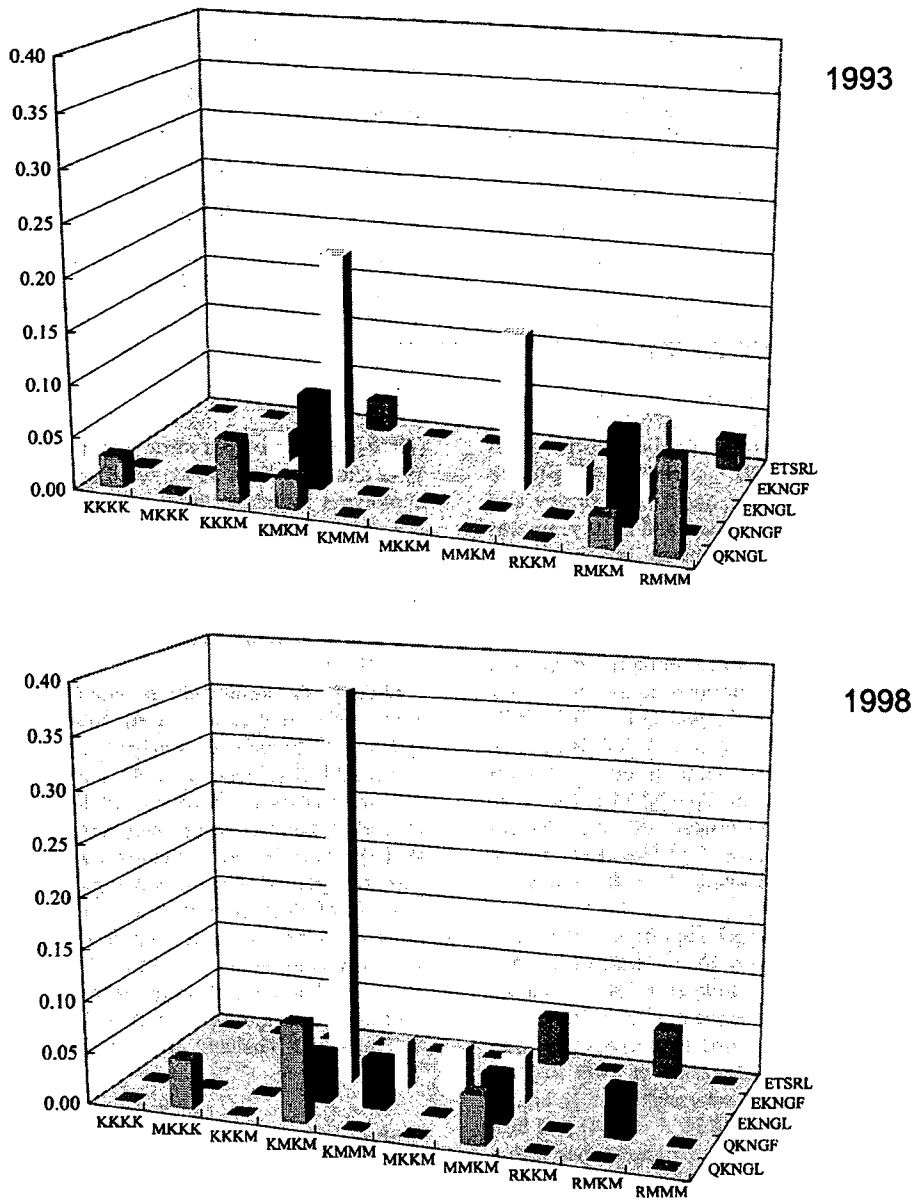

FIGURE 4. Temporal variation in frequency distribution of *P. falciparum msp1* haplotypes between 1993 and 1998. *msp1* haplotypes are unique associations of 5′ recombinant types (x axis) and 3′ sequence types (y axis). Frequencies are shown on the vertical axis.

and 19 of 21 pairs showed LD, indicating limited or little recombination in those areas.[20,24]

DISCUSSION

Intragenic meiotic recombination in the mosquito is a major mechanism of generation of allelic variation in *P. falciparum msp1*. The frequency of recombination in *P. falciparum* generally depends on the intensity of malaria transmission, which varies greatly in different endemic areas.[26,27] In areas of Africa experiencing high perennial transmission, the entomological inoculation rate (the number of infective mosquito bites per person per year) can reach several hundred,[19] whereas it is at least 2 orders of magnitude lower in areas of low and seasonal transmission such as Southeast Asia. Thus, the recombination-driven allelic diversity of *msp1* may be assumed to be higher in an intense transmission area than in a low transmission area. The present study is the first to measure the recombination-driven allelic diversity of *P. falciparum msp1* in Africa. The results demonstrate that the diversity of *msp1* haplotypes in Tanzania is high compared with areas of lower transmission such as Southeast Asia and Melanesia.[20] In the present study, geographic comparisons of *msp1* diversity were performed using a PCR-based typing method, which may lead to underestimation of the frequency of recombination events. Nevertheless, we observed a sub-

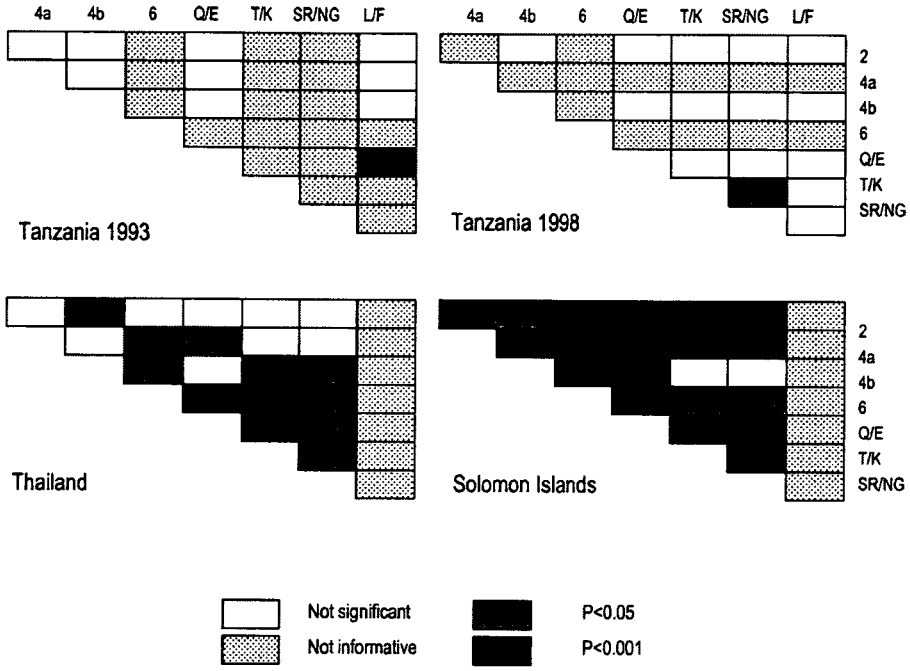

FIGURE 5. Linkage disequilibrium in *P. falciparum msp1* in populations from Tanzania. Pairs of polymorphic blocks 2, 4a, 4b, and 6 and four polymorphic sites (Q/E, T/K, SR/NG, and L/F) in block 17 were subjected to the R^2 test. Non-informative pairs (frequency < 10% in a polymorphic block or nucleotide site) were excluded from the R^2 test. Data from Thailand and Solomon Islands are from Sakihama et al.[20]

stantially high frequency of recombination-driven allelic diversity of *msp1*, suggesting that the extent of recombination-driven allelic diversity of *P. falciparum msp1* is much higher in Africa than we observed.

Although the intensity of transmission is a major factor determining *msp1* allelic diversity, other factors may also be important. These factors include, but are not necessarily limited to, the rate of multiple-genotype infections (polyinfection rate), the mean number of 5′ recombinant type infections per isolate (MORT), and the prevalence of *msp1* haplotypes as well as the parasite-positive rate in a given area. In the Solomon Islands, where the transmission rate is comparable to that of Africa, *msp1* allelic diversity is considerably lower than in Tanzania (Table 3). The polyinfection rate, MORT, and *msp1* haplotype prevalence are relatively limited in the Solomon Islands compared with Tanzania, and therefore the frequencies of out-crossing may be relatively low, resulting in the limited allelic diversity of *msp1* observed in this area.

The frequency distribution of *msp1* haplotypes varied in Nyamisati village between 1993 and 1998. During the same period, frequencies of individual polymorphisms in four polymorphic blocks (blocks 2 to 6) and 4 polymorphic sites (in block 17) remained stable. These two findings appear to contradict each other. However, they are readily reconciled when frequent recombination events are taken into consideration. We observed little linkage disequilibrium in *msp1* in 1993 and 1998, suggesting frequent recombination events in the study area. Therefore, we consider it highly probable that frequent recombination events generate novel *msp1* haplotypes (while simultaneously breaking down previously existing haplotypes), resulting in a temporal variation in their frequency distribution. This explanation is supported by a previous study that showed a rapid decline of linkage disequilibrium along a map distance in *msp1* in highly endemic areas of Africa.[28] Temporal variations in *msp1* polymorphisms in relatively short periods have been reported in Brazil.[29] Epidemic propagations of parasite populations bearing discrete *msp1* alleles along with human movements have been suggested as a likely reason for such temporal variations. Recombination events may play a minor role, if any, in the temporal variation of *msp1* allelic diversity in low transmission areas.

Variation of the frequency distribution of *msp1* haplotypes through time has important implications regarding the parasite's ability to evade the host's immune response. In highly endemic areas, children gradually gain protective immunity to malaria after repeated infections. Although the mechanisms that generate this protective immunity are little understood, it is believed that protective immunity is acquired by cumulative immune responses to multiple antigenic variants after repeated infections.[30–32] Therefore, the extent and prevalence of antigen diversity in a local area is important for the acquisition of protective immunity. MSP-1 is highly immunogenic and induces antibody responses to the entire MSP-1 molecule.[33] Antibodies specific to different regions of MSP-1 inhibit, when combined, parasite growth in an additive manner.[33] Individuals living in endemic areas raise serum antibodies against MSP-1 in an age-dependent manner.[34] The intermittent appearance of novel *msp1* alleles generated by meiotic recombination would produce a number of novel tertiary structure-associated combinational epitopes, and would therefore be likely to induce "epitope"-specific immunity even when frequencies of individual polymorphic blocks and sites are stable. Human antibodies that inhibit merozoite invasion into red cells are known to recognize conformational epitopes.[9] We consider, therefore, that frequent recombination-driven generation of novel *msp1* alleles may affect the

efficiency of acquiring "strain"-specific immunity in highly endemic areas.

In the context of strain-specific immunity, our observation of a significant reduction of MORT from 1993/1998 to 2003 deserves attention. During this period, the age group displaying the highest MORT shifted from those of ages 6–10 years (highest MORT in 1998) to those > 15 years (highest MORT in 2003). This trend was also seen in the polyinfection rates. The reason for this shift is unknown, but it is possibly related to the introduction of insecticide-treated bed nets (ITNs) to the study village in 1999. ITNs have previously been shown to reduce malaria infections substantially in Tanzania.[35] It is also possible that the establishment of a health clinic with continuous monitoring of malaria infections and provision of early treatment of patients contributed to an overall reduction of the mean number of multiple *msp2* genotype infections.[36] The shift of the peak of MORT toward older age groups may be explained in terms of the acquisition of strain-specific immunity. Measures such as ITNs and better health-care facilities will effectively reduce transmission in the areas in which they are deployed. Reduced transmission could lead to an increase in the time it takes an individual to contract, and therefore to develop immunity to, all the different strains present in the area. This would lead to a shift in the peak of MORT to older individuals, as observed in this study. Similarly, the overall reduction of multiple infections may also be a function of reduced transmission.

MSP-1 induces protective antibody responses in individuals living in highly endemic areas.[3,8,9] It may be argued that *msp1* polymorphism is maintained by immune selection, and hence rare polymorphisms increase in frequency over predominant polymorphisms because of the low rates of acquired immunity against them. However, the present study revealed a very stable frequency distribution of *msp1* polymorphisms throughout the period of study (10 years) in Tanzania. Polymorphism in *msp1* has previously been shown to remain stable over a study period of 7 years in the Gambia as determined by typing using monoclonal antibodies.[37] We propose, therefore, that *msp1* polymorphism is not subject to frequency-dependent immune selection.

In conclusion, the present study demonstrates that allelic diversity of *msp1* is higher in Tanzania than in Thailand and the Solomon Islands and suggests that intragenic recombination contributes to the allelic diversity of *P. falciparum msp1* to a greater extent. In Tanzania, frequent recombination events appear to generate novel *msp1* haplotypes intermittently and cause a temporal variation in the frequency distribution of *msp1* haplotypes, whereas the frequencies of individual polymorphisms are stable.

Received October 31, 2006. Accepted for publication December 11, 2006.

Acknowledgments: The authors thank Richard Culleton for reading this manuscript and his comments. We are grateful to the villagers and the research team in Nyamisati who participated in this study.

Financial support: This study was supported by a Grant-in-Aid for Scientific Research on Priority Areas from The Japanese Ministry of Education, Culture, Sports, Science and Technology (18073013), Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (18390131, 18GS03140013), the Japanese Ministry of Health, Labor and Welfare (H17-Sinkou-ippan-019), and the Swedish International Development Agency.

Authors' addresses: Kazuyuki Tanabe and Naoko Sakihama, Laboratory of Malariology, International Research Center of Infectious Diseases, Research Institute for Microbial Diseases Osaka University, 3-1, Yamada-Oka, Suita, 565-0871, Japan, Telephone: +81-6-6879-4260, Fax: +81-6-6879-4262, E-mail: kztanabe@biken.osaka-u.ac.jp. Ingegerd Rooth, Nyamisati Malaria Research Unit, P.O. Box, 663, Dar es Salaam, Tanzania. Anders Björkman and Anna Färnert, Unit of Infectious Diseases, Department of Medicine, Karolinska Institute, Karolinska University Hospital, Solna, SE-17176 Stockholm, Sweden.

Reprint requests: Kazuyuki Tanabe, Laboratory of Malariology, International Research Center of Infectious Diseases, Research Institute for Microbial Diseases Osaka University, 3-1, Yamada-Oka, Suita, 565-0871, Japan. Telephone: +81-6-6879-4260, Fax: +81-6-6879-4262, E-mail: kztanabe@biken.osaka-u.ac.jp.

