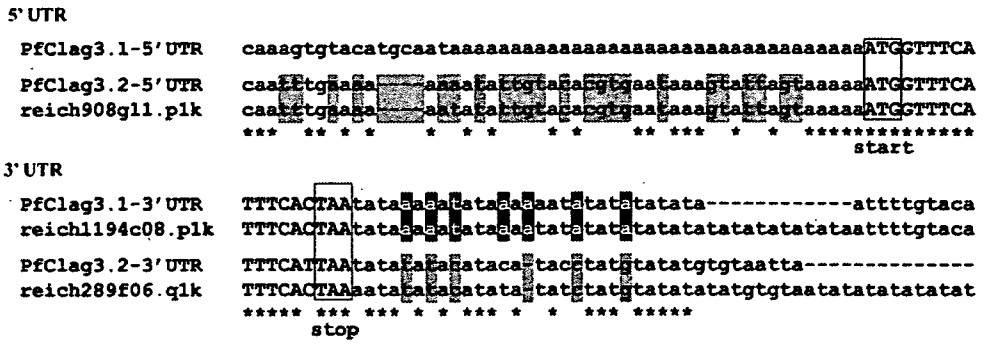

Fig. 6. Nucleotide sequence alignment of the 5′ and 3′ UTRs for *P. falciparum clag3.1* and *3.2* genes and *P. reichenowi* orthologous sequences. A *P. reichenowi* sequence possessing homology with *pfclag3.1* was not found in the current database. Nucleotide sequences corresponding to the ORF and the UTR are shown with upper case and lower case letters, respectively. Putative start and stop codons are boxed. Characteristic nucleotides are displayed in reverse (*clag3.1*) or masked with gray (*clag3.2*).

To date, observation of such high levels of polymorphism for *clag2*, *3.1*, *3.2*, and *8* ($\pi = 0.0053$–0.0164; $d_N = 0.0032$–0.0065) has not been reported for other known malaria rhoptry protein genes. The high polymorphism observed in *clag2*, *3.1*, and *3.2* is consistent with the observation by Kidgell et al. [40] based on the hybridization of genomic DNA from a panel of parasite lines to an oligonucleotide array for the *P. falciparum* genome. In addition, the polymorphism levels are comparable to those of the microneme proteins such as *eba-175* ($\pi = 0.0030$; $d_N = 0.0037$) and *ama-1* ($\pi = 0.0166$; $d_N = 0.0207$), which are exposed to host immune responses [17,18]. Rhoptry proteins are released into the PV and are considered to be minimally exposed to host immunity. If RhopH1/Clag polymorphism is generated by host immune pressure, the questions arises as to how RhopH1/Clag is exposed to host immunity. There are a few possible explanations. First, RhopH1/Clag may be released from the merozoites before attachment to the erythrocyte surface, thereby becoming a target of host immunity. Second, the RhopH complex, which is released into PVs, may be leaked to the surface of infected erythrocytes through the junction between invading parasite and the erythrocyte membrane. Leaked RhopH complex, and therefore parasite-infected erythrocytes, are then potential targets of host immunity. Indeed, the *Pf*RhopH complex and rhoptry-associated protein 2 (RAP-2, RSP-2), another malaria rhoptry protein, have been detected on the erythrocyte surface upon parasite attachment to erythrocytes [41,42]. The last possibility is that RhopH1/Clag, after release into the PV, may be distributed to the parasite-derived membranous network (i.e., Maurer's clefts) in the erythrocyte cytosol, where it is exposed to host immunity. RhopH2 and RhopH3 have recently been observed in materials deriving from Maurer's cleft by proteome analyses, consistent with this possibility [43,44].

There are no obvious associations between particular haplotypes and their geographic origins, and most haplotypes co-exist in different geographic areas, similar to other known polymorphic antigens such as MSP-1 [45,46]. RhopH1/Clag polymorphism might be maintained in natural parasite populations to evade host immunity. Using a T-cell epitope prediction algorithm (SYFPEITHI software) [47], we found that binding of a predicted T-cell epitope peptide to a particular HLA allotype was dramatically affected by the RhopH1/Clag polymorphism in silica. For example, aa positions 1094–1108 of 3D7 line Clag8 (KRISTSIDHISGGKW) was predicted as a T-cell epitope of HLA-DRB1*1101 with a score of 22, but the score for the corresponding region of Camp line Clag8 (MRISSTSTYISNNEW) was 0, emphasizing a potential involvement in immune evasion of RhopH1/Clag polymorphism. We consider the algorithm useful because the score of HLA-DRB1*0701 for the *Pf*CSP Th2R domain, a well characterized malaria polymorphic T-cell epitope peptide, is 22 for the K1 parasite line (KIQYSLSTEWSPCSV) but only 12 for that of the 3D7 line (KIQNSLSTEWSPCSV).

4.2. Evolution of PfRhopH1/Clag polymorphism of the extant P. falciparum population

Gene conversion has been reported for other *P. falciparum* loci, such as *falcipain 2* [48] and *var* [49], as a source of genetic diversity. In this study, we show that *clag3.1/3.2*, which interchange their sequences by gene conversion, evolved more rapidly than *clag2*. The precise function of the RhopH complex remains unknown, and thus whether the gene conversion observed was functionally advantageous or neutral is also unknown; however, gene conversion can be a mechanism for antigenic variation to evade host immunity. Some examples include the *vsg* gene *of Trypanosoma brucei*, causative agent of African sleeping sickness, the *ves* gene of a cattle parasite *Babesia bovis* [50], and *var* genes in *P. falciparum* [51].

