 2 is represented by one of three major types: K1, MAD20, and RO33. The 5′ recombinant types comprise unique combinations of allelic types of blocks 2, 4a, 4b, and 6. To date, no recombination has been found in blocks 6–16.[18,27] Determination of 5′ recombinant types involves primary amplification of a 1-kb region spanning blocks 2–6, followed by nested PCR amplification of block 4a–4b (approximately 100 basepairs). Procedures for determination of 5′ recombinant types are described elsewhere.[26] For the PCR, we used 1 μL of template DNA that was adjusted to be equivalent to 1 μL of the original blood.

We previously reported *msp1* 5′ recombinant types from a Thai population.[18] In that study, 1 μL of 20-fold diluted DNA was used for the PCR, which is an amount not equivalent to the original volume of blood. Furthermore, we later found that the typing method used in that study was neither very sensitive nor specific, and that it produced artifacts due to the relatively weak specificity of the PCR primers used. We therefore improved that method to obtain higher sensitivity and specificity and to avoid artifacts.[26] In the present study, we used the improved method to re-type 5′ recombinant types of the Thai samples. Although there was no significant difference in distribution of 5′ recombinant types between the two methods, the improved method substantially increased the rate of multiple infections of distinct 5′ recombinant types (65% versus 30%) and the mean number of 5′ recombinant types per isolate (3.6 versus 1.8). Determination of 5′ recombinant types was successful in 82 of 90 isolates from the Solomon Islands samples collected in 1995 and 1996 and in 107 of 111 isolates from the Thai samples. These were compared with the 5′ recombinant types previously determined for Solomon Islands isolates collected in 1994 (n = 84)[26] and Vanuatu isolates in 1996–1998 (n = 141).[19]

The 3′ sequence type (block 17), nucleotide sequence of the 3′ region of *P. falciparum msp1*, which encodes the C-terminal 19-kD polypeptide, was determined by direct sequencing. To obtain a clear sequence, we selected only isolates with mono-infections of a single 5′ recombinant type, and excluded isolates of multiple infections. The number of samples sequenced was 30 for the samples collected in 1994 from Guadalcanal, 47 for the samples collected in 1995 and 1996 from Guadalcanal, and 48 for the samples from Thailand. Because the number of mono-infections was limited in our Thai samples (n = 4), it was increased by diluting genomic DNA templates (20-fold). The procedures used for direct sequencing of block 17 are described elsewhere.[19] The PCR products coding for amino acid residues from position 1637 to the C-terminus of MSP-1 (positions are according to Miller and others[9]) were sequenced in both directions using the BigDye Terminator Cycle Sequencing Kit version 3.1 (Applied Biosystems, Foster City, CA) and an ABI 3100 sequencer (Applied Biosystems, Foster City, CA). Sequences were verified by re-sequencing PCR products independently amplified from the same DNA. To date, five amino acid changes have been identified in block 17 in samples obtained from various geographic areas (E or Q at amino acid residue 1644, T or N at 1691, SR or NG at 1700–1701, and L or F at 1716)[21,28] (Figure 1). Hereafter, we refer to combinations of these residues as the 3′ sequence type.

Sequencing of *pfcrt*. Polymorphism at amino acid residues 72–76 in the second exon of *pfcrt*[29] was determined for the Solomon Islands isolates with mono-infection of a 5′ recombinant type from samples collected in 1994 (n = 30) and samples collected in 1995 and 1996 (n = 47). A DNA fragment (468 basepairs) covering the second exon of *pfcrt* was amplified using the method used above for *msp1* with primers CQF4 (5′-CAGATGGCTCACGTTTAGGTGGAGGT-3′) and CQR4 (5′-TGTGTAATGTTTTATATTGGTAG-GTGGA-3′), in a 50-μL reaction mixture containing 1 μL of template genomic DNA. The PCR product was purified and directly sequenced using CQF4 as a sequencing primer.