REFERENCES

1. Holder AA, Guevara Patino JA, Uthaipibull C, Syed SE, Ling IT, Scott-Finnigan T, Blackman MJ, 1999. Merozoite surface protein 1, immune evasion, and vaccine against asexual blood stage malaria. *Parassitologia 41:* 409–414.
2. Mahanty S, Saul A, Miller LH, 2003. Progress in the development of recombinant and synthetic blood-stage malaria vaccines. *J Exp Biol 206:* 3781–3788.
3. Conway DJ, Cavanagh DR, Tanabe K, Roper C, Mikes ZS, Sakihama N, Bojang KA, Oduola AMJ, Kremsner PG, Arnot DE, Greenwood BM, McBride JS, 2000. A principal target of human immunity to malaria identified by molecular population genetic and immunological analyses. *Nat Med 6:* 689–692.
4. Blackman MJ, Ling IT, Nicholls SC, Holder AA, 1991. Proteolytic processing of the *Plasmodium falciparum* merozoite surface protein-1 produces a membrane-bound fragment containing two epidermal growth factor-like domains. *Mol Biochem Parasitol 49:* 29–34.
5. Chang SP, Gibson HL, Lee-Ng CT, Barr PJ, Hui GS, 1992. A carboxyl-terminal fragment of *Plasmodium falciparum* gp195 expressed by a recombinant baculovirus induces antibodies that completely inhibit parasite growth. *J Immunol 149:* 548–555.
6. Kumar S, Yadava A, Keister DB, Tian JH, Ohl M, Perdue-Greenfield KA, Miller LH, Kaslow DC, 1995. Immunogenicity and in vivo efficacy of recombinant *Plasmodium falciparum* merozoite surface protein-1 in *Aotus* monkeys. *Mol Med 1:* 325–333.
7. Egan AF, Morris J, Barnish G, Allen S, Greenwood BM, Kaslow DC, Holder AA, Riley EM, 1996. Clinical immunity to *Plasmodium falciparum* malaria is associated with serum antibodies to the 19-kDa C-terminal fragment of the merozoite surface antigen, PfMSP-1. *J Inf Dis 173:* 765–769.
8. Nwuba R, Sodeinde O, Anumudu C, Omosum Y, Odaibo A, Holder A, Nwagwu M, 2002. The human immune response to *Plasmodium falciparum* includes both antibodies that inhibit merozoite surface protein 1 secondary processing and blocking antibodies. *Infect Immun 70:* 5328–5331.
9. O'Donnel RA, de Koning-Ward TF, Burr RA, Bockarie M, Reeder JC, Cowman A, Crabb B, 2001. Antibodies against merozoite surface protein (MSP)-1_{19} are a major component of the invasion-inhibitory response in individuals immune to malaria. *J Exp Med 193:* 1403–1412.
10. Tanabe K, Mackay M, Goman M, Scaife JG, 1987. Allelic dimorphism in a surface antigen gene of the malaria parasite *Plasmodium falciparum. J Mol Biol 195:* 273–287.
11. Miller LH, Roberts T, Shahabuddin M, McCutchan TF, 1993. Analysis of genetic diversity in the *Plasmodium falciparum* merozoite surface protein-1 (*MSP1*). *Mol Biochem Parasitol 59:* 1–14.
12. Martinelli A, Cheesman S, Hunt P, Culleton R, Raza A, Mackinnon M, Carter R, 2005. A genetic approach to the de novo identification of targets of strain specific immunity in malaria parasites. *Proc Natl Acad Sci USA 102:* 814–819.
13. Cheesman S, Raza A, Carter R, 2006. Mixed strain infections and strain-specific protective immunity in the rodent malaria parasite *Plasmodium chabaudi chabaudi* in mice. *Infect Immun 74:* 2996–3001.

14. Tanabe K, Sakihama N, Kaneko A, 2004. Stable SNPs in malaria antigen genes in isolated populations. *Science 303:* 493.
15. Polley SD, Weedal GD, Thomas AW, Golightly LM, Conway DJ, 2005. Orthologous gene sequences of merozoite surface protein 1 (MSP1) from *Plasmodium reichenowi* and *P. gallinaceum* confirm an ancient divergence of *P. falciparum* alleles. *Mol Biochem Parasitol 142:* 25–31.
16. Ferreira MU, Ribeiro WL, Tonon AP, Kawamoto F, Rich SM, 2003. Sequence diversity and evolution of the malaria vaccine candidate merozoite surface protein-1 (*MSP1*) of *Plasmodium falciparum. Gene 304:* 65–75.
17. Snounou G, Zhu X, Siripoon N, Jarra W, Thaithong S, Brown KN, Viriyakosol S, 1999. Biased distribution of *msp1* and *msp2* allelic variants in *Plasmodium falciparum* populations in Thailand. *Trans R Soc Trop Med Hyg 93:* 369–374.
18. Rooth I, Björkman A, 1992. Fever episodes in a holoendemic area of Tanzania: parasitological and clinical findings and diagnostic aspects related to malaria. *Trans R Soc Trop Med Hyg 86:* 479–482.
19. Hay SI, Rogers DJ, Toomer JF, Snow RW, 2000. Annual *Plasmodium falciparum* entomological inoculation rates (EIR) across Africa: literature survey, internet access and review. *Trans R Soc Trop Med Hyg 94:* 113–127.
20. Sakihama N, Ohmae H, Bakote B, Kawabata M, Hirayama K, Tanabe K, 2006. Limited allelic diversity of *Plasmodium falciparum msp1* from populations in The Solomon Islands, a highly endemic area. *Am J Trop Med Hyg 74:* 31–40.
21. Sakihama N, Kimura M, Hirayama K, Kanda T, Na-Bangchang K, Jongwutiwes S, Conway D, Tanabe K, 1999. Allelic recombination and linkage disequilibrium within *Msp1* of *Plasmodium falciparum*, the malignant human malaria parasite. *Gene 230:* 47–54.
22. Tanabe K, Sakihama N, Färnert A, Rooth I, Björkman A, Walliker D, Ranford-Cartwright L, 2002. In vitro recombination during PCR of *Plasmodium falciparum* DNA: a potential pitfall in molecular population genetic analysis. *Mol Biochem Parasitol 122:* 211–216.
23. Qari SH, Shi YP, Goldman IF, Nahlen BL, Tibayrenc M, Lal AA, 1998. Predicted and observed alleles of *Plasmodium falciparum* merozoite surface protein-1 (MSP-1), a potential malaria vaccine antigen. *Mol Biochem Parasitol 92:* 241–252.
24. Tanabe K, Sakihama N, Nakamura Y, Kaneko O, Kimura M, Ferreira MU, Hirayama K, 2000. Selection and genetic drift of polymorphisms within the merozoite surface protein-1 gene of *Plasmodium falciparum. Gene 241:* 325–331.
25. Ferreira MU, Liu Q, Kimura M, Tanabe K, Kawamoto F, 1998. Allelic diversity in the merozoite surface protein-1 and epidemiology of multiple-clone *Plasmodium falciparum* infections in northern Tanzania. *J Parasitol 84:* 1286–1289.
26. Babiker H, Walliker D, 1997. Current views on the population structure of *Plasmodium falciparum*: implications for control. *Parasitol Today 13:* 262–267.
27. Mu J, Awadalla P, Duan J, McGee KM, Joy DA, McVean GAT, Su AZ, 2005. Recombination hot spots and population structure in *Plasmodium falciparum. PLoS Biol 3:* 1734–1741.
28. Conway DJ, Roper C, Oduola AM, Arnot DE, Kremsner PG, Grobusch MP, Curtis CF, Greenwood BM, 1999. High recombination rate in natural populations of *Plasmodium falciparum. Proc Natl Acad Sci USA 96:* 4506–4511.
29. Silva NS, Silveira LA, Machado RL, Povoa MM, Ferreira MU, 2000. Temporal and spatial distribution of the variants of merozoite surface protein-1 (MSP-1) in *Plasmodium falciparum* populations in Brazil. *Ann Trop Med Parasitol 94:* 675–688.
30. Beck HP, Felger I, Huber W, Steiger S, Smith T, Weiss N, Alonso P, Tanner M, 1997. Analysis of multiple *Plasmodium falciparum* infections in Tanzanian children during the phase III trials of the malaria vaccine SPf66. *J Inf Dis 175:* 921–926.
31. Contamin H, Fandeur T, Rogier C, Bonnefoy S, Trape JF, Mercereau-Puijalon O, 1996. Different genetic characteristics of *Plasmodium falciparum* isolates collected during successive clinical malaria episodes in Senegalese children. *Am J Trop Med Hyg 54:* 632–643.
32. Färnert A, Rooth I, Svensson A, Snounou G, Bjorkman A, 1999. Complexity of *Plasmodium falciparum* infections is consistent over time and protects against clinical disease in Tanzanian children. *J Infect Dis 179:* 989–995.
33. Woehlbier U, Epp C, Kauth CW, Lutz R, Long CA, Coulibaly B, Kouyaté B, Arevalo-Herrera M, Herrera S, Bujard H, 2006. Analysis of antibodies directed against merozoite surface protein 1 of the human malaria parasite *Plasmodium falciparum. Infect Immun 74:* 1313–1322.
34. Ekaka MT, Jouin H, Lekoulou F, Issifou S, Mercereau-Puijalon O, Ntoumi F, 2002. *Plasmodium falciparum* merozoite surface protein 1 (MSP1) genotyping and humoral responses to allele-specific variants. *Acta Trop 81:* 33–46.
35. Fraser-Hurt N, Felger I, Edoh D, Steiger S, Mashaka M, Masanja H, Smith T, Mbena F, Beck HP, 1999. Effect of insecticide-treated bed nets on haemoglobin values, prevalence and multiplicity of infection with *Plasmodium falciparum* in a randomized controlled trial in Tanzania. *Trans R Soc Trop Med Hyg 93 (Suppl 1):* 47–51.
36. Bereczky S, Liljander A, Rooth I, Faraja L, Montgomery SM, Färnert A, 2007. Multiclonal asymptomatic *Plasmodium falciparum* infections predict a reduced risk of malaria disease in a Tanzanian population. *Microbes Infect 9:* 103–110.
37. Conway DJ, Greenwood BM, McBride JS, 1992. Longitudinal study of *Plasmodium falciparum* polymorphic antigens in a malaria-endemic population. *Infect Immun 60:* 1122–1127.

Review TRENDS in Parasitology Vol.23 No.4

Parasitology in Japan

Progress in the molecular biology of malaria and the immunology of nematode infections

Naoki Arizono[1], Kenji Nakanihsi[2], Toshihiro Horii[3] and Kazuyuki Tanabe[3]

[1] Department of Medical Zoology, Kyoto Prefectural University of Medicine, Kawaramachi-Hirokoji, Kyoto 602-8566, Japan
[2] Department of Immunology and Medical Zoology, Hyogo College of Medicine, Hyogo 663-8501, Japan
[3] Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871, Japan

Japan is one of a small number of countries to have successfully controlled or eliminated major parasitic diseases, including malaria, filariasis, schistosomiasis and enteric parasitoses. Of particular importance in this success was a close collaboration between primary research and public health efforts. Many Japanese researchers continue to study malaria, particularly the areas of genetics and immunology, and this should contribute to global parasite eradication strategies. Furthermore, studies of immunoregulation of nematode infection using the interleukin-18 pathway, most of which have been conducted in Japan, are helping to improve researchers' understanding of human immune mechanisms and host–parasite interactions.

A brief history of the control of malaria and enteric parasites in Japan

At the end of the 19th century, no fewer than 200 000 malaria cases were recorded in Japan annually. The parasites that caused these cases were *Plasmodium vivax* in the main islands, and *P. vivax* and *Plasmodium falciparum* in the Okinawa islands. After extensive drug administration and vector control, the incidence of malaria declined gradually and the last endemic malaria focus was eliminated from Japan in the mid-1950s (following a brief resurgence immediately after World War II). Researchers had a pivotal role in this decline, often being directly involved in control operations and in generating new findings, devising new tools and evaluating control interventions. One of the most important research accomplishments in Japan in the 1970s was the work done by Aikawa on various aspects of malaria biology, starting with his pioneering work on the mechanisms by which malaria parasites invade red blood cells [1].

Japan also faced a major problem with intestinal parasites, with a prevalence of ascariasis as high as 60% until the mid-20th century [2]. A comprehensive program to eradicate intestinal parasites began after World War II and involved schools, local health centers and nongovernmental organizations. This program achieved great success, including a decline in intestinal parasite prevalence during the 1960s and 1970s, and this coincided with the rapid industrialization of the country. Today, it is rare to encounter intestinal parasitoses in Japan. Indicative of the potential global impact of the Japanese experience is the thick smear method, which was devised by Kato and Miura [3], which greatly contributed to the detection of parasite ova. Indeed, an improved version of this method has now been adopted as the world standard for quantitative diagnosis of parasite ova. Following the successful control of malaria and intestinal parasitoses, many Japanese researchers continue to be engaged in studies to elucidate the molecular and immunological mechanisms of these diseases (Table 1).

Malaria research in Japan

Molecular mechanisms of invasion

The molecular mechanisms of parasite invasion of host cells, particularly in the sexual stage and liver-infective stage, are a 'hot topic' in malaria research. Chinzei and colleagues have analyzed ookinete and sporozoite expressed sequence tags in the malaria parasite of rodents *Plasmodium berghei* [4,5]. The functions of several novel molecules in the sexual and liver-infective stages have been identified using phenotype analysis of parasites that have undergone targeted gene disruption [4]. Chinzei and colleagues have shown that ookinete-specific molecules (e.g. circumsporozoite protein thrombospondin-related anonymous protein (CTRP), calcium-dependent protein kinase (CDPK3) and membrane attack ookinete protein (MAOP) [5–7]) are essential for ookinete migration and invasion of the mosquito midgut epithelium (Figure 1). Another important finding is that the accumulation of sporozoites in the salivary glands of mosquitoes is mediated by the apical membrane antigen-1 erythrocyte binding-like protein (MAEBL) [8]. Sporozoites that are introduced into the vertebrate host liver must pass through the sinusoidal layer to reach the hepatocytes. Three novel proteins, sporozoite protein essential for cell traversal (SPECT, SPECT2) and cell traversal protein expressed in ookinete and sporozoite (CelTOS) [7–11], are essential for sporozoites to pass through the Kupffer cells in the sinusoidal layer. Six-cys family proteins [e.g.

Corresponding author: Arizono, N. (arizonon@koto.kpu-m.ac.jp).
Available online 22 February 2007.

www.sciencedirect.com 1471-4922/$ – see front matter doi:10.1016/j.pt.2007.02.005

Table 1. Malaria research in Japan[a]

Research topics	Year	Research group	Refs
Ultrastructural studies of malaria parasites	1966–2003	M. Aikawa and colleagues	[1]
Identification of function of *Plasmodium berghei* oocyst and sporozoite proteins	1999–present	Y. Chinzei and colleagues	[3–11]
Polymorphism study of *msp1* of *Plasmodium falciparum* and *Plasmodium vivax*	1987–present	K. Tanabe and colleagues	[12–16]
Involvement of Treg cells in immunity to malaria	2004–present	H. Hisaeda and colleagues	[17,18]
TLR-mediated innate immune responses to malaria	2001–present	S. Akira and colleagues	[19–23]
Clinical development of the SE36 malaria vaccine	1992–present	T. Horri and colleagues	[24–27]
P. vivax malaria TBV research	1988–present	T. Tsuboi, M. Torii and colleagues	[28–32]
Malaria eradication in Vanuatu	1998–present	A. Kaneko and colleagues	[33,34]

[a]For information on recent research of malaria biochemistry and drug development in Japan, see Ref. [61], a forthcoming article in the Parasitology in Japan series.

the 36kDa surface protein of *Plasmodium berghei* gametes and zygotes and a related protein (Pbs36, Pbs36p)] enable sporozoites to recognize hepatocytes as the target cells [12]. These parasite molecules are important for infection of host cells and are, therefore, candidates for the development of antimalarial drugs and vaccines.