Based on the shared hybridization pattern between *clag3.1* and *3.2*, Chung et al. [36] proposed that these genes are alleles of the same locus; however, because the origin of two *rhoph1/clag* loci on chr 3 appear to predate the *P. falciparum–P. reichenowi* divergence, these should be categorized as paralogous genes but not the same gene. Shared features between these loci detected by Southern blot hybridization by Chung et al. can be simply explained by the gene conversion identified in this study. *clag3h* could be generated by an unequal crossover between *clag3.1* and *3.2* of a set of chromosomes during meiosis. If such crossover had occurred, parasite lines possessing three *rhoph1/clag* on chr 3 would be expected, with a molecule having its 5′ end derived from *clag3.1* and its 3′ end from *clag3.2* (Supplemental Fig. S5, model 1); however, this type of *rhoph1/clag* was not detected in this study. KMWII line appears to possess 3 *rhoph1/clag*

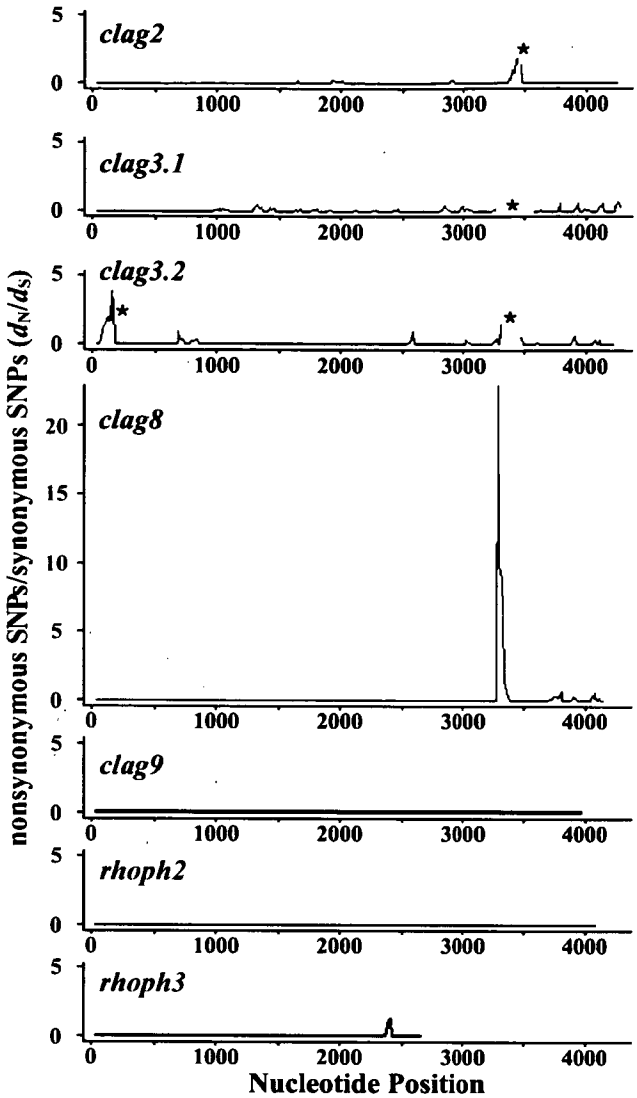

Fig. 7. Sliding window plot of d_N/d_S ratio for seven genes of the *Pf*RhopH complex. For optimum sequence alignment, nt positions 3433–3435 was excluded from *clag2*, nt positions 3337–3447 from *clag3.1*, and nt positions 88–99 and 3343–3444 from *clag3.2* for the analysis (asterisks), because these regions were highly polymorphic with indels. Nucleotide numbers are those of 3D7 line sequences. Window length is 50 bp, and step size is 10 bp.

genes on chr 3, but the third *rhoph1/clag* on chr 3 appears not to be generated by the mechanism described above, because this *rhoph1/clag* was obviously a duplicated *clag3.1* gene amplified with the *clag3.1*-specific primer set. Thus, *clag3h* is more likely a product of a recombination event between *clag3.1* and *3.2* on the same chromosome (Supplemental Fig. S5, model 2). Because a unique amino acid of Clag3H (e.g., Ala at aa position 1116) suggests a relatively old origin of *clag3h*, recombination events between *clag3.1* and *3.2* might be rare in the natural population.

Four highly polymorphic *rhoph1/clag* genes contained unexpectedly large numbers of synonymous substitutions. Based on the ratio of interspecific distance to intraspecific distance, the TMRCA of the polymorphism of *P. falciparum clag2* and *8* were estimated to be 1.89 (95% CI, 1.02–3.18) and 0.87 (95% CI, 0.42–1.54) mya, respectively. Although there is still controversy surrounding its accuracy, TMRCA of the extant *P. falciparum* population was estimated to be approximately 0.1–0.2 mya based on the genetic distance in nuclear genome housekeeping genes between *P. falciparum* and *P. reichenowi* ([52], Tanabe, unpublished data). Thus, polymorphism of *clag2* and *8* appears to be generated between the divergence of *P. falciparum* and *P. reichenowi* and TMRCA of the extant *P. falciparum* populations. Early origins of the polymorphism have been suggested for merozoite surface proteins *Pf*MSP-1 and *Pf*MSP-2, for which the origin of the polymorphism was proposed to predate the *P. falciparum–P. reichenowi* divergence (thus termed 'ancient origin'), or TMRCA of the extant *P. falciparum* population, respectively [53,54]. Early origins of the polymorphism older than TMRCA of extant *P. falciparum* populations would suggest that *rhoph1/clag* polymorphisms confer an advantage to the parasite and were positively selected for during the recent evolution of *P. falciparum*.

In summary, four factors appear to affect current *rhoph1/clag* polymorphism; (i) older origin than TMRCA of the extant *P. falciparum* population; (ii) gene conversion and (iii) copy number polymorphism for *rhoph1/clag* on chr 3; and (iv) positive diversifying selection. Multigene families play important roles in many aspects of malaria biology, e.g., responsibility for redundancy of erythrocyte invasion or antigenic variation of parasite-infected erythrocytes. Given the abundance of multigene families in the *P. falciparum* genome [55], combination of the mechanisms described in this study can be a powerful driving force to generate high biologic redundancy for parasite survival.

Acknowledgements

We thank NIAID intramural editor Brenda Rae Marshall for assistance. Sequence data of *P. reichenowi* were obtained from The Sanger Centre website (http://www.sanger.ac.uk/Projects/P_reichenowi/). This work was supported in part by Grants-in-Aid for Scientific Research 15406015 (to MT), 18390131 (to KT), and 17590372 and 17406009 (to OK) from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and by the Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, USA (to XS).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molbiopara.2007.11.004.