Statistical analyses. Frequency distributions of *msp1* 5′ recombinant types, 3′ sequence types, and *msp1* haplotypes in different parasite populations were compared using the chi-square test with Yates' correction for data sets less than 10 and Fisher's exact test. Differences in the rate of multiple 5′ recombinant type infections were tested for significance using Student's *t*-test, and differences in the number of multiple 5′ recombinant types per person (i.e., multiplicity) were tested for significance using a two-tailed Mann-Whitney U test. Pairs of polymorphic blocks 2, 4a, 4b, and 6 and polymorphic sites in block 17 were subjected to an R^2 test to assess linkage disequilibrium, as described elsewhere.[21] Non-informative pairs (frequency < 10% in a polymorphic block or nucleotide site) were excluded from the R^2 test. Significance of linkage disequilibrium was assessed using an m × n chi-square test and two-tailed Fisher's exact probability test. The haplotype diversity index (h) of *msp1* haplotypes was calculated using formula (8-5) of Nei[30] $h = \{n/(n - 1)\}x\{1 - \Sigma pi^2\}$ where p and i are the frequency and number of *msp1* haplotypes, respectively. Variance (V) of h was calculated using the formula $V = \{2/n(n - 1)\}[2(n - 2)\{\Sigma pi^3 - (\Sigma pi^2)^2\} + \Sigma pi^2 - (\Sigma pi^2)^2]$ that was modified from formula (8-12) of Nei[30] for a haploid genome. A P value less than 0.05 was considered statistically significant.

RESULTS

Diversity and multiplicity of 5′ recombinant types (blocks 2–6). Our typing method can potentially differentiate between 24 distinct 5′ recombinant types. Table 1 lists the distribution of 5′ recombinant types of the Solomon Islands samples collected in 1995 and 1996 and the Thai samples, together with our previously published data for Solomon Islands samples collected in 1994[26] and samples from Vanuatu.[19] The number of distinct 5′ recombinant types found in the Solomon Islands samples was 9 (n = 6 in 1994 and n =

TABLE 1

Prevalence of *Plasmodium falciparum* merozoite surface protein 1 (*msp1*) 5′ recombinant types in the Solomon Islands in comparison with Thailand and Vanuatu*

5′ recombinant type	Solomon Islands (1994)†	Solomon Islands (1995–1996)				Thailand	Vanuatu‡
	Area B	Area A	Area B	Area C	All		
#1 KKKK	36	9	7	5	21	37	0
#2 MKKK	0	1	0	0	1	79	0
#3 RKKK	0	0	0	0	0	1	0
#13 KKKM	0	0	1	0	1	1	0
#14 MKKM	0	0	0	0	0	1	0
#15 RKKM	0	0	0	0	0	4	0
#16 KMKM	2	0	0	0	0	9	13
#17 MMKM	6	5	17	8	30	89	0
#18 RMKM	17	4	4	2	10	34	6
#20 MKMM	0	0	0	0	0	6	0
#21 RKMM	0	1	1	2	4	43	0
#22 KMMM	0	0	0	0	0	57	0
#23 MMMM	38	11	5	6	22	24	20
#24 RMMM	46	13	5	9	27	1	103
Total	145	44	40	32	116	386	142
Number of 5′ recombinant types	6	7	7	6	8	14	4
Rate of multiple infection (%)	51/84 (60.7)	8/35 (22.9)	11/15 (44.0)	10/22 (45.5)	29/82 (35.4)	103/107 (96.3)	1/141 (0.7)
Mean number of 5′ recombinant types	1.73	1.26	1.60	1.40	1.41	3.6	1.00–1.03§

* 5′ recombinant types are defined as associations of allelic types in msp1 blocks 2, 4a, 4b, and 6. K, M, and R denote K1, MAD20, and RO33 allelic types, respectively. Ten 5′ recombinant types were not identified in any of the three geographic areas: #4, KMKK; #5, MMKK; #6, RMKK; #7, KKMK; #8, MKMK; #9, RKMK; #10, KMMK; #11, MMMK; #12, RMMK; #19, KKMM.
† Sakihama and others.[26]
‡ Sakihama and others.[19]
§ Range of values separately calculated for the four islands of Vanuatu studied.