Antigen polymorphism

A thorough understanding of the causes and consequences of antigen polymorphism in malaria parasites is crucial for the development of effective vaccines. In a landmark article, Tanabe *et al.* proposed that meiotic recombination is the major genetic mechanism that underlies the diversity observed in the gene that encodes merozoite surface protein-1 (msp1), a leading vaccine candidate [13]. Tanabe *et al.* have continued to investigate the underlying genetics of *msp1* polymorphism in parasite populations from various geographic locations. For example, they have shown that levels of *msp1* allelic diversity do not necessarily correlate with the intensity of transmission in Southeast Asia and Melanesia [14,15] and that this is in contrast to the relationship that is observed in highly endemic areas in Africa. Their recent report on the stability of single nucleotide polymorphisms in isolated parasite populations in Vanuatu [16] has positive implications for the development of vaccines. Tanabe and colleagues have recently extended their studies to include *P. vivax msp1* and have shown that the diversity seen in this *msp1* is generated by meiotic recombination events throughout the gene (in contrast to restricted regions in *P. falciparum msp1*). This is the case even in areas where *P. vivax* prevalence is low and malaria transmission is seasonal, such as Thailand [17].

Immune evasion mechanisms and innate immunity

In addition to antigen polymorphism, malaria parasites have several strategies to evade host immunity: for example, immune suppression. Hisaeda *et al.* recently proposed a novel immune-evasion mechanism of malaria parasites, the activation of regulatory T (Treg) cells. Immune-suppressive $CD4^+CD25^+$ Treg cells are

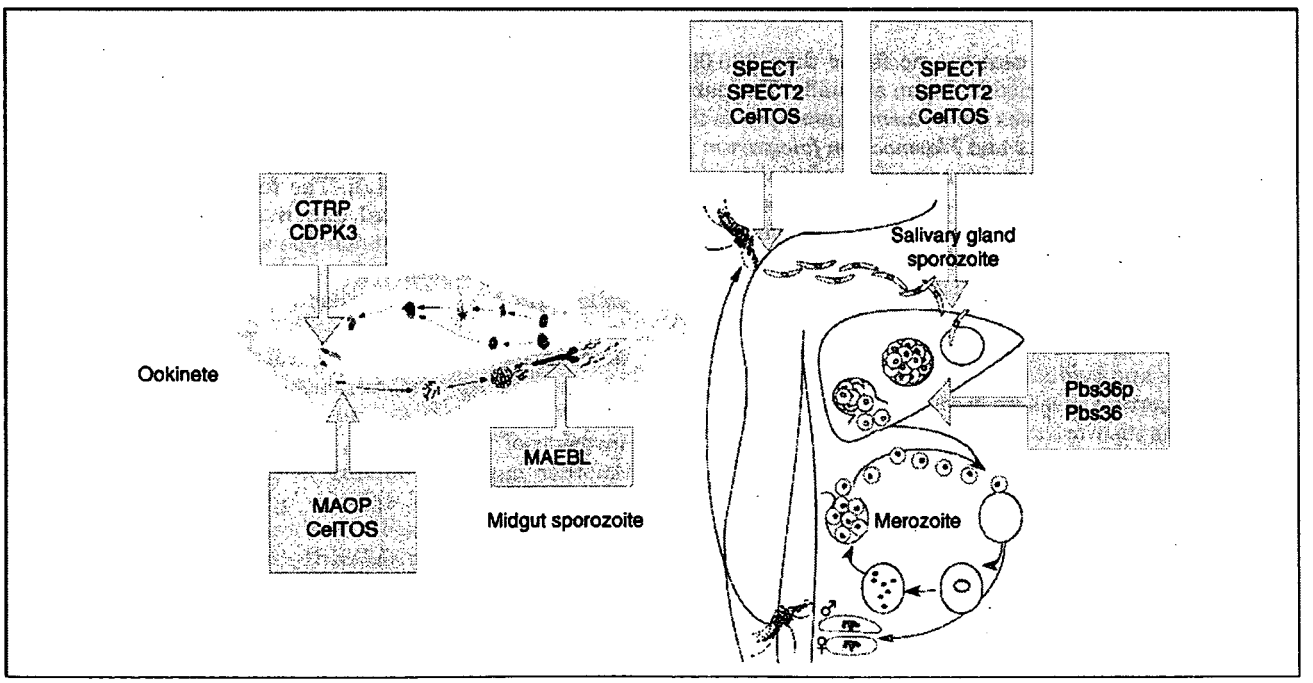

Figure 1. Novel proteins that are involved in host-cell invasion in the sexual and liver-infective stages of malaria parasites. Ookinete-specific proteins (CTRP, CDPK3 and MAOP) enable ookinete infection of the midgut epithelial cell. The midgut sporozoite protein MAEBL is important for sporozoite invasion of the salivary gland (SG). SG sporozoite proteins (e.g. SPECT, SPECT2, CelTOS, Pbs36p and Pbs36) function in both sporozoite migration and infection of the skin and the liver. The sexual cycle of *Plasmodium* in the mosquito is shown on the left hand side and the asexual cycle in the human is shown on the right hand side. Figure courtesy of Y. Chinzei.

preferentially activated during infection, which results in a failure to develop immune responses [18,19]. Mice that were infected with a nonlethal strain of *Plasmodium yoelii* (PyNL) developed T-cell immunity at early stages of infection, whereas those infected with a lethal strain (PyL) showed impaired immune responses in association with an increase in Treg cell numbers. Infection with PyL, but not with PyNL, enhanced the suppressive activity exerted by Treg cells. Furthermore, depletion of Treg cells enabled mice to survive infection with PyL. These results indicate that the immune suppression that is observed in lethal malaria is associated with activation of Treg cells. It has recently been confirmed that Treg cells are activated in *P. falciparum* infection, which indicates that activation of these cells is a central mechanism by which malaria parasites subvert host immune systems.

Effective treatment and prevention of malaria infection requires an understanding of host innate immunity. Toll-like receptors (TLRs) have been extensively studied in recent years and have an important role not only in direct killing and proinflammatory responses but also in eliciting efficient adaptive (acquired) immune responses [20]. Nakanishi and colleagues were the first to report that myeloid differentiation factor 88, an essential adaptor molecule for most TLR signaling, is involved in proinflammatory responses and liver injury, both of which are caused by *P. berghei* infection [21]. Akira and colleagues have searched for possible ligands of TLRs that are derived from malaria parasites. They found that hemozoin (malaria pigment) is a TLR9 agonist that activates TLR9-expressing dendritic cells to produce proinflammatory cytokines and chemokines [22,23]. Moreover, they found that glycosylphosphatidylinositols (GPIs) activate macrophages using TLR2 and, to a lesser extent, TLR4 [24]. Interestingly, GPIs that contain two or three fatty acids are recognized by distinct heterodimers of TLRs: TLR2–TLR6 or TLR2–TLR1, respectively. Further studies of innate immune responses should elucidate some of the complex host–parasite interactions that are associated with malaria.

Malaria vaccine development

Several malaria vaccines are currently being developed in Japan. Horii and colleagues are developing a vaccine using the recombinant protein SE36, which consists of the N-terminal domain of the serine repeat antigen (SERA)5 of *P. falciparum* [25,26]. Cross-sectional field studies to examine the degree to which naturally acquired immunity to SE36 can contribute to protection against malaria have shown that the anti-SE36 IgG3 titer has strong negative correlations with symptoms and blood parasitemia [27,28]. In addition, seroconversion rates for SE36 were $\leq$50% for adults and <10% for younger subjects in the cohorts. These findings strongly indicate that the N-terminal domain of SERA is a hidden protective antigen that could be the Achilles' heel of the malaria parasite. Clinical-grade Biken (BK)-SE36 (i.e. purified SE36 adsorbed onto aluminum hydroxide gel; Biken, Osaka University: http://www.biken.osaka-u.ac.jp/e/) that was prepared under high-quality laboratory conditions was safe and highly immunogenic in chimpanzees. BK–SE36 provided significant protection for squirrel monkeys against malaria parasite challenge (T. Horii *et al.*, unpublished). In immunized monkeys, antibody titers that had been induced by the vaccine were boosted following infection of the monkeys with malaria parasites. The safety and immunogenicity of BK–SE36 in humans have been confirmed in a Phase I clinical trial in Japan (T. Horii *et al.*, unpublished).

Transmission-blocking vaccines (TBVs) against malaria, using ookinete surface proteins as candidates, are being developed by Tsuboi and Torii [29–33]. For many malarial regions outside Africa, the development of effective vaccines requires coverage against both *P. falciparum* and *P. vivax*. The *P. vivax* TBV candidate genes, *P. vivax* surface proteins 25kDa and 28kDa (*pvs25* and *pvs28*), have been cloned from the *P. vivax* Salvador I (Sal I) strain [29]. Mice that were vaccinated with recombinant Pvs25 or Pvs28 adsorbed onto alum developed strong antibody responses, and oocyst development of Sal I was completely inhibited by antisera from the mice [30]. Antibodies that were raised against *P. vivax* Sal I-based vaccines are not affected by polymorphisms of Pvs25 and Pvs28 in *P. vivax* isolates [31]. Recombinant Pvs25 and Pvs28 are potent vaccines when used for intranasal immunization [32] and when the genes are used for DNA vaccination [33]. The first Phase I clinical trial of a Pvs25-based TBV was recently conducted and it indicated a correlation between the antibody concentration and the degree of inhibition. In humans, recombinant Pvs25 generates transmission-blocking immunity against *P. vivax* and demonstrates the potential of this antigen as a component of a TBV.

Eradication of malaria in Vanuatu

In Vanuatu, the malaria eradication program that was headed by Kaneko *et al.* was successful [34]. Malaria control programs must be adapted to the local epidemiological characteristics. Vanuatu consists of 80 islands in Melanesia with hypo- to meso-endemic malaria [35] – conditions that are suitable for sustainable parasite elimination. In 1991 on Aneityum island (~700 inhabitants), Kaneko's team conducted weekly mass drug administration (MDA) using chloroquine, pyrimethamine–sulfadoxine and primaquine over a nine-week period. Permethrin-impregnated bed nets were distributed with an annual reimpregnation cycle, and fish were introduced into breeding sites of *Anopheles farauti* to eat the mosquito larvae. The effects of these interventions on malaria were monitored over an eight-year period. Two islands, one with and one without malaria transmission, were used for comparisons. In Aneityum, sustained interruption of malaria transmission was achieved because of high levels of community involvement, MDA compliance (88.3%) and provision of bed nets (0.94 nets per villager). Surveys showed the absence of *P. falciparum* after MDA and of *P. vivax* from 1996 onwards, except for two imported infections in 1999. This shows that malaria transmission can be interrupted on an isolated island using appropriate short-term MDA and sustained vector control if there is a high degree of community participation [33].

Nematode research in Japan

Strongyloides venezuelensis *infection in mice*

There have been many detailed studies to elucidate immunoprotective mechanisms against helminth infections

[36]. In general terms, it is thought that protective immunity against helminth infection is closely associated with T helper (Th)2-cell generation in the host, as has been observed in schistosomiasis, ascariasis, filariasis and several experimental helminth infections in rodents. A typical Th2 response activates a set of effector cells and defense mechanisms that protects against helminth infection, including eosinophils, mast cells, basophils, mucin and enhancement of intestinal smooth muscle contractility [36]. Nawa *et al.* conducted one of the earliest studies that provided evidence that a single effector-cell type can efficiently protect against helminth infections [37]. They showed, using mast-cell-deficient W/W^v mice, that intestinal mast cells have a crucial role in protection against *Strongyloides ratti*. Further studies indicated that mast cells and basophils also have a protective role against infectious exoparasites such as ticks [38]. These studies were aided by the discovery of mast-cell-deficient mice and by the elucidation of the ontogeny of mast cells by Kitamura *et al.* [39]. More recently, Koyasu and colleagues [40] demonstrated that phosphatidylinositol-3-kinase-deficient mice that are infected with *Strongyloides venezuelensis* respond by delaying the expulsion of nematodes. The fact that these mice are characterized by poor mast-cell development in the intestine [caused by dominant interleukin (IL)-12 production by dendritic cells] illustrates the importance of intestinal mast cells.

Helminth-induced activation of intestinal mast cells

Studies by Nawa and colleagues of the protective role of intestinal mucosal mast cells (MMCs) in *S. venezuelensis* expulsion indicate that these cells produce glycosaminoglycans that have a crucial role in the expulsion of this parasite [41]. In addition, *S. venezuelensis* expulsion is closely associated with an increased number of intestinal MMCs and an increased release of mouse mast-cell protease-1 (mMCP-1), both of which are hallmarks of infection with gastrointestinal nematodes [42–45]. mMCP-1, which is selectively expressed in intestinal MMCs, participates in the effector-phase response to intestinal nematode expulsion [43–45]. Indeed, mMCP-1-deficient mice fail to expel gastrointestinal nematodes [46]. mMCP-1 is not detectable in IL-3-driven bone-marrow-derived mast cells; however, these cells begin to produce mMCP-1 when incubated with IL-9, stem cell factor (SCF) and transforming growth factor (TGF)-β *in vitro* [47]. Thus, Th2 cells that are generated in helminth-infected mice seem to produce IL-3 and IL-9, which (in collaboration with SCF and TGF-β from the gut epithelium) induce and activate MMCs to produce glycosaminoglycans and mMCP-1.

IL-18- and IL-2-induce intestinal MMCs that produce mMCP-1

In general terms, the binding of a ligand to a T-cell receptor (TCR) is required for Th1 and Th2 cells to produce interferon (IFN)-γ and IL-4, respectively. IL-18 was the first cytokine shown to activate Th1 cells to produce IFN-γ without TCR–ligand binding when administered with IL-12 [48]. The resultant IFN-γ then activates macrophages to produce nitric oxide, which leads to the eradication of the intracellular pathogen. Thus, the combination of IL-18 and IL-12 has a protective effect against intracellular pathogens such as *Leishmania major* [49,50]. However, IL-18 without IL-12 induces the production of Th2 cytokines by T cells, mast cells or basophils [51–54] (Figure 2). Following daily injection of a mixture of IL-2 and IL-18, mice had increased serum levels of IL-3, IL-4, IL-9 and IL-13 [53]. IL-3 and IL-9 are potent mast-cell growth factors [55] and are essential for the induction of mMCP-1 [47]. To study this induction, mice were injected with a mixture of IL-2 and IL-18, then their intestinal MMCs were counted and the level of mMCP-1 in their serum was measured. The results indicate that IL-18 and IL-2 increase the number of MMCs in the intestine and increase the level of mMCP-1 in the serum in a dose-dependent manner [56].

After being treated with IL-2 and IL-18, wild-type mice exhibited intestinal mastocytosis (i.e. intestinal accumulation of MMCs). By contrast, wild-type mice that were depleted of $CD4^+$ T cells by pretreatment with an anti-CD4 antibody, and recombination activating gene-deficient ($Rag2^{-/-}$) mice lacking both T and B cells did not exhibit intestinal mastocytosis after treatment with IL-2 and IL-18. Interestingly, signal transducer and activator of transcription-deficient ($Stat6^{-/-}$) mice also exhibited intestinal mastocytosis after being treated with IL-2 and IL-18. Thus, induction of intestinal mastocytosis by IL-18 and IL-2 is dependent on $CD4^+$ T cells but is independent of STAT6 activation [56]. Because mice that were treated with a combination of IL-18 and IL-2 produced IL-3 and IL-9, mice that were given a daily injection of IL-3 and IL-9 were examined for intestinal mastocytosis. The results indicate that this treatment induced intestinal accumulation of MMCs that contain and produce mMCP-1 in a dose-dependent manner [56]. Thus, treatment with IL-2 and IL-18 induces production of $mMCP\text{-}1^+$ MMCs by first inducing production of IL-3 and IL-9 by $CD4^+$ T cells (Figure 3).