References

[1] Cowman AF, Crabb BS. Invasion of red blood cells by malaria parasites. Cell 2006;124:755–66.
[2] Kaneko O. Erythrocyte invasion: vocabulary and grammar of the *Plasmodium* rhoptry. Parasitol Int 2007;56:255–62.
[3] Bannister LH, Mitchell GH, Butcher GA, Dennis ED. Lamellar membranes associated with rhoptries in erythrocytic merozoites of *Plasmodium knowlesi*: a clue to the mechanism of invasion. Parasitology 1986;92:291–303.

[4] Sam-Yellowe TY, Perkins ME. Interaction of the 140/130/110 kDa rhoptry protein complex of *Plasmodium falciparum* with the erythrocyte membrane and liposomes. Exp Parasitol 1991;73:161–71.
[5] Rungruang T, Kaneko O, Murakami Y, et al. Erythrocyte surface glycosylphosphatidyl inositol anchored receptor for the malaria parasite. Mol Biochem Parasitol 2005;140:13–21.
[6] Lustigman S, Anders RF, Brown GV, Coppel RL. A component of an antigenic rhoptry complex of *Plasmodium falciparum* is modified after merozoite invasion. Mol Biochem Parasitol 1988;30:217–24.
[7] Cowman AF, Baldi DL, Duraisingh M, et al. Functional analysis of proteins involved in *Plasmodium falciparum* merozoite invasion of red blood cells. FEBS Lett 2000;476:84–8.
[8] Holder AA, Freeman RR, Uni S, Aikawa M. Isolation of a *Plasmodium falciparum* rhoptry protein. Mol Biochem Parasitol 1985;14:293–303.
[9] Brown HJ, Coppel RL. Primary structure of a *Plasmodium falciparum* rhoptry antigen. Mol Biochem Parasitol 1991;49:99–110.
[10] Shirano M, Tsuboi T, Kaneko O, Tachibana M, Adams JH, Torii M. Conserved regions of the *Plasmodium yoelii* rhoptry protein RhopH3 revealed by comparison with the *P. falciparum* homologue. Mol Biochem Parasitol 2001;112:297–9.
[11] Ling IT, Kaneko O, Narum DL, et al. Characterisation of the *rhoph2* gene of *Plasmodium falciparum* and *Plasmodium yoelii*. Mol Biochem Parasitol 2003;127:47–57.
[12] Kaneko O, Tsuboi T, Ling IT, et al. The high molecular mass rhoptry protein, RhopH1, is encoded by members of the *clag* multigene family in *Plasmodium falciparum* and *Plasmodium yoelii*. Mol Biochem Parasitol 2001;118:237–45.
[13] Holt DC, Gardiner DL, Thomas EA, et al. The cytoadherence linked asexual gene family of *Plasmodium falciparum*: are there roles other than cytoadherence? Int J Parasitol 1999;29:939–44.
[14] Ling IT, Florens L, Dluzewski AR, et al. The *Plasmodium falciparum clag9* gene encodes a rhoptry protein that is transferred to the host erythrocyte upon invasion. Mol Microbiol 2004;52:107–18.
[15] Kaneko O, Yim Lim BY, Iriko H, et al. Apical expression of three RhopH1/Clag proteins as components of the *Plasmodium falciparum* RhopH complex. Mol Biochem Parasitol 2005;143:20–8.
[16] Ghoneim A, Kaneko O, Tsuboi T, Torii M. The *Plasmodium falciparum* RhopH2 promoter and first 24 amino acids are sufficient to target proteins to the rhoptries. Parasitol Int 2007;56:31–43.
[17] Polley SD, Conway DJ. Strong diversifying selection on domains of the *Plasmodium falciparum* apical membrane antigen 1 gene. Genetics 2001;158:1505–12.
[18] Baum J, Thomas AW, Conway DJ. Evidence for diversifying selection on erythrocyte-binding antigens of *Plasmodium falciparum* and *P. vivax*. Genetics 2003;163:1327–36.
[19] Siddiqui WA, Tam LQ, Kramer KJ, et al. Merozoite surface coat precursor protein completely protects *Aotus* monkeys against *Plasmodium falciparum* malaria. Proc Natl Acad Sci USA 1987;84:3014–8.
[20] Cooper JA, Ingram LT, Bushell GR, et al. The 140/130/105 kilodalton protein complex in the rhoptries of *Plasmodium falciparum* consists of discrete polypeptides. Mol Biochem Parasitol 1988;29:251–60.
[21] Doury JC, Bonnefoy S, Roger N, Dubremetz JF, Mercereau-Puijalon O. Analysis of the \ weight rhoptry complex of *Plasmodium falciparum* using monoclonal antibodies. Parasitology 1994;108:269–80.
[22] Trager W, Jensen JB. Human malaria parasites in continuous culture. Science 1976;193:673–5.
[23] Jongwutiwes S, Tanabe K, Nakazawa S, Uemura H, Kanbara H. Coexistence of GP195 alleles of *Plasmodium falciparum* in a small endemic area. Am J Trop Med Hyg 1991;44:299–305.