8 in 1995–1996), which is intermediate between the numbers obtained from Thailand (n = 14) and Vanuatu (n = 4). Distribution of 5′ recombinant types differed greatly among the three geographic areas (Table 1) ($P < 10^{-10}$ for all pairs compared). These differences are due mainly to sharp contrasts in frequencies of some 5′ recombinant types; e.g., the frequency of type 2 was 0.205, 0–0.023 and 0 in Thailand, the Solomon Islands, and Vanuatu, respectively, and the frequency of type 24 was 0.003, 0.125–0.317, and 0.725 in Thailand, the Solomon Islands, and Vanuatu, respectively. Five 5′ recombinant types (#3, #14, #15, #20, and #22) were found only in Thailand. The prevalence of infections with multiple 5′ recombinant types varied considerably among the three geographic areas (Table 1). The rate of multiple 5′ recombinant type infections in the Solomon Islands was 23–61%, which is intermediate between Thailand (96%) and Vanuatu (1%). All differences between pairs of geographic areas were significant ($P < 0.001$). The mean number of 5′ recombinant types per person (i.e., multiplicity) in the Solomon Islands (1.4–1.7) was also intermediate between Thailand (3.6) and Vanuatu (1.0). The differences in multiplicity were highly significant ($P < 10^{-4}$ for all pairs).

In contrast to these results, distribution of 5′ recombinant types did not differ significantly among areas A, B and C in Guadalcanal (Table 1), but differed significantly between samples collected in 1994 and samples in 1995 and 1996 in area B ($P < 0.0005$). This difference was due mainly to temporal fluctuations of five major 5′ recombinant types (#1, #17, #18, #23, and #24) (Table 1). A slight variation in the prevalence of multiple 5′ recombinant type infections was observed. In the samples collected in 1995 and 1996, the rate of multiple infections and multiplicity were higher in rural areas (areas B and C) than in Honiara City: multiple infection rate = 44–46% versus 23%, respectively; multiplicity = 1.4–1.6 versus 1.26, respectively. However, these differences were not significant. In area B, there was temporal variation in multiplicity between the samples from 1994 and the samples from 1995 and 1996 (1.7 versus 1.4, respectively), but the difference was not significant.

We examined effects of transmission season, host age, and parasite density on *msp1* diversity for samples (n = 35) collected in area A (Honiara) in 1995 and 1996. The rate of multiple infection and multiplicity were slightly higher in the rainy season (n = 11) than in the dry season (n = 24): multiple infection rate = 27.3% versus 20.8%, respectively; multiplicity = 1.36 versus 1.25, respectively. However, these differences were not significant. The multiple infection rate and multiplicity did not differ significantly between subjects less than 15 years of age and those at least 15 years of age (n = 15 and 20, respectively). There was no difference in multiple infection rate or multiplicity between a parasite density of at least 4,000 parasites/μL and a parasite density less than 4,000 parasites/μL (n = 17 and 18, respectively). Similar analysis was not performed for populations in areas B and C because of limited or biased distribution of samples among seasons, ages, and parasite densities. In samples from area B collected in January 1994 (rainy season), the multiple infection rate and multiplicity did not differ significantly between subjects less than 15 years of age and those at least 15 years old (n = 57 and 27, respectively).