Expulsion of S. venezuelensis *by MMCs activated by IL-18 and IL-2*

To examine the functional role of intestinal MMCs produced in response to the combination of IL-18 and IL-2, gastrointestinal nematodes were surgically implanted in mice that had been pretreated with IL-2 and/or IL-18 for 13 days. When the parasites were recovered 16 hours after implantation [41], wild-type mice that were treated with IL-18 and IL-2 had rejected the implanted worms almost completely, whereas mice that received saline, IL-2 or IL-18 alone were heavily parasitized. Mast-cell-deficient W/W^v mice that were treated with IL-2 and IL-18 failed to reject the nematode, which confirmed a previous finding that MMCs have a crucial role in the rapid expulsion of implanted adult worms [41]. $Stat6^{-/-}$ mice that were pretreated with IL-2 and IL-18 also exhibited the capacity to reject implanted parasites. These results indicate that IL-18 and IL-2 stimulate $CD4^+$ T cells to produce IL-3 and IL-9, which, in turn, induce intestinal mastocytosis that is independent of STAT6 activation (Figure 3). STAT6 activation is essential for induction of the Th2 response [57], which indicates that IL-18 and IL-2 induce production of IL-3 and IL-9 by T cells without Th2 cell development.

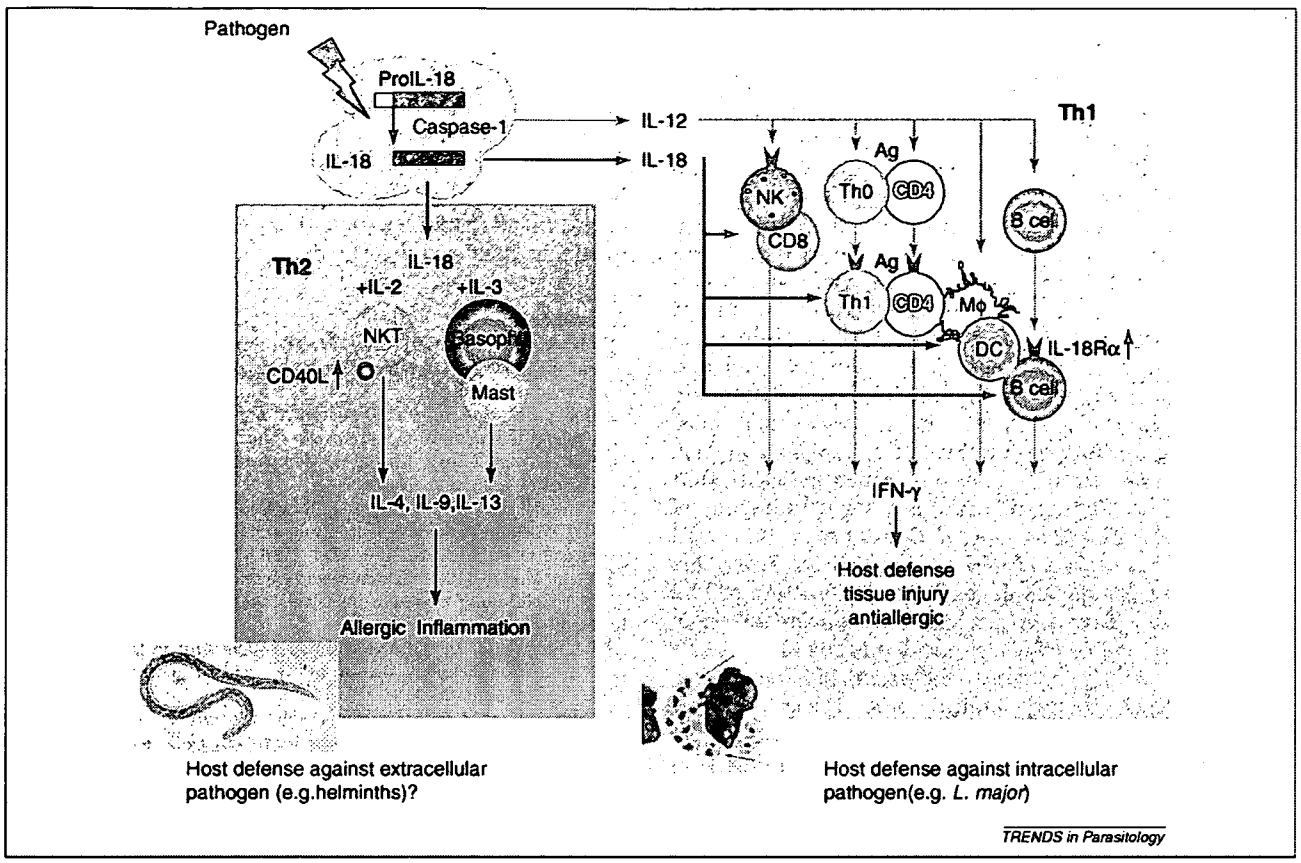

Figure 2. IL-18 stimulates both Th1 and Th2 responses, depending on the cytokine environment. The combination of IL-18 and IL-12 strongly stimulates various types of cell to produce IFN-γ, which, in turn, activates macrophages to produce nitric oxide, leading to the eradication of intracellular pathogens such as *Leishmania major*. However, without IL-12, IL-18 promotes production of Th2 cytokines by T cells, basophils and mast cells. Thus, IL-18 can regulate both Th1 (pink box) and Th2 (yellow box) responses. The expulsion of some types of gastrointestinal nematode depends on Th2 responses, which indicates that IL-18 has a protective function against helminth infection through activation of T cells, basophils and mast cells to produce Th2 cytokines. Abbreviations: Ag, antigen; DC, dendritic cell; MΦ, macrophage; NKT, natural killer T cell.

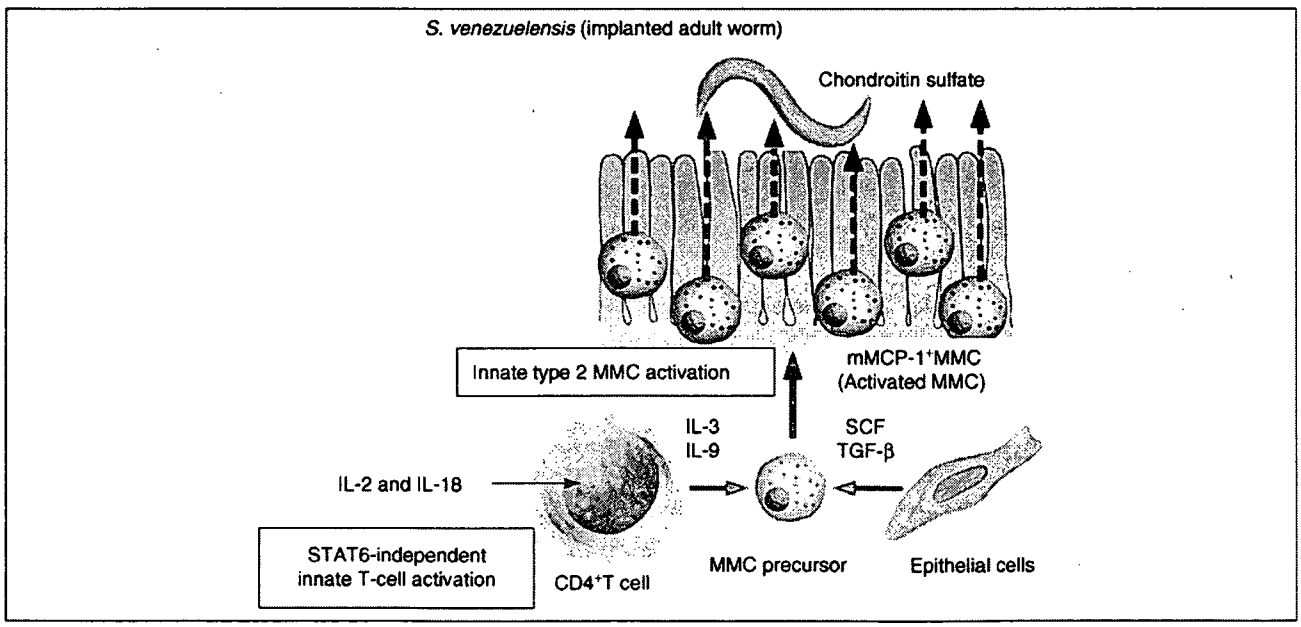

Figure 3. Protective role of IL-18-induced intestinal MMC in expulsion of *Strongyloides venezuelensis*. *In vivo* treatment with a combination of IL-18 and IL-2 stimulates $CD4^+$ T cells to produce IL-3 and IL-9 in a STAT6-independent manner. IL-3 and IL-9 from $CD4^+$ T cells, and SCF and TGF-β from epithelial cells induce precursor cells to develop into mMCP-1^+ MMC. These activated mast cells promptly expel implanted adult *S. venezuelensis* by producing chondroitin sulfate. This is thought to work by interfering with the attachment of the parasite to the host [38].

Expulsion of S. venezuelensis *by MMCs activated by endogenous IL-18*

Having demonstrated the protective effect of exogenous IL-18, it is important to examine the protective role of endogenous IL-18. In one study [56], mice were inoculated with *S. venezuelensis* third-stage larvae, and the serum levels of IL-18 in the mice were measured. Wild-type mice had significantly increased serum levels of IL-18 on days four to 14 after inoculation. The period required for worm expulsion was also compared among wild-type, IL-18-deficient ($Il18^{-/-}$) and IL-18 receptor α chain-deficient ($Il18r\alpha^{-/-}$) mice. Wild-type mice exhibited complete worm expulsion within 12 days after inoculation. By contrast, $Il18^{-/-}$ and $Il18r\alpha^{-/-}$ mice required 16 days for complete worm expulsion. These *Il18*- and *Il18rα*-deficient mice exhibited significantly reduced levels of mMCP-1 on days four, seven and ten after infection, which indicates that endogenous IL-18 is important for early induction of mMCP-1 [56]. The *Il18*- and *Il18rα*-deficient mice had increased serum levels of mMCP-1 by day 14. Thus, this late mMCP-1 production depends on the function of Th2 cells that might have been generated late during infection. Indeed, $Il18^{-/-}$ mice normally develop a Th2 response by day ten following helminth infection [56,58]. Taken together, these results indicate the involvement of two types of intestinal MMC activation: prompt IL-18-dependent (innate type-2) MMC activation and late Th2-cell-dependent (acquired type-2) MMC activation. Because IL-18 induces MMC activation independently of antigen stimulation, prompt IL-18-dependent activation can be designated as innate type-2 MMC activation. This distinguishes it from Th2-cell-dependent acquired type-2 MMC activation [56].

This article describes the available evidence regarding the IL-18-dependent pathway of mMCP-1$^+$ MMC generation and its important role in *S. venezuelensis* rejection. However, it seems unlikely that a single mechanism is sufficient to protect against all intestinal nematodes. For instance, mast cells do not effectively expel the intestinal nematode *Nippostrongylus brasiliensis*. Studies indicate that rejection of *N. brasiliensis* is closely associated with mucus secretion [59], changes in terminal sugar residues of mucin [59], enhancement of intestinal smooth muscle contractility and changes in intestinal epithelial function [36]. Indeed, the expression levels of several intestinal epithelial-cell-related genes were reported to be altered during nematode infections in mice [60]. Therefore, it is not surprising that effector mechanisms against helminth parasites vary according to parasite species, host species and the internal environment of the host.

Concluding remarks

The holistic approach that Japan took proved successful in eliminating malaria and other diseases. More recently, advancements in molecular parasitology and immunology in Japan have resulted in many successes in studies of malaria and nematodes. The subjects under study include the search for novel molecules that are related to *Plasmodium* invasion of mosquito cells, novel immune-evasion mechanisms and vaccine development. These studies will contribute to both a better understanding of host–parasite interactions and global parasite eradication strategies. In addition, Japanese researchers are making important advances in clarifying the IL-18-dependent pathway of immunoprotection against helminths, an important element of the host–parasite interaction. Japanese research findings, coupled with the Japanese experience in controlling infectious diseases, are now being employed to help shape effective disease control and elimination strategies in Africa, Asia and the Pacific.

References

1 Aikawa, M. *et al.* (1978) Erythrocyte entry by malarial parasites: a moving junction between erythrocyte and parasite. *J. Cell Biol.* 77, 72–82
2 Kobayashi, A. *et al.* (2006) Historical aspects for the control of soil-transmitted helminthiases. *Parasitol. Int.* 55, S289–S291
3 Kato, I. and Miura, M. (1954) Comparative examinations. *Jap. J. Parasitol* 3, 35
4 Yuda, M. *et al.* (1999) Targeted disruption of the *Plasmodium berghei* CTRP gene reveals its essential role in malaria infection of the vector mosquito. *J. Exp. Med.* 190, 1711–1716
5 Yuda, M. *et al.* (1999) Structure and expression of an adhesive protein-like molecule of mosquito invasive-stage malarial parasite. *J. Exp. Med.* 189, 1947–1952
6 Ishino, T. *et al.* (2006) A calcium-dependent protein kinase regulates *Plasmodium* ookinete access to the midgut epithelial cell. *Mol. Microbiol.* 59, 1175–1184
7 Kadota, K. *et al.* (2004) Essential role of membrane-attack protein in malarial transmission to mosquito host. *Proc. Natl. Acad. Sci. U. S. A.* 101, 16310–16315
8 Kariu, T. *et al.* (2002) MAEBL is essential for malarial sporozoite infection of the mosquito salivary gland. *J. Exp. Med.* 195, 1317–1323
9 Ishino, T. *et al.* (2004) Cell-passage activity is required for the malarial parasite to cross the liver sinusoidal cell layer. *PLoS Biol.* 2, 77–84
10 Ishino, T. *et al.* (2005) A *Plasmodium* sporozoite micronemal protein with a membrane attack complex-related domain is required for breaching the liver sinusoidal cell layer prior to hepatocyte infection. *Cell. Microbiol.* 7, 199–208
11 Kariu, T. *et al.* (2006) CelTOS, a novel malarial protein that mediates transmission to mosquito and vertebrate hosts. *Mol. Microbiol.* 59, 1369–1379
12 Ishino, T. *et al.* (2005) Two proteins with 6-cys motifs are required for malarial parasites to commit to infection of the hepatocyte. *Mol. Microbiol.* 58, 1264–1275
13 Tanabe, K. *et al.* (1987) Allelic dimorphism of a surface antigen gene of the malaria parasite *Plasmodium falciparum*. *J. Mol. Biol.* 195, 273–287
14 Tanabe, K. *et al.* (2000) Selection and genetic drift of polymorphisms within the merozoite surface protein-1 gene of *Plasmodium falciparum*. *Gene* 241, 325–331
15 Sakihama, N. *et al.* (2006) Limited allelic diversity of *Plasmodium falciparum msp1* from populations in the Solomon Islands. *Am. J. Trop. Med. Hyg.* 74, 31–40
16 Tanabe, K. *et al.* (2004) Stable SNPs in malaria antigen genes in isolated populations. *Science* 303, 493
17 Putaporntip, C. *et al.* (2002) Mosaic organization and heterogeneity in frequency of allelic recombination of the *Plasmodium vivax* merozoite surface protein-1 locus. *Proc. Natl. Acad. Sci. U. S. A.* 99, 16348–16353
18 Hisaeda, H. *et al.* (2004) Escape of malaria parasites from host immunity requires CD4$^+$CD25$^+$ regulatory T cells. *Nat. Med.* 10, 29–30
19 Hisaeda, H. *et al.* (2005) Resistance of regulatory T cells to glucocorticoid-induced TNFR family-related protein during *Plasmodium yoelii* infection. *Eur. J. Immunol.* 35, 3516–3524
20 Akira, S. *et al.* (2006) Pathogen recognition and innate immunity. *Cell* 124, 783–801
21 Adachi, K. *et al.* (2001) *Plasmodium berghei* infection in mice induces liver injury by an IL-12- and toll-like receptor/myeloid differentiation factor 88-dependent mechanism. *J. Immunol.* 167, 5928–5934
22 Coban, C. *et al.* (2002) Purified malaria pigment (hemozoin) enhances dendritic cell maturation and modulates the isotype of antibodies induced by a DNA vaccine. *Infect. Immun.* 70, 3939–3943