[24] Kaneko O, Soubes SC, Miller LH. *Plasmodium falciparum*: invasion of *Aotus* monkey red blood cells and adaptation to *Aotus* monkeys. Exp Parasitol 1999;93:116–9.
[25] Su X, Ferdig MT, Huang Y, et al. A genetic map and recombination parameters of the human malaria parasite *Plasmodium falciparum*. Science 1999;286:1351–3.
[26] Mu J, Ferdig MT, Feng X, et al. Multiple transporters associated with malaria parasite responses to chloroquine and quinine. Mol Microbiol 2003;49:977–89.
[27] Thompson JD, Higgins DG, Gibson TJ, Clustal W. improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 1994;22:4673–80.
[28] Kumar S, Tamura K, Nei M. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform 2004;5:150–63.
[29] Nei M, Gojobori T. Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. Mol Biol Evol 1986;3:418–26.
[30] Rozas J, Sanchez-DelBarrio JC, Messeguer X, Rozas R. DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics 2003;19:2496–7.
[31] McDonald JH, Kreitman M. Adaptive protein evolution at the *Adh* locus in *Drosophila*. Nature 1991;351:652–4.
[32] Tajima F. Simple methods for testing the molecular evolutionary clock hypothesis. Genetics 1993;135:599–607.
[33] Haubold B, Wiehe T. Statistics of divergence times. Mol Biol Evol 2001;18:1157–60.
[34] Betrán E, Rozas J, Navarro A, Barbadilla A. The estimation of the number and the length distribution of gene conversion tracts from population DNA sequence data. Genetics 1997;146:89–99.
[35] Mu J, Duan J, Makova KD, et al. Chromosome-wide SNPs reveal an ancient origin for *Plasmodium falciparum*. Nature 2002;418:323–6.
[36] Chung WY, Gardiner DL, Anderson KA, Hyland CA, Kemp DJ, Trenholme KR. The CLAG/RhopH1 locus on chromosome 3 of *Plasmodium falciparum*: two genes or two alleles of the same gene? Mol Biochem Parasitol 2007;151:229–32.
[37] Escalante AA, Ayala FJ. Phylogeny of the malarial genus *Plasmodium*, derived from rRNA gene sequences. Proc Natl Acad Sci USA 1994;91:11373–7.
[38] Goodman M. The genomic record of Humankind's evolutionary roots. Am J Hum Genet 1999;64:31–9.
[39] Hughes AL, Nei M. Pattern of nucleotide substitution at major histocompatibility complex class I loci reveals overdominant selection. Nature 1988;335:167–70.
[40] Kidgell C, Volkman SK, Daily J, et al. A systematic map of genetic variation in *Plasmodium falciparum*. PLoS Pathog 2006;2:e57.
[41] Sam-Yellowe TY, Shio H, Perkins ME. Secretion of *Plasmodium falciparum* rhoptry protein into the plasma membrane of host erythrocytes. J Cell Biol 1988;106:1507–13.
[42] Douki JB, Sterkers Y, Lepolard C, et al. Adhesion of normal and *Plasmodium falciparum* ring-infected erythrocytes to endothelial cells and the placenta involves the rhoptry-derived ring surface protein-2. Blood 2003;101:5025–32.
[43] Sam-Yellowe TY, Fujioka H, Aikawa M, Hall T, Drazba JA. A *Plasmodium falciparum* protein located in Maurer's clefts underneath knobs and protein localization in association with Rhop-3 and SERA in the intracellular network of infected erythrocytes. Parasitol Res 2001;87:173–85.
[44] Vincensini L, Richert S, Blisnick T, et al. Proteomic analysis identifies novel proteins of the Maurer's clefts, a secretory compartment delivering *Plasmodium falciparum* proteins to the surface of its host cell. Mol Cell Proteomics 2005;4:582–93.
[45] Ferreira MU, Kaneko O, Kimura M, Liu Q, Kawamoto F, Tanabe K. Allelic diversity at the merozoite surface protein-1 (MSP-1) locus in natural *Plasmodium falciparum* populations: a brief overview. Mem Inst Oswaldo Cruz 1998;93:631–8.
[46] Sakihama N, Ohmae H, Bakote B, Kawabata M, Hirayama K, Tanabe K. Limited allelic diversity of *Plasmodium falciparum* merozoite surface protein 1 gene from populations in the Solomon Islands. Am J Trop Med Hyg 2006;74:31–40.
[47] Rammensee H, Bachmann J, Emmerich NP, Bachor OA, Stevanovic S. SYFPEITHI: database for MHC ligands and peptide motifs. Immunogenetics 1999;50:213–9.
[48] Nielsen KM, Kasper J, Choi M, et al. Gene conversion as a source of nucleotide diversity in *Plasmodium falciparum*. Mol Biol Evol 2003;20:726–34.