3′ sequence types (block 17). The Solomon Islands samples contained all five previously reported nucleotide polymorphisms;[21,28] and their sequence types were Q-KNG-L, Q-KNG-F, E-KNG-L, and E-TSR-L (Table 2). All of these sequence types were also found in both Thailand and Vanuatu. Distribution of these 3′ sequence types differed greatly between the Solomon Islands and other areas: $P < 10^{-6}$ and $P < 10^{-10}$ for comparison with Thailand and Vanuatu, respectively. In Guadalcanal, there was no significant difference in distribution of 3′ sequence types among areas A, B, and C or between samples collected in 1994 and samples in 1995 and 1996. However, when samples were pooled to make two major groups for the Fisher's exact test, a significant difference appeared between 1994 and 1995–1996 ($P = 0.01$).

Diversity of *msp1* haplotypes. Among the Solomon Islands samples, there were six distinct *msp1* haplotypes in the 1994 samples and eight distinct *msp1* haplotypes in the 1995–1996 samples (Table 2). These numbers are comparable to those for Vanuatu (n = 6), but are much lower than those for Thailand (n = 16). Haplotype diversity (h) of the Solomon Islands populations (0.78–0.80) was intermediate between those of Thailand (0.89) and Vanuatu (0.43–0.68). There were marked differences in distribution of *msp1* haplotypes among the three geographic areas ($P < 10^{-10}$) (Figure 3). Consistently, of 19 distinct *msp1* haplotypes observed in these areas, only one haplotype (MMMM-ETSRL) was found in all areas. The Solomon Islands populations shared four haplotypes with Thailand and three haplotypes with Vanuatu. Only one haplotype was shared by Thailand and Vanuatu. These distributions suggest that the Solomon Islands populations comprise a transitional mixture between those of Thailand and Vanuatu.

In Guadalcanal, distribution of *msp1* haplotypes did not significantly differ among areas A, B, and C, but differed significantly between the 1994 samples and the 1995–1996 samples. This temporal difference is due largely to fluctuations in frequencies of *msp1* haplotypes in the two populations (Figure 3), similar to temporal fluctuations observed for frequencies of some 5′ recombinant types (Table 1).

Linkage disequilibrium in *msp1*. We analyzed linkage disequilibrium for the Solomon Islands area A 1995–1996 samples (n = 27) and area B 1994 samples (n = 30). Of the 15 informative pairs within and between polymorphic blocks 2–6 and polymorphic nucleotides in block 17, only one pair was not significant for both the Solomon Islands 1994 samples and 1995–1996 samples (Figure 4), which indicated strong linkage disequilibrium throughout the entire *msp1* gene. In contrast, the Thai population (n = 33) showed limited linkage disequilibrium: 7 of 15 pairs were not significant.

Polymorphism in *pfcrt*. All of the Solomon Islands isolates (n = 77) had an allele coding for SVMNT at residues 72–76 in the second exon of *pfcrt*; this is a Papua New Guinea-type chloroquine-resistant allele of *pfcrt*.[23] Recently, we reported the monomorphic prevalence of this resistant type in all four islands in Vanuatu,[31] suggesting monomorphic prevalence of the Papua New Guinea-type chloroquine-resistant *pfcrt* allele in the southwestern Pacific.

DISCUSSION

Several genetic mechanisms are involved in the generation of allelic diversity of *P. falciparum* genes: a point mutation causing a single nucleotide polymorphism; an insertion/

TABLE 2

Distribution of *Plasmodium falciparum* merozoite surface protein 1 (*msp1*) haplotypes in the Solomon Islands in comparison with Thailand and Vanuatu*