23 Coban, C. *et al.* (2005) Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin. *J. Exp. Med.* 201, 19–25
24 Krishnegowda, G. *et al.* (2005) Induction of proinflammatory responses in macrophages by the glycosylphosphatidylinositols of *Plasmodium falciparum*: cell signaling receptors, glycosylphosphatidylinositol (GPI) structural requirement, and regulation of GPI activity. *J. Biol. Chem.* 280, 8606–8616
25 Soe, S. *et al.* (2002) *Plasmodium falciparum* serine repeat protein, a new target of monocyte-dependent antibody-mediated parasite killing. *Infect. Immun.* 70, 7182–7184
26 Li, J. *et al.* (2002) Characterization of proteases involved in the processing of *Plasmodium falciparum* serine repeat antigen (SERA). *Mol. Biochem. Parsitol.* 120, 177–186
27 Okech, B. *et al.* (2006) High titers of IgG antibodies against *Plasmodium falciparum* serine repeat antigen 5 (SERA5) are associated with protection against severe malaria in Ugandan children. *Am. J. Trop. Med. Hyg.* 74, 191–197
28 Okech, B.A. *et al.* (2001) Natural human IgG subclass responses to *Plasmodium falciparum* serine repeat antigen (SERA) in Uganda. *Am. J. Trop. Med. Hyg.* 65, 912–917
29 Tsuboi, T. *et al.* (1998) Sequence polymorphism in two novel *Plasmodium vivax* ookinete surface proteins, Pvs25 and Pvs28, that are malaria transmission-blocking vaccine candidates. *Mol. Med.* 4, 772–782
30 Hisaeda, H. *et al.* (2000) Antibodies to malaria vaccine candidates Pvs25 and Pvs28 completely block the ability of *Plasmodium vivax* to infect mosquitoes. *Infect. Immun.* 68, 6618–6623
31 Sattabongkot, J. *et al.* (2003) Blocking of transmission to mosquitoes by antibody to *Plasmodium vivax* malaria vaccine candidates Pvs25 and Pvs28 despite antigenic polymorphism in field isolates. *Am. J. Trop. Med. Hyg.* 69, 536–541
32 Arakawa, T. *et al.* (2003) Serum antibodies induced by intranasal immunization of mice with *Plasmodium vivax* Pvs25 co-administered with cholera toxin completely block parasite transmission to mosquitoes. *Vaccine* 21, 3143–3148
33 Kongkasuriyachai, D. *et al.* (2004) Potent immunogenicity of DNA vaccines encoding *Plasmodium vivax* transmission-blocking vaccine candidates Pvs25 and Pvs28-evaluation of homologous and heterologous antigen-delivery prime-boost strategy. *Vaccine* 22, 3205–3213
34 Kaneko, A. *et al.* (2000) Malaria eradication on islands. *Lancet* 356, 1560–1564
35 Kaneko, A. *et al.* (1998) Malaria epidemiology, glucose 6-phosphate dehydrogenase deficiency and human settlement in Vanuatu archipelago. *Acta Trop.* 70, 285–302
36 Finkelman, F.D. *et al.* (2004) Interleukin-4- and interleukin-13-mediated host protection against intestinal nematode parasites. *Immunol. Rev.* 201, 139–155
37 Nawa, Y. *et al.* (1985) Defective protective capacity of W/W^v mice against *Strongyloides ratti* infection and its reconstitution with bone marrow cells. *Parasite Immunol.* 7, 429–438
38 Matsuda, H. *et al.* (1985) Inability of genetically mast cell-deficient W/W^v mice to acquire resistance against larval *Haemaphysalis longicornis* ticks. *J. Parasitol.* 71, 443–448
39 Kitamura, Y. *et al.* (1978) Decrease of mast cells in W/W^v mice and their increase by bone marrow transplantation. *Blood* 52, 447–452
40 Fukao, T. *et al.* (2002) Selective loss of gastrointestinal mast cells and impaired immunity in PI3K-deficient mice. *Nat. Immunol.* 3, 295–304
41 Maruyama, H. *et al.* (2000) A role of mast cell glycosaminoglycans for the immunological expulsion of intestinal nematode, *Strongyloides venezuelensis*. *J. Immunol.* 164, 3749–3754
42 Lantz, C.S. *et al.* (1998) Role for interleukin-3 in mast-cell and basophil development and in immunity to parasites. *Nature* 392, 90–93
43 Scudamore, C.L. *et al.* (1997) Mast cell heterogeneity in the gastrointestinal tract: variable expression of mouse mast cell protease-1 (mMCP-1) in intraepithelial mucosal mast cells in nematode-infected and normal BALB/c mice. *Am. J. Pathol.* 150, 1661–1672
44 Grencis, R.K. (1997) Th2-mediated host protective immunity to intestinal nematode infections. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 352, 1377–1384
45 Wastling, J.M. *et al.* (1997) Constitutive expression of mouse mast cell protease-1 in normal BALB/c mice and its up-regulation during intestinal nematode infection. *Immunology* 90, 308–313
46 Knight, P.A. *et al.* (2000) Delayed expulsion of the nematode *Trichinella spiralis* in mice lacking the mucosal mast cell-specific granule chymase, mouse mast cell protease-1. *J. Exp. Med.* 192, 1849–1856
47 Miller, H.R. *et al.* (1999) A novel function for transforming growth factor-β 1: upregulation of the expression and the IgE-independent extracellular release of a mucosal mast cell granule-specific β-chymase, mouse mast cell protease-1. *Blood* 93, 3473–3486
48 Nakanishi, K. *et al.* (2001) Interleukin-18 regulates both Th1 and Th2 responses. *Annu. Rev. Immunol.* 19, 423–474
49 Ohkusu, K. *et al.* (2000) Potentiality of interleukin-18 as a useful reagent for treatment and prevention of *Leishmania major* infection. *Infect. Immun.* 68, 2449–2456
50 Wei, X.Q. *et al.* (1999) Altered immune responses and susceptibility to *Leishmania major* and *Staphylococcus aureus* infection in IL-18-deficient mice. *J. Immunol.* 163, 2821–2828
51 Yoshimoto, T. *et al.* (1999) IL-18, although antiallergic when administered with IL-12, stimulates IL-4 and histamine release by basophils. *Proc. Natl. Acad. Sci. U. S. A.* 96, 13962–13966
52 Yoshimoto, T. *et al.* (2000) IL-18 induction of IgE: dependence on $CD4^+$ T cells, IL-4 and STAT6. *Nat. Immunol.* 1, 132–137
53 Yoshimoto, T. *et al.* (2003) Nonredundant roles for CD1d-restricted natural killer T cells and conventional $CD4^+$ T cells in the induction of immunoglobulin E antibodies in response to interleukin 18 treatment of mice. *J. Exp. Med.* 197, 997–1005
54 Tsutsui, H. *et al.* (2004) Induction of allergic inflammation by interleukin-18 in experimental animal models. *Immunol. Rev.* 202, 115–138
55 Galli, S.J. and Hammel, I. (1994) Mast cell and basophil development. *Curr. Opin. Hematol.* 1, 33–39
56 Sasaki, Y. *et al.* (2005) IL-18 with IL-2 protects against *Strongyloides venezuelensis* infection by activating mucosal mast cell-dependent type 2 innate immunity. *J. Exp. Med.* 202, 607–616
57 Takeda, K. *et al.* (1996) Essential role of STAT6 in IL-4 signaling. *Nature* 380, 627–630
58 Takeda, K. *et al.* (1998) Defective NK cell activity and Th1 response in IL-18-deficient mice. *Immunity* 8, 383–390
59 Nawa, Y. *et al.* (1994) Selective effector mechanisms for the expulsion of intestinal helminths. *Parasite Immunol.* 16, 333–338
60 Knight, P.A. *et al.* (2004) Expression profiling reveals novel innate and inflammatory responses in the jejunal epithelial compartment during infection with *Trichinella spiralis*. *Infect. Immun.* 72, 6076–6086
61 Kita, K. *et al. Parasitology in Japan:* Advances in drug discovery and biochemical studies. *Trends Parasitol.* (in press)

Available online at www.sciencedirect.com
ScienceDirect
Molecular & Biochemical Parasitology 156 (2007) 74–79
MOLECULAR & BIOCHEMICAL PARASITOLOGY

Short communication

Recent independent evolution of *msp1* polymorphism in *Plasmodium vivax* and related simian malaria parasites☆

Kazuyuki Tanabe[a,*], Ananias Escalante[b], Naoko Sakihama[a], Masanori Honda[c], Nobuko Arisue[d], Toshihiro Horii[d], Richard Culleton[a], Toshiyuki Hayakawa[a], Tetsuo Hashimoto[e], Shirley Longacre[f], Sisira Pathirana[g], Shiroma Handunnetti[g,h], Hirohisa Kishino[c]

[a] *Laboratory of Malariology, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamada-Oka, Suita, Osaka 565-0871, Japan*
[b] *School of Life Sciences, Arizona State University, Tempe, AZ, USA*
[c] *Graduate School of Agriculture and Life Sciences, University of Tokyo, Tokyo 113-8657, Japan*
[d] *Department of Molecular Protozoology, Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871, Japan*
[e] *Institute of Biological Sciences, University of Tsukuba, Tsukuba 305-8572, Japan*
[f] *Laboratorie de Vaccinologie, Institut Pasteur, Paris Cedex 15, France*
[g] *Malaria Research Unit, Department of Parasitology, Faculty of Medicine, University of Colombo, Colombo, Sri Lanka*
[h] *Institute of Biochemistry, Molecular Biology and Biotechnology, University of Colombo, Colombo, Sri Lanka*

Received 28 February 2007; received in revised form 2 July 2007; accepted 2 July 2007
Available online 10 July 2007

Abstract

The *Plasmodium* MSP-1 is a promising malaria vaccine candidate. However, the highly polymorphic nature of the MSP-1 gene (*msp1*) presents a potential obstacle for effective vaccine development. To investigate the evolutionary history of *msp1* polymorphism in *P. vivax*, we construct phylogenetic trees of *msp1* from *P. vivax* and related monkey malaria parasite species. All *P. vivax msp1* alleles cluster in the *P. vivax* lineage and are not distributed among other species. Similarly, all *P. cynomolgi msp1* alleles cluster in the *P. cynomolgi* lineage. This suggests that, in contrast to presumed ancient origin of *P. falciparum msp1* polymorphism, the origin of *P. vivax msp1* polymorphism is relatively recent. We observed positive selection in the *P. vivax* lineage but not in *P. cynomolgi*. Also, positive selection acts on different regions of *msp1* in *P. vivax* and *P. falciparum*. This study shows that the evolutionary history of *msp1* differs greatly among parasite lineages.

Keywords: Malaria; *Plasmodium vivax*; *Plasmodium cynomolgi*; *msp1*; Polymorphism; Evolution

Plasmodium vivax is the most widely distributed of the four malaria parasites that infect man and a major cause of morbidity in large areas of the world [1]. The 200 kD merozoite surface protein 1 (MSP-1) of *Plasmodium* plays an important role in erythrocyte invasion by the merozoite and is a leading candidate for malaria vaccines [2]. Polymorphism in the gene encoding MSP-1 (*msp1*) is strongly associated with strain-specific immunity, and allele-linked protective immunity has been demonstrated in experimental animals [3,4]. Thus, immune pressures may have favoured the selection of polymorphic alleles of *msp1*, accelerating antigenic diversity. In order to assess how easily the parasite can generate antigenic diversity, investigations of the evolution of current antigen polymorphisms are crucial.

Our recent observations show that single nucleotide polymorphisms (SNPs) in *msp1* of *P. falciparum* are stable for at least 30 years in isolated populations [5]. Additionally, a phylogenetic study of *msp1* from *P. falciparum* and *Plasmodium reichenowi* (a parasite of chimpanzees and the most closely related species to *P. falciparum*) suggested an ancient origin for *msp1* polymorphism at about 27 million years (Myr) ago [6], the time far exceeding the divergence time of the two species 6 Myr

Abbreviations: MSP-1, merozoite surface protein 1; *msp1*, merozoite surface protein 1 gene; nt, nucleotide; aa, amino acid; Myr, million years; ML, maximum likelihood; LRT, likelihood ratio test; TMRCA, time to the most recent common ancestor

☆ *Note*: Nucleotide sequence data reported in this paper have been submitted to DDBJ/EMBL/GenBank with accession numbers: AB266180–AB266196.

* Corresponding author. Tel.: +81 6 6879 4260; fax: +81 6 6879 4262.
E-mail address: kztanabe@biken.osaka-u.ac.jp (K. Tanabe).

0166-6851/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.molbiopara.2007.07.002

ago. However, the ancient origin of *msp1* polymorphism and the persistence of ancestral *msp1* alleles in both species (i.e. trans-species polymorphism) cannot be conclusively proven because comparative polymorphism analysis is impossible due to the existence of only a single isolate of *P. reichenowi*. *P. vivax* also exhibits extensive diversity at the *msp1* locus [7] but the pattern of polymorphism is clearly different to that seen in *P. falciparum* [8,9]. *P. falciparum msp1* can be divided into two major allelic types, designated K1 and MAD20. The diversity between these two major allelic types is very high with amino acid sequence similarity as low as ~50%, but it is limited within each allelic type. However, such clear dimorphism is not apparent for *P. vivax msp1*. It seems likely, therefore, that the evolutionary history of *msp1* polymorphism is different for each of these two parasites.

Recent phylogenetic studies have suggested that *P. vivax* became a parasite of man only relatively recently (when compared to the divergence of *P. falciparum* and *P. reichenowi* 6 Myr ago) as a result of a host switch from an Asian macaque parasite species [10,11]. If the timing of divergence among the *msp1* alleles of *P. vivax* is ancient, prior to *P. vivax* speciation, then several *msp1* allelic types would presumably be shared between *P. vivax* and *P. vivax*-related monkey malaria species. If *msp1* polymorphism in *P. vivax* originated after the host switch, then the polymorphism must have generated relatively rapidly. A comparative phylogenetic analysis would reveal whether the timing of the origin of *msp1* polymorphism is ancient or recent. In the present study, to estimate the time to the most recent common ancestor (TMRCA) of the extant *P. vivax msp1* alleles, we have conducted comparative molecular evolutionary and population genetic analyses of *msp1* sequences from *P. vivax* and seven *P. vivax*-related monkey malaria parasite species, including 11 isolates of *P. cynomolgi*. *P. cynomolgi* is known to be phylogenetically close to *P. vivax* [10], and has been used as an animal model of *P. vivax* for vaccine studies [12].