[49] Freitas-Junior LH, Bottius E, Pirrit LA, et al. Frequent ectopic recombination of virulence factor genes in telomeric chromosome clusters of *P. falciparum*. Nature 2000;407:1018–22.
[50] Dzikowski R, Deitsch K. Antigenic variation by protozoan parasites: insights from *Babesia bovis*. Mol Microbiol 2006;59:364–6.
[51] Kraemer SM, Kyes SA, Aggarwal G, et al. Patterns of gene recombination shape *var* gene repertoires in *Plasmodium falciparum*: comparisons of geographically diverse isolates. BMC Genomics 2007;8:45.
[52] 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.
[53] Polley SD, Weedall 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.
[54] Roy SW, Ferreira MU, Hartl DL. Evolution of allelic dimorphism in malarial surface antigens. Heredity 2008;100:103–10.
[55] Michon P, Stevens JR, Kaneko O, Adams JH. Evolutionary relationships of conserved cysteine-rich motifs in adhesive molecules of malaria parasites. Mol Biol Evol 2002;19:1128–42.

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.*

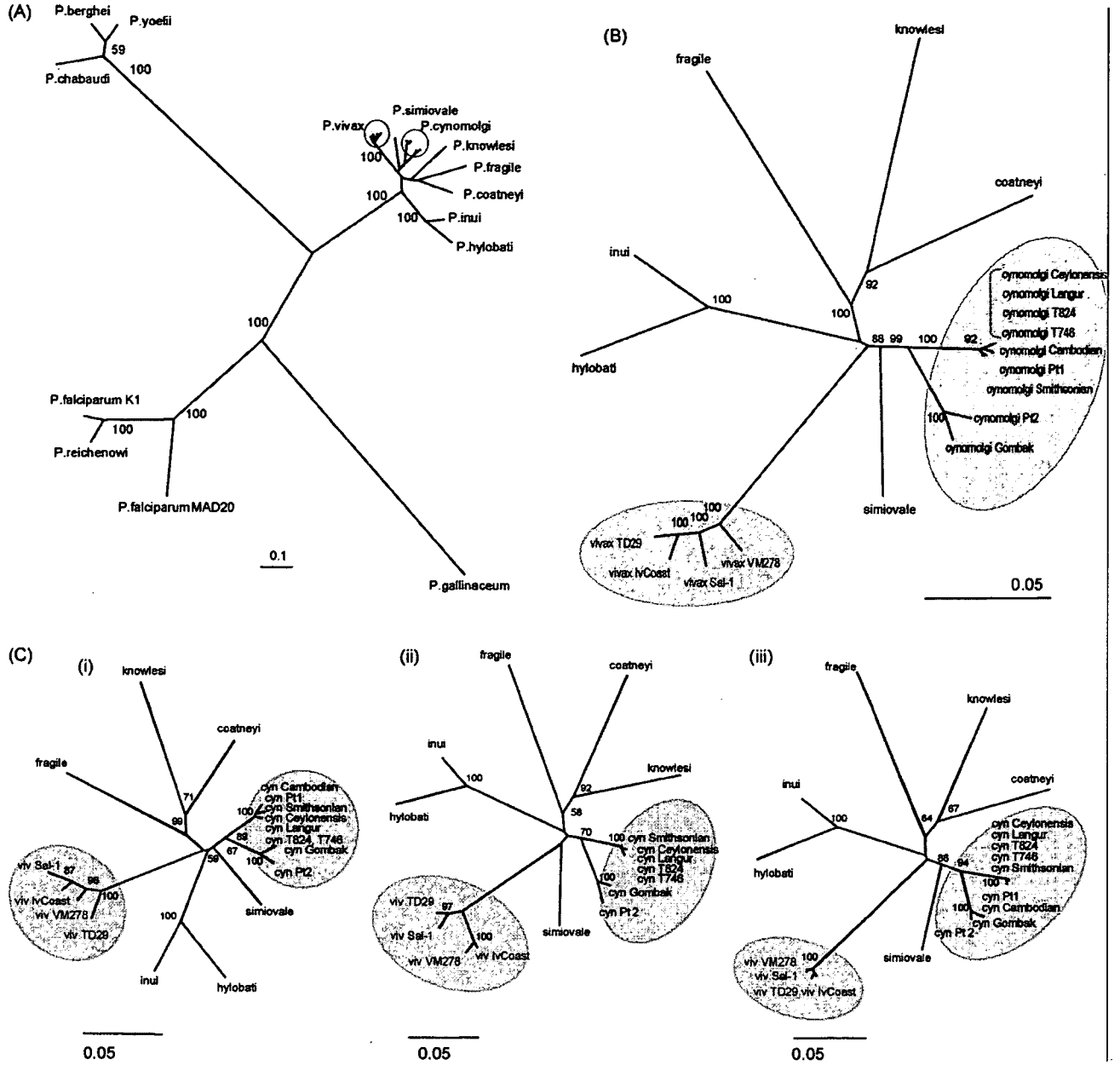

Fig. 1. ML phylogenetic tree of *msp1*. (A) Tree of *msp1* amino acid sequences from human, monkey, rodent and avian *Plasmodium* species. The numbers shown along nodes represent bootstrap values, of which only those of >50% are listed. (B) Tree of *msp1* nucleotide sequences from *P. vivax* and *P. 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

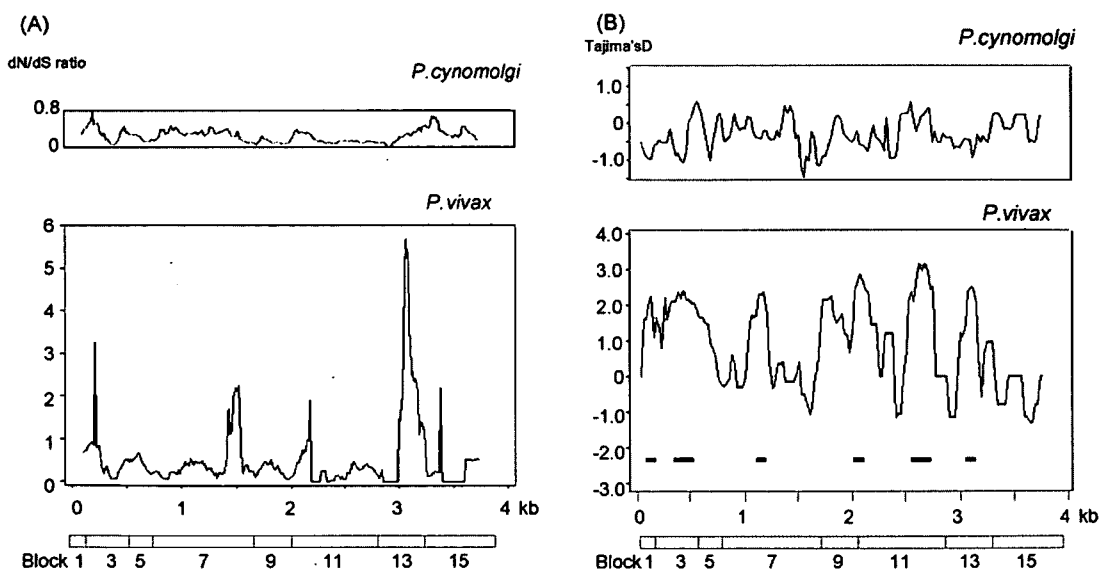

Fig. 2. Diversifying selection in *P. vivax msp1*. (A) Sliding window plot of dN/dS ratios of *msp1* from *P. cynomolgi* and *P. vivax*. A window size of 200 and a step of 10 nucleotides were used. (B) Sliding window plot of Tajima's D index of *msp1* from *P. cynomolgi* and *P. vivax*. A window size 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.