5′ recombinant type	3′ sequence type					No. of msp1 haplotypes	$h \pm SE$
	QKNGL	QKNGF	EKNGL	ETSRL	Total		
Solomon Islands (1994)							
KKKK	10	0	0	0	10		
MMKM	0	0	0	1	1		
RMKM	0	0	0	5	5		
MMMM	0	0	9	1	10		
RMMM	0	0	0	4	4		
Total	10	0	9	11	30	6	0.78 ± 0.04
Solomon Islands (1995–1996)							
KKKK	9	0	0	0	9		
MMKM	1	0	0	13	14		
RMKM	0	0	0	1	1		
MMMM	0	1	5	3	9		
RMMM	0	0	0	14	14		
Total	10	1	5	31	47	8	0.80 ± 0.03
Thailand							
KKKK	2	0	0	0	2		
MKKK	9	0	4	0	13		
KMKM	0	0	0	1	1		
MMKM	0	2	12	2	16		
RMKM	0	0	3	0	3		
RKMM	0	0	3	1†	4		
KMMM	1	1	2	3	7		
MMMM	0	0	1	1	2		
Total	12	3	25	8	48	16	0.89 ± 0.03
Vanuatu‡							
KMKM	0	0	13	0	13		
RMKM	0	0	0	6	6		
MMMM	0	0	0	19	19		
RMMM	0	69	6	27	102		
Total	0	69	19	52	140	6	0.43 ± 0.90–0.68 ± 0.07§

* *msp1* haplotype is defined as an association between 5′ recombinant type and 3′ sequence type. See Materials and methods for details of 5′ recombinant types and 3′ sequence type.
† One isolate with ETSRL type.
‡ Sakihama and others.[19]
§ Range of h separately calculated for the four islands of Vanuatu studied.

deletion of repetitive sequences, resulting in a repeat-length polymorphism; and meiotic recombination that produces novel alleles in progeny that differ from the parental alleles. Repeat length polymorphism is a prominent feature of *P. falciparum* antigen genes such as *msp1*, *msp2*, and glurp. Accordingly, repeat sequences in those loci have been widely used as polymorphic markers for genotyping of *P. falciparum*.[32] Repeat length polymorphism is probably generated by a mitotic replication slippage mechanism (slipped strand mispairing),[33] and is presumed to evolve rapidly.[26,31] Unequal crossing-over or gene conversion at meiosis may also be involved in the generation of repeat length polymorphism. Diversity of *msp1* haplotypes examined in the present study is distinct from that of repeat length polymorphisms, and is presumably generated by meiotic recombination in non-repetitive sequences. The present method identifies recombinant types in the 5′ region (block 2–6), sequence types in the 3′ regions (block 17), and *msp1* haplotypes (unique associations of 5′ recombinant types and 3′ sequence types). Simultaneously, this method monitors the number and distribution of 5′ recombinant types in a parasite population, and multiplicity of infections (the number of 5′ recombinant types per person). In the present study, we analyzed recombination-generated diversity of *msp1* and multiplicity of infections in a hyperendemic area in Guadalcanal, the Solomon Islands, and compared them with those of mesoendemic areas in Thailand and Vanuatu. We selected these geographic areas because all analysis of samples from these areas was performed under the same experimental conditions.

The diversity of 5′ recombinant types and multiplicity of infections in Guadalcanal *P. falciparum* populations were lower than those of northwestern Thailand, despite the high endemicity in Guadalcanal. It is generally believed that diversity and multiplicity of infection increase as transmission frequency increases. Therefore, the present results are not consistent with this presumed correlation. A similar discrepancy has been reported in Papua New Guinea, where malaria transmission is more intense than in Thailand but the diversity of *msp2* and multiplicity of infections are relatively low.[34] The present results also indicate that linkage disequilibrium within *msp1* is greater in Guadalcanal than in Thailand. Linkage disequilibrium may derive from clustering of particular alleles. To avoid potential bias due to such clustering, we selected samples only from a local population (area A 1995–1996 samples and area B 1994 samples) and did not combine populations from different areas of Guadalcanal. Nevertheless, we detected strong linkage disequilibrium in *msp1*, indicating that limited recombination events are the most likely cause of this linkage disequilibrium. Although the annual entomologic inoculation rates (EIRs) of the present study areas were not available, previous reports indicate that areas close to area B have a very high EIR: 584–1,022 bites per person per year,[35] a level comparable with those of high-transmission