The macaque malaria parasite species and isolates used to obtain *msp1* sequences were *P. cynomolgi* (11 isolates: Ceylonensis, Gombak, B, M (Mulligan), Langur, Smithsonian, Pt1, Pt2 (Berok), Cambodian, T746 and T824; the latter two from wild toque monkeys in Sri Lanka), *P. simiovale*, *P. fragile* (Hackeri), *P. knowlesi* (Malayan), *P. hylobati*, *P. coatneyi* and *P. inui* (Celebes). Most of the *Plasmodium* species used here have been described previously [10]. The isolates of *P. cynomolgi* underlined above were obtained from the American Type Culture Collection. Both amino acid and nucleotide sequences were aligned using CLUSTAL X [13] with manual corrections. Sequences were divided into conserved and variable blocks (Supplemental Fig. 1) and regions of variable blocks and gaps were excluded from further analysis. Departure from neutrality was analyzed for *msp1* sequences from *P. vivax* and *P. cynomolgi* using several population genetic tests. The mean numbers of synonymous substitutions per synonymous site (dS) and nonsynonymous substitutions per nonsynonymous site (dN) were estimated by the Nei and Gojobori method with the Jukes and Cantor correction using MEGA version 3.1 [14]. Sliding window plot of dN/dS ratios and Tajima's D index [15] were conducted by DnaSP [16]. The McDonald and Kreitman test [17] was applied after calculating the numbers of synonymous and nonsynonymous changes within and between species using DnaSP. Positive selection sites were estimated using the LRT of CODEML program implemented in PAML [18], in which two random sites models, M7 and M8, were used. Positive selection sites were also predicted by the Suzuki and Gojobori's parsimony method implemented in CODEML. The time to the most recent common ancestor (TMRCA) of the *P. vivax msp1* alleles we used was estimated measuring genetic distances between species and within species. Since *msp1* is likely to be under positive selection, we chose only those branches that showed rate constancy using the third position of the codon (codon 3). We tested evolutionary rate constancy using Tajima's relative rate test for the molecular clock hypothesis [19]. Because an appropriate out-group was not available for *P. vivax* and related species (see Fig. 1), we conducted multiple analyses by sequentially assigning each of the species under comparison as an out-group. When lineages showing rate constancy were identified, the genetic distance between them was calculated and compared with the distance between individual *P. vivax* isolates. Calculation methods for TMRCA were described previously [20].

The maximum likelihood (ML) phylogenetic tree of amino acid sequences of *msp1* from human, monkey, rodent and avian *Plasmodium* species identifies four major clades: (i) *P. falciparum* and *P. reichenowi*, (ii) *P. gallinaceum*, (iii) rodent malaria species, *P. yoelii*, *P. chabaudi* and *P. berghei* and (iv) *P. vivax* and related monkey *Plasmodium* species, *P. knowlesi*, *P. coatneyi*, *P. fragile*, *P. hylobati*, *P. inui*, *P. cynomolgi* and *P. simiovale* (Fig. 1A). *P. vivax* is clearly a member of clade iv, being consistent with recent evidence indicating that *P. vivax* is derived from an ancestral macaque malaria parasite via a host switch from macaque to man [10,11]. Except for the close relationship between *P. hylobati* and *P. inui*, the phylogenetic relationships among *P. vivax* and *P. vivax*-related monkey malaria species do not have sufficiently high bootstrap values (<50%). The tree includes four *msp1* alleles from *P. vivax*, which form a monophyletic group (100% bootstrap value), suggesting that the polymorphism observed within this species generated after the speciation of *P. vivax*. Nine isolates of *P. cynomolgi* are clustered in a *P. cynomolgi* group, although bootstrap support is not convincing.

The ML tree based on nucleotide sequences of *msp1* from *P. vivax* and *P. vivax*-related monkey malaria species reveals four distinct sub-clades: (i) *P. knowlesi*, *P. coatneyi* and *P. fragile*, (ii) *P. inui* and *P. hylobati*, (iii) *P. simiovale* and *P. cynomolgi* and (iv) *P. vivax* (Fig. 1B). These four sub-clades radiated from a common ancestor. This nucleotide sequence tree is robust, as compared with the ML tree of amino acid sequences because bootstrap values are very high at all nodes of the four sub-clades. Of the species for which we analyzed more than one isolate, we found no evidence of trans-species polymorphism: all four *P. vivax msp1* alleles cluster in the *P. vivax* lineage and are not distributed among other species. Similarly, all nine *P. cynomolgi msp1* alleles cluster in the *P. cynomolgi* lineage. The two sequences (one of which is partial) of *P. knowlesi* cluster together (data not shown). Average pairwise genetic distance between *P.*

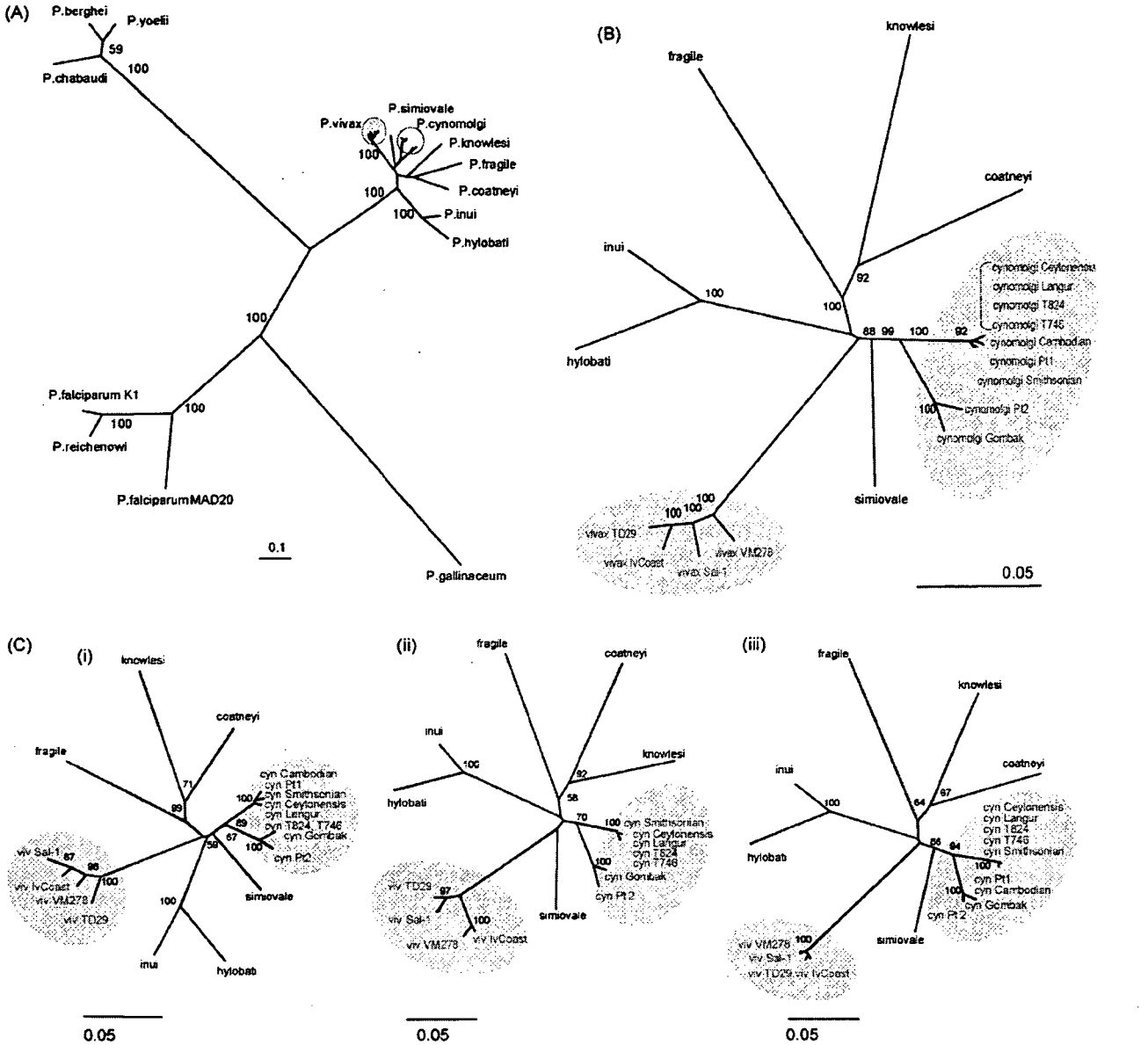

Fig. 1. ML phylogenetic tree of *msp1*. (A) Tree of *msp1* amino acid sequences from human, monkey, rodent and avian *Plasmodium* species. The numbers shown along nodes represent bootstrap values, of which only those of >50% are listed. (B) Tree of *msp1* nucleotide sequences from *P. vivax* and *P. vivax*-related monkey malaria parasite species. This tree was constructed with 1000 heuristic replicates under a GTR + Γ model ($\alpha = 0.58$). The numbers shown along nodes represent bootstrap values, of which only those of >50% are listed. Four *P. vivax* isolates and nine *P. cynomolgi* isolates are colored. (C) Tree of three separate regions of *msp1* nucleotide sequences: (i) the 5′ regions (1687 nt from conserved blocks 1, 3, 5 and 7), (ii) the central region (1083 nt from conserved blocks 9 and 11) and (iii) the 3′ region (1065 nt from conserved blocks 13 and 15). See Supplemental Fig. 1 for the eight conserved blocks. Trees were constructed under a GTR + Γ model ($\alpha = 0.59$) for 5′ region and a TvM + Γ model ($\alpha = 0.59$) for the central and 3′ regions. The numbers shown along nodes represent bootstrap values of which only those of >50% are listed. Abbreviations: viv: *P. vivax* and cyn: *P. cynomolgi*. *Methods*: Parasite genomic DNA was extracted using a QIAamp DNA Blood Mini Kit (QIAGEN, Germany). Complete nucleotide sequences of *msp1* were obtained by direct sequencing of both directions after PCR amplification using primers that covered target regions in both directions (Supplementary Table 2). PCR conditions and sequencing procedures were as previously described [20]. A partial sequence of *P. knowlesi* (from a Thai patient) (GenBank accession #DQ220743), 31 previously reported *P. vivax msp1* sequences [7] and one sequence from Africa (DQ220742) were included for comparison. The *msp1* sequences of other species included here are: *P. falciparum* (K1: X03371 and MAD20: X05624), *P. reichenowi* (AJ786604), *P. gallinaceum* (AJ809338) from a chicken, and three rodent malaria species, *P. chabaudi* (L22982), *P. berghei* (U43521) and *P. yoelii* (J04668). Among 32 *P. vivax* sequences, the four most distantly related alleles (Ivory Coast, TD29, VM278 and Sal-1) were used for phylogenetic reconstruction analysis. The *msp1* sequences of the B, M and Pt1 isolates of *P. cynomolgi* showed complete identity, thus, we used nine *P. cynomolgi msp1* sequences for further analysis. *msp1* tree based on amino acid sequences (1234 sites, Supplemental Fig. 1) was constructed using the ProtTest version 1.3 [27] with 1000 heuristic replicates under a JTT + Γ + F model ($\alpha = 0.97$). Among-site-rate heterogeneity was taken into consideration by using discrete gamma distribution with four categories. Bootstrap analysis was done by applying PROML to 100 re-sampled datasets produced by SEQBOOT program in PHYLIP version 3.6 [28]. Aligned nucleotide sequences of *P. vivax* and *P. vivax*-related monkey malaria species (3834 sites, Supplemental Fig. 1) were analyzed using ML method performed with PAUP* 4.0 β10 [29]. The appropriate nucleotide substitution model was first determined using the Modeltest (version 3.7) estimations [30]. For branch support of the ML tree, bootstrap probability was estimated from 1000 heuristic replicates. All trees were reconstructed with TreeView 1.6.6 [31].

vivax and *P. cynomolgi* sequences was 0.1387 ± 0.0061, whereas that within *P. vivax* and *P. cynomologi* were 0.0238 ± 0.0017 and 0.0314 ± 0.0020, respectively: the interspecific distance is, therefore, much higher than the intraspecific distances, which suggests that the *msp1* polymorphism observed in the two species is not trans-specific. To investigate the possibility that trans-species polymorphism may exist in particular *regions* of *msp1*, we constructed ML trees based on three separate regions of the gene: the 5′ region (1687 bp), the central region (1083 bp) and the 3′ region (1065 bp) (Fig. 1C). Three phylogenetic trees show no evidence for dispersal of alleles across species, confirming that all *msp1* alleles have been generated after the divergence of *P. vivax* and *P. cynomolgi*.

We estimated TMRCA of the *P. vivax msp1* alleles we used. Tajima's relative rate test did not reject the molecular clock hypothesis for a comparison between *P. vivax* and *P. knowlesi* with *P. fragile* as an out-group ($P = 0.551$–1.0 for all 32 *P. vivax* alleles). To calculate the rate of synonymous nucleotide substitution, we used a time frame of 6 Myr as the divergence time between *P. vivax* and *P. knowlesi* (T. Hayakawa, unpublished). The evolutionary rate of the mitochondrial genome is constant among those parasite lineages. The synonymous substitution rate of *msp1* was $(3.08 \pm 0.17) \times 10^{-8}$ per site per year. Using this substitution rate, TMRCA of *P. vivax msp1* was estimated to be $594{,}000 \pm 76{,}000$ (S.E.) years ago. This estimate contradicts the ancient origin of *P. vivax msp1* polymorphism (6 Myr ago) proposed by others, who have assumed the co-speciation of *P. vivax* and *P. knowlesi* with their respective hosts (Hominoidea and Cercopithecoidea Old World monkeys) 20–25 Myr ago [21], the timing not supported by a recent host-parasite co-phylogeny study [11].

We applied five population genetic analyses of neutrality to *msp1* sequences of *P. vivax* and *P. cynomolgi*. Sliding window plot of dN/dS ratios revealed sharp peaks greater than 1.0, a value indicative of positive selection. *P. vivax* showed five such peaks of $dN/dS > 1$ with a window size of 200 bp. No dN/dS peaks of greater than 1.0 were observed for *P. cynomolgi* (Fig. 2A). Notably, the highest dN/dS ratio (5.6) was observed in the part of the gene encoding the N-terminal 33 kD fragment, a product produced after secondary processing of the C-terminal 42 kD region of MSP-1, which is a promising vaccine candidate for both *P. falciparum* and *P. vivax*, but not in the 19 kD region, the other processed product. Those peaks were reproducible after reducing a window size to 100 bp, though three additional peaks and two new peaks appeared in *P. vivax* and *P. cynomolgi*, respectively (data not shown).

Sliding window plot of Tajima's D index revealed six regions with significantly high positive D in *msp1* of *P. vivax* but not in *P. cynomolgi* (Fig. 2B), indicating diversifying selection in *P. vivax msp1*. Two peaks (the fourth and sixth peaks) overlap the third and fourth peaks in the sliding window plot of dN/dS ratio (Fig. 2A), the latter residing in the MSP-1 33 kD fragment. The McDonald and Kreitman test, in which the numbers of synonymous and nonsynonymous substitutions for interspecific fixed differences were compared with intraspecific differences (polymorphisms), detected a significant excess of nonsynonymous polymorphisms within *P. vivax* (Table 1), suggesting positive selection in *P. vivax msp1*. This positive selection is evident in the central and 3′ regions, the latter containing the 33 kD fragment of MSP-1. No excess of nonsynonymous substitution polymorphism was seen in *P. cynomolgi msp1*.