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

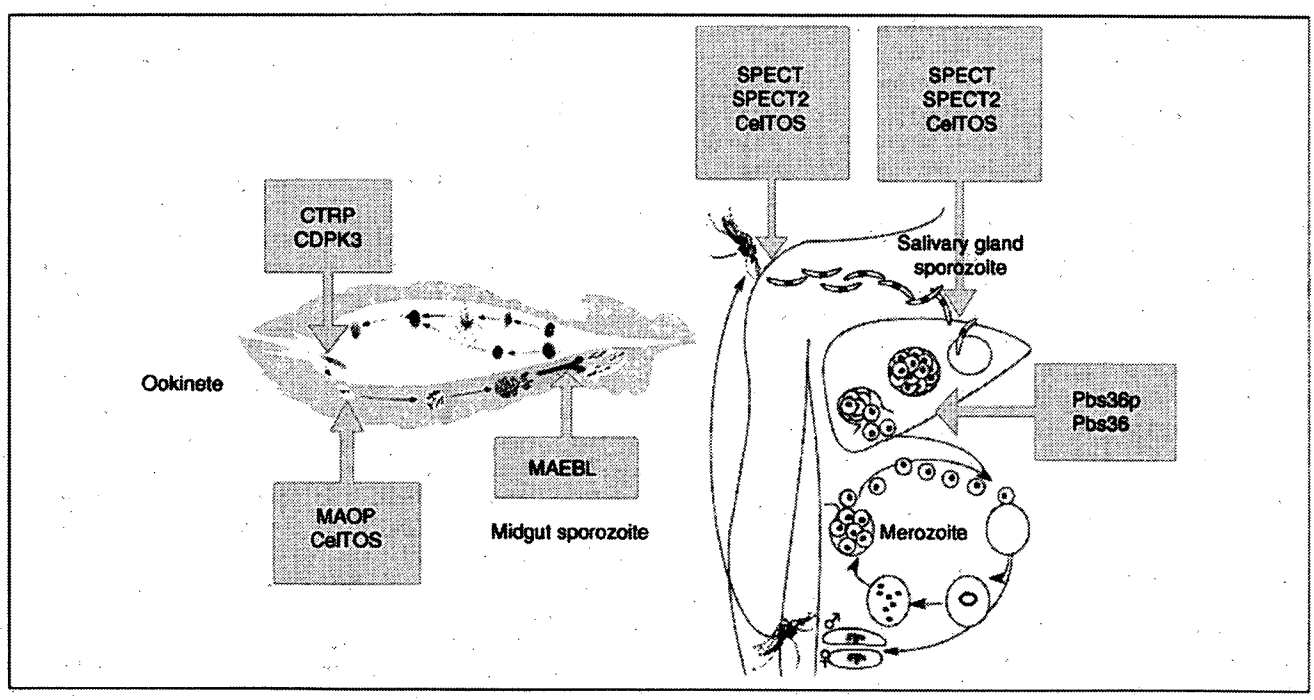

Figure 1. Novel proteins that are involved in host-cell invasion in the sexual and liver-infective stages of malaria parasites. Ookinete-specific proteins (CTRP, CDPK3 and MAOP) enable ookinete infection of the midgut epithelial cell. The midgut sporozoite protein MAEBL is important for sporozoite invasion 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.

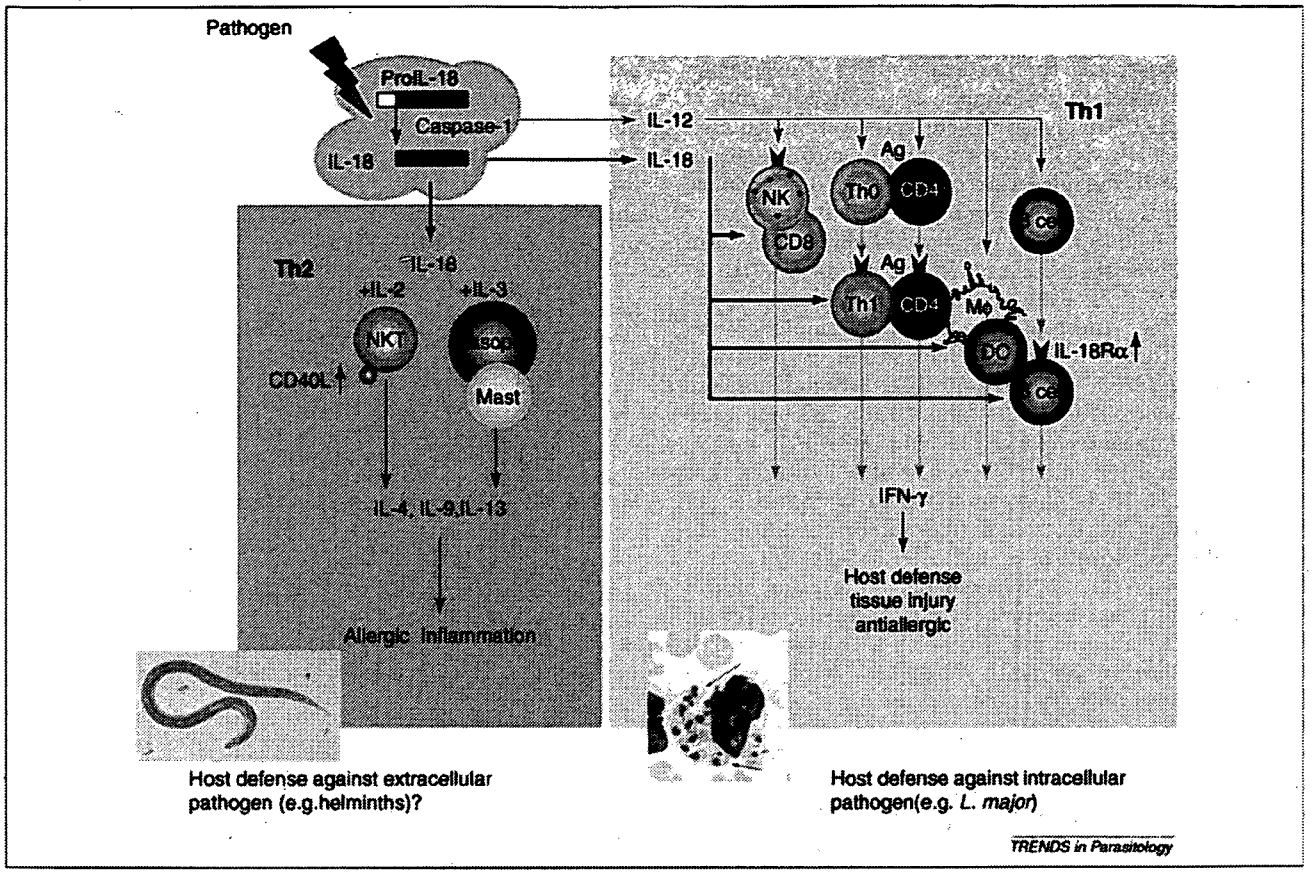

Figure 2. IL-18 stimulates both Th1 and Th2 responses, depending on the cytokine environment. The combination of IL-18 and IL-12 strongly stimulates various types of cell to produce IFN-γ, which, in turn, activates macrophages to produce nitric oxide, leading to the eradication of intracellular pathogens 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.

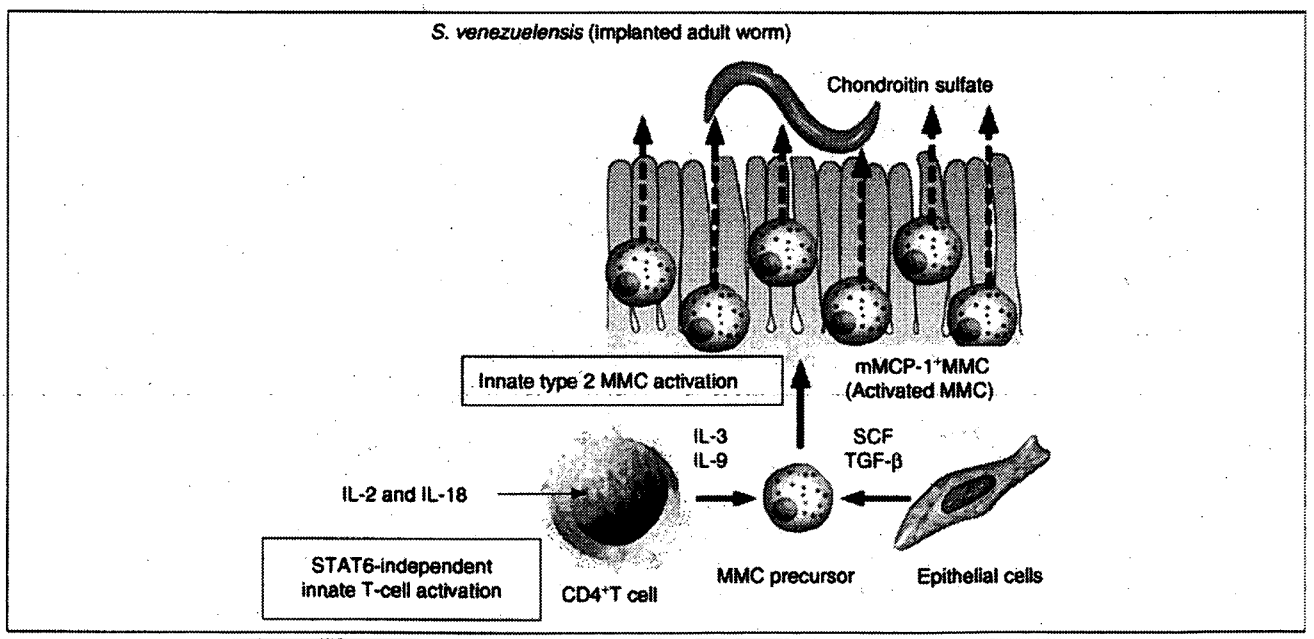

Figure 3. Protective role of IL-18-induced intestinal MMC in expulsion of *Strongyloides venezuelensis*. *In vivo* treatment with a combination of IL-18 and IL-2 stimulates $CD4^+$ T cells to produce IL-3 and IL-9 in a STAT6-independent manner. IL-3 and IL-9 from $CD4^+$ T cells, and SCF and TGF-β from epithelial cells induce precursor cells to develop into $mMCP\text{-}1^+$ 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
Parasitology International 56 (2007) 185–194
www.elsevier.com/locate/parint

Allelic diversity in the merozoite surface protein 1 gene of *Plasmodium falciparum* on Palawan Island, the Philippines

Naoko Sakihama [a], Masatoshi Nakamura [b], Antonio A. Palanca Jr. [c], Renee A. Argubano [c], Ester P. Realon [c], Alan L. Larracas [d], Ronald L. Espina [d], Kazuyuki Tanabe [a,*]

[a] *Laboratory of Malariology, International Research Center of Infectious Diseases, Research Institute for Microbial Diseases, Osaka University, 3-1, Yamada-Oka, Suita, Osaka 565-0871, Japan*
[b] *Department of Tropical Medicine and Parasitology, Dokkyo Medical University, Tochigi, Japan*
[c] *Palawan Provincial Health Office, Puerto Princesa, Palawan, Philippines*
[d] *Center for Health Development IVB, Palawan Extention Office, Puerto Princesa, Palawan, Philippines*

Received 28 November 2006; received in revised form 29 January 2007; accepted 31 January 2007
Available online 8 February 2007

Abstract

Allelic diversity of the *Plasmodium falciparum* merozoite surface protein 1 gene (*msp1*) is mainly generated by meiotic recombination at the mosquito stage. We investigated recombination-based allelic diversity of *msp1* in a *P. falciparum* population from Palawan Island, the Philippines, where malaria transmission is moderate. We identified the 5′ recombinant types, 3′ sequence types and *msp1* haplotypes (unique combinations of 5′ recombinant type and 3′ sequence type), and compared them with those of *P. falciparum* from the Solomon Islands, where malaria transmission is high. The mean number of 5′ recombinant types per patient in Palawan was 1.44, which is comparable to the number for the Solomon Islands (1.41). The Palawan parasite population had 15 *msp1* haplotypes, whereas the Solomon Islands population had only 8 haplotypes. The Palawan population showed strong linkage disequilibrium between polymorphic blocks/sites within *msp1*, which is comparable to the results for the Solomon Islands. These findings support our hypothesis that the extent of allelic diversity of *msp1* is determined not only by the transmission intensity but also by the number of *msp1* alleles prevalent in the local parasite population and the extent of mixed-allele infections. Contribution of a high prevalence of the chloroquine (CQ)-sensitive allele of *P. falciparum* CQ resistance transporter (*pfcrt*) to the relatively high *msp1* diversity in the Palawan population is discussed.

Keywords: Malaria; *msp1*; Polymorphism; Recombination; *pfcrt*

1. Introduction

Malaria caused by *Plasmodium falciparum* is a major public health problem in the tropics. Due to the worldwide spread of drug-resistant malaria, there is an urgent need for the development of malaria vaccines. The 200-kDa *P. falciparum* merozoite surface protein 1 (MSP-1), which plays an important role in erythrocyte invasion by the merozoite, is a target of human immune responses [1,2], and is thus considered a good candidate vaccine target [1,3]. However, the gene encoding MSP-1 (*msp1*) is highly polymorphic, and this polymorphism is a major obstacle to the development of effective malaria vaccines based on MSP-1. In animal models, protective immunity directed against MSP-1 is strain-specific [4–6]. *P. falciparum msp1* consists of 17 blocks, with varying degrees of sequence conservation among alleles [7,8]. Sequence variation in *msp1* is dimorphic (*i.e.*, one or the other of two major allelic forms represented by K1 allele and MAD20 allele) in all variable blocks except for block 2, which has three major allelic forms (see Fig. 2). A major source of allelic diversity of *msp1* is meiotic recombination, which only occurs in the *Anopheles* mosquito stage. These facts suggest that the extent of *msp1* allelic diversity is largely dependent on the frequency of recombination events and the intensity of malaria transmission.