The likelihood ratio test (LRT) of positive selection rejected the null model of neutrality in *P. vivax* lineages ($P < 10^{-5}$) and predicted 51 positively selected amino acid sites in *P. vivax msp1* (Supplementary Table 1). In contrast, the test did not reject the null model for *P. cynomolgi* lineages ($P = 0.08$) and predicted no positive selection sites. The Suzuki and Gojobori's parsimony method predicted five positive selection sites in *P. vivax* lineages

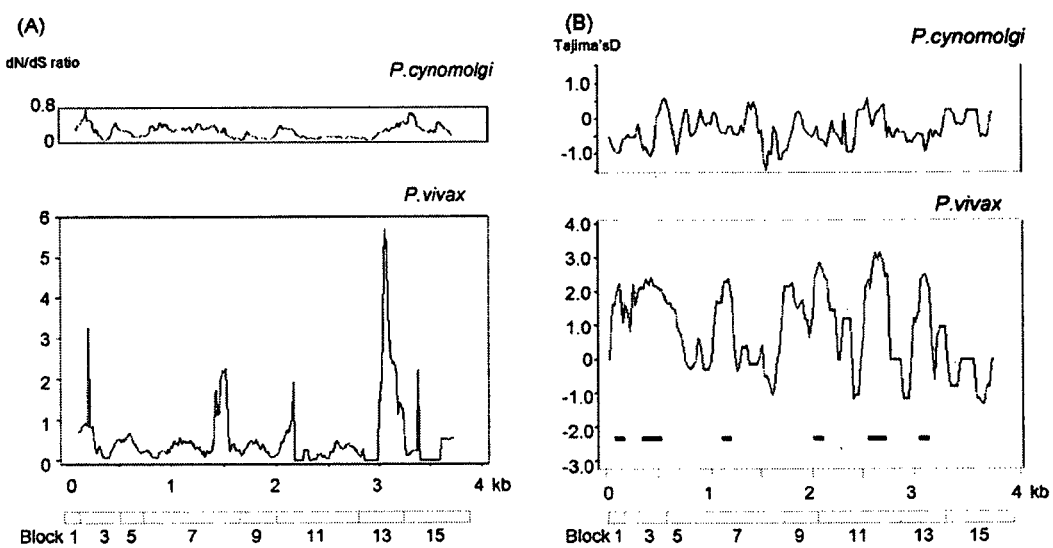

Fig. 2. Diversifying selection in *P. vivax msp1*. (A) Sliding window plot of dN/dS ratios of *msp1* from *P. cynomolgi* and *P. vivax*. A window size of 200 and a step of 10 nucleotides were used. (B) Sliding window plot of Tajima's D index of *msp1* from *P. cynomolgi* and *P. vivax*. A window size of 100 and a step of 25 nucleotides were used. Six regions with significantly high positive D values are marked with bars. Positions of eight conserved blocks used for comparison in (A) and (B) are shown at the bottom. See Supplemental Fig. 1 for the eight conserved blocks.

Table 1
The McDonald and Kreitman test for *msp1* of *P. vivax* and *P. cynomolgi*

Region	Change	Interspecific fixed difference	Intraspecific difference	
			P. vivax	*P. cynomolgi*
Whole gene	Synonymous	152	99	175
	Nonsynonymous	124	128	144
			($P=0.012$)	($P=1.0$)
5′ region (1686 bp)	Synonymous	49	63	73
	Nonsynonymous	50	73	66
			($P=0.525$)	($P=0.646$)
Central region (1083 bp)	Synonymous	50	30	58
	Nonsynonymous	22	38	34
			($P=0.003$)	($P=0.391$)
3′ region (1065 bp)	Synonymous	53	6	44
	Nonsynonymous	52	17	44
			($P=0.034$)	($P=0.947$)

at amino acid positions 197 (with amino acid polymorphism of (T/I/G), 714 (A/K), 1046 (D/T), 1047 (G/A) and 1050 (T/A/E), but no positive selection sites in *P. cynomolgi* (for positions, see Supplemental Fig. 2). These five sites are located in regions with a dN/dS ratio of >2.0 (Fig. 2A), and are included in the 51 sites predicted by the LRT. Importantly, sites 1046, 1047 and 1050 are located in the 33 kD region fragment. Taken together, these results clearly indicate strong positive selection in *P. vivax* but not in *P. cynomolgi*. The G+C content and codon usage did not differ significantly between *P. vivax* and *P. cynomolgi*, thus, excluding the possibility that compositional differences contributed to the detection of positive selection.

At least five positively selected amino acid sites were predicted in *P. vivax msp1*. It is conceivable that the polymorphism at the five sites identified is essential in immune evasion by the parasite. Using a T cell epitope-prediction algorithm (SYFPEITHI software) [22], the prediction of which was consistent with experimentally identified T-cell epitopes of *P. falciparum* circumsporozite protein (data not shown), we observed differential bindings of polymorphic peptides to class II MHC allotypes. For example, a binding of a 15-mer peptide, ETKWKALGAEIEELK (positive selection site at 714 underlined), to DRB1*0101 is substantially reduced by a replacement from A to K with a score from 28 to 20. Conversely, the binding of the peptide to DRB1*1101 is augmented by a replacement from A to K with a score increasing from 17 to 25. These differential bindings were also observed in other polypeptides that included one or more of the positive selection sites (data not shown). Polymorphism in T-cell epitope regions may well enable parasites to escape host immune responses. Consequently, these polymorphisms would be maintained in *P. vivax* populations. Therefore, we consider that host immune responses play a role in maintaining *P. vivax msp1* alleles.

It is notable that the highest peak of dN/dS ratio is located in an *msp1* region encoding the 33 kD fragment, a processed product of the C-terminal 42 kD fragment of MSP-1. The 42 kD and 19 kD fragments are a promising vaccine candidate for both *P. falciparum* and *P. vivax* [23–25]. *P. vivax msp1* shows positive selection in the 33 kD fragment but not in the 19 kD fragment, in which *P. falciparum* shows strong positive selection [26]. Thus, we consider that positive selection acts on different regions of *msp1* in *P. vivax* and *P. falciparum*. Interestingly, three of the five positively selected amino acid sites cluster in the 33 kD fragment of MSP-1, but none of the sites occurs in the C-terminal 19 kD region.

In conclusion, the present study reveals the TMRCA of the extant *P. vivax msp1* alleles is relatively recent, compared with that of *P. falciparum msp1*. We observed strong positive selection in the *P. vivax* lineage but not in *P. cynomolgi*. Also, positive selection acts on different regions of *msp1* in *P. vivax* and *P. falciparum*. This study presents evidence showing that the evolutionary history of *msp1* polymorphism differs greatly among *P. vivax*, *P. falciparum* and *P. cynomolgi*, and highlights the importance of understanding the protective immunity to MSP-1 in a species-specific context.

Acknowledgements

We thank D. Conway for comments, and C. Amaratunga and W. Wijayalath for preparing Toque monkey parasite DNA samples. This work was supported by MEXT (18073013) and JSPS (18390131, 17-05495, 18GS03140013).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molbiopara.2007.07.002.

References

[1] Mendis K, Sina BJ, Marchesini P, Carter R. The neglected burden of *Plasmodim vivax*. Am J Trop Med Hyg 2001;64:97–106.
[2] Holder AA, Guevara Patino JA, Uthaipibull C, et al. Merozoite surface protein 1, immune evasion, and vaccines against asexual blood stage malaria. Parassitologia 1999;41:409–14.
[3] Martinelli A, Cheesman S, Hunt P, et al. A genetic approach to the de novo identification of targets of strain-specific immunity in malaria parasites. Proc Natl Acad Sci USA 2005;102:814–9.

[4] Cheesman S, Raza A, Carter R. Mixed strain infections and strain-specific protective immunity in the rodent malaria parasite *Plasmodium chabaudi chabaudi* in mice. Infect Immun 2006;74:2996–3001.
[5] Tanabe K, Sakihama N, Kaneko A. Stable SNPs in malaria antigen genes in isolated populations. Science 2004;303:493.
[6] Polley SD, Weedal GD, Thomas AW, Golightly LM, Conway DJ. Orthologous gene sequences of merozoite surface protein 1 (MSP-1) from *Plasmodium reichenowi* and *P. gallinaceum* confirm an ancient divergence of *P. falciparum* alleles. Mol Biochem Parasitol 2005;142:25–31.
[7] Putaporntip C, Jongwutiwes S, Sakihama N, et al. Mosaic organization and heterogeneity in frequency of allelic recombination of the *Plasmodium vivax* merozoite surface protein-1 locus. Proc Natl Acad Sci USA 2002;99:16348–53.
[8] Tanabe K, Mackay M, Goman M, Scaife J. Allelic dimorphism in a surface antigen gene of the malaria parasite *Plasmodium falciparum*. J Mol Biol 1987;195:273–87.
[9] Miller LH, Roberts T, Shahabuddin M, McCutchan TF. Analysis of sequence diversity in the *Plasmodium falciparum* merozoite surface protein-1 (MSP-1). Mol Biochem Parasitol 1993;59:1–14.
[10] Escalante AA, Cornejo OE, Freeland D, et al. A monkey's tale: the origin of *Plasmodium vivax* as a human malaria parasite. Proc Natl Acad Sci USA 2005;102:1980–5.
[11] Mu J, Joy DA, Duan J, et al. Host switch leads to emergence of *Plasmodium vivax* malaria in humans. Mol Biol Evol 2005;22:1686–93.
[12] Lakshman Perera KLR, Handunneti SM, Holm I, Longacre S, Mendis K. Baculovirus merozoite surface protein 1 C-terminal recombinant antigens are highly protective in a natural primate model for human *Plasmodium vivax* malaria. Infect Immun 1998;66:1500–6.
[13] Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl Acids Res 1997;24:4876–82.
[14] Kumar S, Tamura K, Nei M. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform 2004;5:150–63.
[15] Tajima F. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 1989;123:585–95.
[16] Rozas J, Sanchez-DelBarrio JC, Messeguer X, Rozas R. DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinform 2003;19:2496–7.
[17] McDonald JH, Kreitman M. Adaptive protein evolution at the *Adh* locus in *Drosophila*. Nature 1991;351:652–4.
[18] Yang Z. PAML: a program package for phylogenetic analysis by maximum likelihood. Comput Appl Biosci 1997;13:555–6.
[19] Tajima F. Simple methods for testing the molecular evolutionary clock hypothesis. Genetics 1993;135:599–607.
[20] Tanabe K, Sakihama N, Hattori T, et al. Genetic distance in housekeeping genes between *Plasmodium falciparum* and *Plasmodium reichenowi* and within *P. falciparum*. J Mol Evol 2004;59:687–94.
[21] Putaporntip C, Jongwutiwes S, Iwasaki T, Kanbara H, Hughes AL. Ancient common ancestry of the merozoite surface protein 1 of *Plasmodium vivax* as inferred from its homologue in *Plasmodium knowlesi*. Mol Biochem Parasitol 2005;146:105–8.
[22] Rammensee H-G, Bachmann J, Emmerich NN, Bachor OA, Stevanovic S. SYFPEITHI: database for MHC ligands and peptide motifs. Immunogenetics 1999;50:213–9.
[23] Collins WE, Kaslow DC, Sullivan JS, et al. Testing the efficacy of a recombinant merozoite surface protein (MSP-119) of *Plasmodium vivax* in *Saimiri boliviensis* monkeys. Am J Trop Med Hyg 1999;60:350–6.
[24] Chang SP, Case SE, Gosnell WL, et al. A recombinant baculovirus 42-kilodalton C-terminal fragment of *Plasmodium falciparum* merozoite surface protein 1 protects *Aotus* monkeys against malaria. Infect Immun 1996;64:253–61.
[25] Kumar S, Yadava A, Keister DB, et al. Immunogencity and in vivo efficacy of recombinant *Plasmodium falciparum* merozoite surface protein-1 in *Aotus* monkeys. Mol Med 1995;1:325–32.
[26] Pacheco MA, Poe AC, Collins WE, Lal AA, Tanabe K, et al. A comparative study of the genetic diversity of the 42 kDa fragment of the merozoite surface protein 1 in *Plasmodium falciparum* and *P. vivax*, Inf Gen Evol 7:180–187.
[27] Abascal F, Zardoya R, Posada D. ProtTest: selection of best-fit models of protein evolution. Bioinform 2005;21:2104–5.
[28] Felsenstein J. PHYLIP (Phylogeny Inference Package) version 3.6. Seattle: Department of Genome Sciences, University of Washington; 2004.
[29] Swofford DL. PAUP*. version 4. Sunderland, Masacchusetts: Sinauer Associates; 2002.
[30] Posada D, Crandall KA. MODELTEST: testing the model of DNA substitution. Bioinform 1998;14:817–8.
[31] Page RDM. TreeView: an application to display phylogenetic trees on personal computers. Comput Appl Biosci 1996;12:357–8.

Available online at www.sciencedirect.com
ScienceDirect
Molecular & Biochemical Parasitology 158 (2008) 11–21
MOLECULAR & BIOCHEMICAL PARASITOLOGY

Diversity and evolution of the *rhoph1/clag* multigene family of *Plasmodium falciparum*☆

Hideyuki Iriko[a,b,1], Osamu Kaneko[a,*], Hitoshi Otsuki[a], Takafumi Tsuboi[b,c], Xin-zhuan Su[d], Kazuyuki Tanabe[e], Motomi Torii[a]

[a] *Department of Molecular Parasitology, Ehime University Graduate School of Medicine, Shitsukawa, Toon, Ehime 791-0295, Japan*
[b] *Venture Business Laboratory, Ehime University, Matsuyama, Ehime 790-8577, Japan*
[c] *Cell-free Science and Technology Research Center, Ehime University, Matsuyama, Ehime 790-8577, Japan*
[d] *Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA*
[e] *Laboratory of Malariology, International Research Center for Infectious Diseases, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565-0871, Japan*

Received 4 June 2007; received in revised form 5 November 2007; accepted 6 November 2007
Available online 17 November 2007

Abstract

A complex of high-molecular-mass proteins (*Pf*RhopH) of the human malaria parasite *Plasmodium falciparum* induces host protective immunity and therefore is a candidate for vaccine development. Understanding the level of polymorphism and the evolutionary processes is important for advancements in both vaccine design and knowledge of the evolution of cell invasion in this parasite. In the present study, we sequenced the entire open reading frames of seven genes encoding the proteins of the *Pf*RhopH complex (*rhoph2*, *rhoph3*, and five *rhoph1/clag* gene paralogs). We found that four *rhoph1/clag* genes (*clag2*, *3.1*, *3.2*, and *8*) were highly polymorphic. Amino acid substitutions and indels are predominantly clustered around amino acid positions 1000–1200 of these four *rhoph1/clag* genes. An excess of nonsynonymous substitutions over synonymous substitutions was detected for *clag8* and *9*, indicating positive selection. The McDonald–Kreitman test with a *Plasmodium reichenowi* orthologous sequence also supports positive selection on *clag8*. Based on the ratio of interspecific genetic distance to intraspecific distance, the time to the most recent common ancestor of the *clag2* and *8* polymorphisms was estimated to be 1.89 and 0.87 million years ago, respectively, assuming divergence of *P. falciparum* and *P. reichenowi* 6 million years ago. In addition to a copy number polymorphism, gene conversion events were detected for the *rhoph1/clag* genes on chromosome 3, which likely play a role in increasing the diversity of each locus. Our results indicate that a high diversity of the *Pf*RhopH1/Clag multigene family is maintained by diversifying selection forces over a considerably long period.

Keywords: Gene conversion; Malaria; Polymorphism; Rhoptry; Selection

1. Introduction

Malaria infects more than 300 million people and kills 1–2 million each year. Efforts have been made to develop effective malaria vaccines, but none is available so far. Malaria is caused by the obligate intracellular protozoan *Plasmodium* parasites; and entry into erythrocytes is prerequisite for the growth in the mammalian host. After contact with the erythrocyte surface, parasite discharge the content of the microorganelles called the micronemes to establish a tight junction with the erythrocyte surface molecules; parasite then invaginates into a nascent parasitophorous vacuole (PV) [1,2]. During formation of the PV, the parasite discharges the contents of another pair of microorganelles, the rhoptries [3]. The molecules located within these

Abbreviations: aa, amino acid; chr, chromosome; *clag*, cytoadherence-linked asexual gene(s); CI, confidence interval; mya, million years ago; nt, nucleotide; ORF, open reading frame(s); PCR, polymerase chain reaction; PV, parasitophorous vacuole; PVM, PV membrane; S.E., standard error; TMRCA, time to the most recent common ancestor; UTR, untranslated region(s).

☆ *Note:* Sequence data from this article have been deposited with the GenBank™/EMBL/DDBJ databases under accession numbers AB250801–AB250912.

* Corresponding author. Present address: Department of Protozoology, Institute of Tropical Medicine, Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan. Tel.: +81 95 819 7838; fax: +81 95 819 7805.

[1] Current address: Division of Medical Zoology, Department of Microbiology and Immunology, Faculty of Medicine, Tottori University, Yonago, Tottori 683-8503, Japan.

0166-6851/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.molbiopara.2007.11.004

organelles play a key role in erythrocyte invasion and have been studied as vaccine targets, with the aim to induce antibodies to block invasion. One erythrocyte-binding molecule in the rhoptry is a complex of high-molecular-mass proteins called the RhopH complex [4,5]. The RhopH complex is distributed throughout the erythrocyte and PV membrane (PVM) and has been detected in ring-stage parasites [6], suggesting an important role during PV establishment. The importance of the complex has further been emphasized by the failure of attempts to disrupt the *pfrhoph3* gene locus, suggesting its necessity for parasite survival [7].

The RhopH complex comprises three distinct components: RhopH1, RhopH2, and RhopH3 [8–12]. The genes encoding RhopH1 are members of the *rhoph1/clag* gene family, which was originally defined by the cytoadherence-linked asexual gene (*clag*) on chromosome 9 in *Plasmodium falciparum* (*clag9*) and consists of at least three members; *clag2*, *3.1*, and *9* [13–15]. Although not yet determined experimentally, molecules encoded by *clag3.2* and *8* are likely parts of the RhopH complex as judged by their similarity in amino acid sequence and transcription pattern with other members [15]. Because only one RhopH1/Clag participates to form a single RhopH complex [15,16], five types of *Pf*RhopH complex are expected to exist, each of which contains one *rhoph1/clag* gene product. In this report we employ 'RhopH1/Clag' (protein) and '*rhoph1/clag*' (gene) as the family name, and 'Clag' (protein) and '*clag*' (gene) for each member.

Erythrocyte-binding proteins discharged from *P. falciparum* merozoites are considered to be targets of host immune responses. Strong diversifying selections on microneme proteins have been detected (e.g., AMA-1 and EBA-175), suggesting that polymorphism of these proteins has been maintained to evade host immunity in parasite populations [17,18]. Antibodies against the *Pf*RhopH complex partially inhibit the growth of *P. falciparum* in vitro and in vivo, consistent with its potential as a vaccine target [19–21]. Although the RhopH complex has been shown to induce host protective immunity and is likely to be under host immune pressure, the genetic diversity and immunologic characteristics of this complex are not fully understood. Here, we analyzed sequence polymorphism in five *rhoph1/clag* members, *rhoph2*, and *rhoph3* and show that some of the *rhoph* genes are under positive/diversifying selection. In addition, we assessed a population genetic mechanism that might drive the evolution of the *rhoph1/clag* multigene family.

2. Materials and methods

2.1. Malaria parasites

All cloned lines of *P. falciparum* were maintained in vitro, essentially as described previously [22]. The parasite lines examined originated from Southeast Asia (Dd2, FVO, Camp, T9/96, T9/102, K1, and Thai838), Papua New Guinea (MAD20), Central and South America (HB3, 7G8, DIV17, DIV29, DIV30, PC49, PC54, Santa Lucia, and Haiti), and Africa (RO33, 123/5, 128/4, SL/D6, LF4/1, 102/1, M2, M5, Fab9, 713, P13, and KMWII) and have been previously described [23–25]. Their geographic origins have also been previously described [26].

2.2. DNA and RNA isolation

Genomic DNA was obtained as described previously [24]. Total RNA was isolated from schizont stage-enriched HB3 and Dd2 parasite lines using the RNeasy mini kit (Qiagen, Valencia, CA). Complementary DNA was synthesized using random hexamers and an Omniscript reverse transcription kit (Qiagen) after DNase treatment.

2.3. Polymerase chain reaction (PCR) amplification and sequencing

Nucleotide sequences corresponding to open reading frame (ORF) were determined for five *pfrhoph1/clag* genes, *rhoph2*, and *rhoph3* in four parasite lines: Dd2, HB3, 7G8, and FVO. DNA fragments were PCR amplified with KOD-Plus DNA polymerase (Toyobo, Japan) using a panel of oligonucleotides specific for the genes (Supplemental Table 1) and sequenced directly using an ABI PRISM® 310 genetic analyzer (Applied Biosystems, Foster City, CA) or sequenced after cloning into pGEM-T Easy® plasmid (multiple plasmid clones sequenced for each DNA fragment; Promega, Madison, WI). To PCR amplify DNA fragments including the entire ORF of *clag3.1* or *3.2*, LA *Taq* DNA polymerase (TaKaRa, Japan) was used with oligonucleotide primers 3.1F (5′-TGTGCAATATATCAAAGTGTACATGC-3′) and 3.1R (5′-TAGAAAATATTAGAATTGCTATTATGTAC-3′) or 3.2F (5′-AATAGTTGAGTACGCACTAATATGTC-3′) and 3.2R (5′-ACACAAATTCTTAATAATTATATAAAACC-3′), respectively. A highly polymorphic region identified in *clag2*, *3.1*, *3.2*, and *8* in this study was further analyzed by increasing the number of parasite lines ($n=25$) from different geographic areas.

2.4. Plasmodium reichenowi sequences

A TBLASTN search was performed against the *P. reichenowi* preliminary genome shotgun database (Dennis strain; Sanger Centre, UK) using Clag2, 3.1, 3.2, and 8 amino acid sequences as queries. For *prclag2* and *prclag8*, sequences were assembled using SeqMan II accompanied with Lasergene software (DNASTAR Inc., Madison, WI) with manual corrections. Regions covered by at least two independent reads and showing identical sequences were selected and used for analysis (Supplemental Figs. S1 and S2). The generated sequences were 3273 bp long for *prclag2*, corresponding to nucleotide (nt) positions 193–870, 1021–1902, 2458–3432, and 3448–4185 of *pfclag2* (3D7), and 2175 bp long for *prclag8*, corresponding to nt positions 1459–4173 of *pfclag8* (3D7). For Clag3 orthologs in the *P. reichenowi* genome, only sequences possessing homology with the 5′ untranslated region (UTR) (reich908g11.plk) or 3′ UTR (reich1194c08.plk and reich289f06.qlk) were used.

2.5. Sequence alignment and analysis

The entire ORFs for the 7 *Pf*RhopH complex-related genes (5 *rhoph1/clag* genes, *rhoph2*, and *rhoph3*) in four culture-adapted

P. falciparum lines—Dd2 (Southeast Asia), 7G8 (Brazil), HB3 (Honduras), and FVO (Vietnam)—were aligned with those retrieved from a genome database (3D7 line, presumably African in origin) using a CLUSTAL W program [27] with manual corrections; nucleotide diversity (π) and its standard error (S.E.) were computed with the Jukes and Cantor method using MEGA 3.1 software [28] after excluding insertions/deletions (indels) and highly polymorphic regions. The mean numbers of synonymous substitutions per synonymous site (d_S) and nonsynonymous substitutions per nonsynonymous site (d_N) and their standard errors were computed using the Nei and Gojobori method [29] with the Jukes and Cantor correction, implemented in MEGA 3.1. The statistical difference between d_S and d_N was tested using a one-tailed Z-test with 500 bootstrap pseudosamples using MEGA 3.1. A value of d_N significantly higher or lower than d_S at the 95% confidence level was taken as evidence for positive or purifying selection, respectively. The d_N:d_S ratio was evaluated using a sliding window method (50 bases with a step size of 10 bases) in DnaSP 4.0 [30]. Positive selection was also evaluated using the McDonald–Kreitman test [31]. Before estimating the time to the most recent common ancestor (TMRCA) for *P. falciparum clag2* and *8* polymorphism, the evolutionary rate constancy of *clag2* and *8* between *P. falciparum* and *P. reichenowi* was validated using a *Plasmodium yoelii* ortholog *Py*RhopH1A (accession number AB060734) as an outgroup using Tajima's relative rate test [32] implemented in MEGA 3.1. Mean and 95% confidence intervals (CI) for estimated TMRCA were computed based on the model assuming the distribution of the distance and the substitution rate were Gamma-distributed [33]. Gene conversion was evaluated for each exon using an algorithm by Betrán et al. [34] implemented in DnaSP 4.0.

Unrooted dendrograms of the *pfrhoph1/clag* members were constructed using the neighbor-joining and maximum parsimony methods in MEGA 3.1, and Tajima's relative rate test was used to evaluate the evolutionary rate among members. Indels and highly polymorphic regions could not be satisfactorily aligned and were therefore excluded from the analysis. The sequences (3D7 parasite line) used to construct trees and the evolutionary rate were as follows: nt positions 154–312, 331–573, 727–1122, 1207–1266, 1324–1560, 1609–2988, 3004–3288, and 3382–3924 for *clag3.2*; nt positions 160–318, 337–579, 733–1128, 1213–1272, 1330–1566, 1615–2994, 3010–3294, and 3388–3930 for *clag3.1*; nt positions 223–381, 400–642, 799–1194, 1279–1338, 1390–1626, 1696–3075, 3091–3375, and 3553–4095 for *clag2*; nt positions 130–288, 307–549, 706–1101, 1186–1245, 1300–1536, 1606–2985, 3001–3285, and 3415–3957 for *clag8*; and nt positions 82–240, 265–507, 652–1047, 1132–1191, 1276–1512, 1582–2961, 2977–3261, and 3394–3936 for *clag9*.

3. Results

3.1. Polymorphism of the PfRhopH complex-related genes

All seven *Pf*RhopH complex-related genes showed greater nucleotide diversity levels than the average (+2 S.E.) of 204 ORFs on *P. falciparum* chromosome (chr) 3 [35] (Table 1). Among the seven genes, *clag2*, *3.1*, *3.2*, and *8* are highly polymorphic with nucleotide diversity ($\pi = 0.0053$–0.0164) comparable to malaria vaccine candidate antigen protein genes such as *eba-175* ($\pi = 0.0030$) and *ama-1* ($\pi = 0.0166$) [17,18]. The observed nucleotide diversity levels of *clag2*, *3.1*, and *3.2* should be taken as minimum estimates, because indels and highly polymorphic regions were excluded from this analysis to obtain reliable alignments. The highly polymorphic nature of four *rhoph1/clag* genes at the nucleotide level extends to the amino acid level, which is represented by high d_N values (Table 1). Thus, the genes encoding RhopH1/Clag are more polymorphic than RhopH2 and RhopH3.

Among the four RhopH1/Clag showing high polymorphism (Clag2, 3.1, 3.2, and 8), the majority of polymorphic sites are clustered in a region at amino acid (aa) positions 1000–1200

Table 1
Nucleotide diversity of the *Pf*RhopH complex genes[a]

Gene	*n*	Indel	Sites	π	π (S.E.)	d_N	d_N (S.E.)	d_S	d_S (S.E.)	d_N/d_S	P^b
clag2[c]	5	(+)	4,317	0.0053	(0.0008)	0.0032	(0.0007)	0.0133	(0.0028)	0.24	(0.0003)
clag3.1[c]	5	(+)	4,140	0.0164	(0.0015)	0.0062	(0.0011)	0.0582	(0.0058)	0.11	($<10^{-10}$)
clag3.2[c]	5	(+)	4,134	0.0138	(0.0011)	0.0063	(0.0011)	0.0445	(0.0050)	0.14	($<10^{-10}$)
clag8	5	(−)	4,182	0.0066	(0.0007)	0.0065	(0.0011)	0.0069	(0.0020)	0.94	ns
clag9	5	(−)	4,020	0.0009	(0.0003)	0.0011)	(0.0004)	0.0000	(0.0000)	∞	0.002
rhoph2	5	(−)	4,134	0.0009	(0.0003)	0.0010	(0.0004)	0.0005	(0.0005)	2.00	ns
rhoph3	5	(−)	2,691	0.0013	(0.0004)	0.0012	(0.0005)	0.0015	(0.0010)	0.80	ns
clag2[d]	24	(+)	522	0.0131	(0.0032)	0.0114	(0.0042)	0.0192	(0.0074)	0.60	ns
clag8[d]	26	(−)	585	0.0267	(0.0042)	0.0305	(0.0060)	0.0132	(0.0061)	2.31	0.020
Chr 3[e]	5		202,069	0.00044	(0.00006)	0.00039	(0.0060)	0.00068	(0.00010)	0.57	

[a] *n*, Number of sequences sampled; sites, sites analyzed excluding noncoding sequences and alignment gaps; π, pairwise nucleotide diversity; d_N, number of nonsynonymous substitutions over numbers of nonsynonymous sites; d_S, number of synonymous substitutions over numbers of synonymous sites; S.E., standard error computed using the Nei-Gojobori method with Jukes-Cantor correction. S.E. was estimated using the bootstrap method with 500 replication.

[b] *P*-value indicates that d_N is significantly greater than d_S. Those shown in parenthesis indicate that d_S are significantly greater than d_N. The statistical difference between d_S and d_N was tested using an one-tail Z-test with 500 bootstrap pseudosamples implemented in MEGA 3.1. ns indicate not significant ($P > 0.05$).

[c] For optimal sequence alignment, nt 3433–3435 was excluded from *clag2*, nt 3337–3447 from *clag3.1*, and nt 88–99 and 3343–3444 from *clag3.2* for the analysis. Nucleotide numbering are after the 3D7 line sequences.

[d] nt 3022–3606 of *clag8* and nt 3106–3420 and 3436–3642 of *clag2* were used.

[e] Data from 204 ORF on *P. falciparum* chr 3 using five parasite lines [35].