Abbreviations: MSP-1, merozoite surface protein 1; *msp1*, merozoite surface protein 1 gene; CQ, chloroquine; *pfcrt*, *Plasmodium falciparum* chloroquine resistance transporter; LD, linkage disequilibirium; EGF, epidermal growth factor; GPI, glycosylphosphatidylinositol.

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

1383-5769/$ - see front matter © 2007 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.parint.2007.01.011

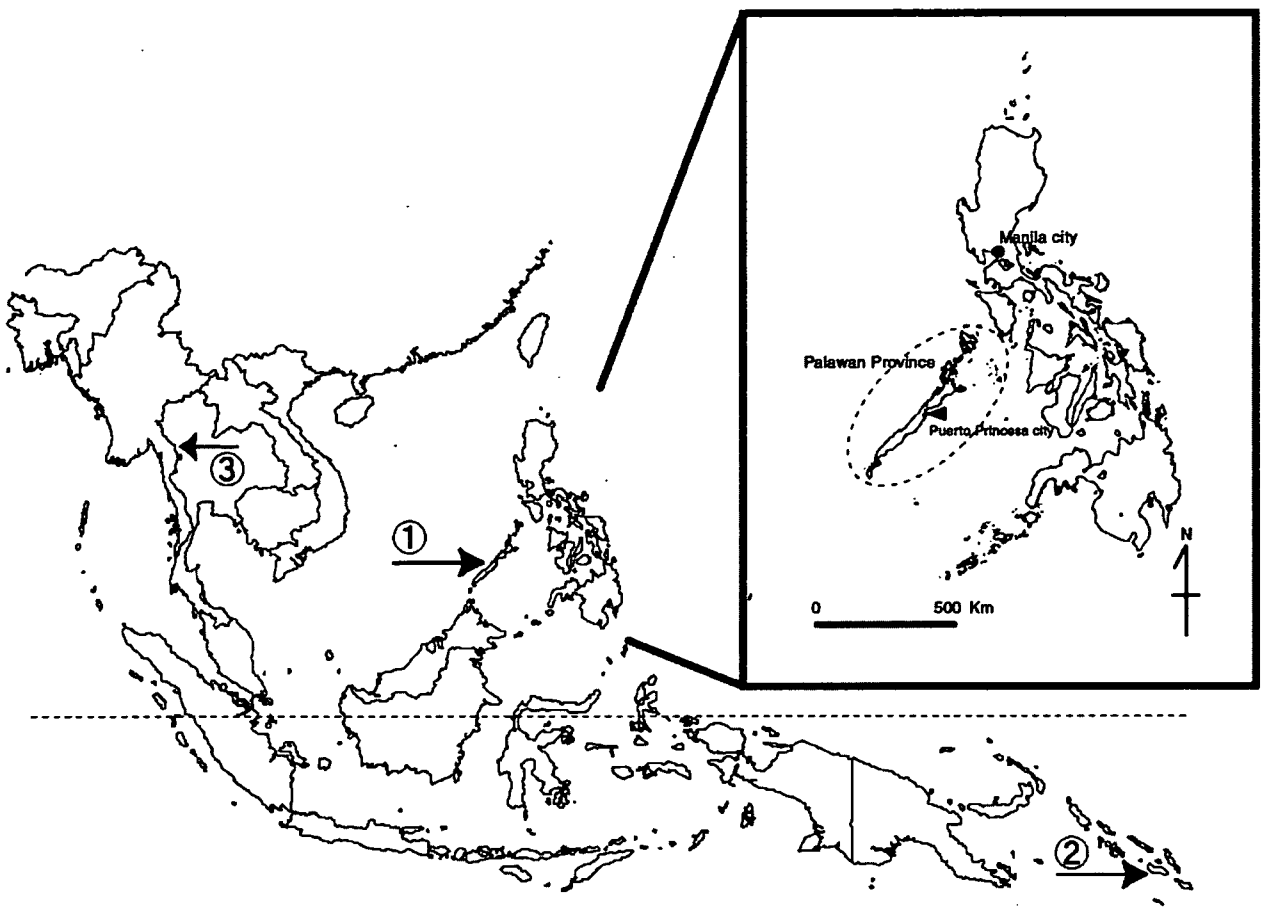

Fig. 1. Location of the study area. (1) Palawan Island in the Philippines, (2) northern Guadalcanal in The Solomon Islands and (3) Mae Sod in northwestern Thailand. Major islands of Palawan Province are circled with broken line in the box. Puerto Princesa is indicated by an arrowhead. Manila City is indicated by a closed circle.

In high-transmission areas in Africa, a high frequency of intragenic recombination events in *msp1* has been observed [9].

To investigate allelic diversity of *msp1* in natural *P. falciparum* populations, we have chosen *msp1* haplotypes as a polymorphic genotype marker [10–12]. *msp1* haplotypes are defined as unique combinations of 5′ recombinant types (combinations of allelic types in blocks 2, 4a, 4b, and 6) and 3′ sequence types (block 17). The extent of *msp1* allelic diversity is estimated by the number of distinct *msp1* haplotypes prevalent in a population and the haplotype diversity (*h*) [13], which is a population genetic index of allelic diversity. The frequency of recombination events in *msp1* can be inferred from analysis of linkage disequilibrium (LD) between polymorphic blocks/sites within *msp1*. Previous studies suggest that the extent of *msp1* allelic diversity and the frequency of recombination events in *msp1* are generally high in high-transmission areas and low in low-transmission areas [12,14,15]. However, not all studies have shown such an association between *msp1* allelic diversity and the intensity of malaria transmission. In a recent study, we observed relatively low allelic diversity of *msp1* in *P. falciparum* populations from the Solomon Islands, where the intensity of malaria transmission is very high [16].

We are interested in *msp1* allelic diversity on islands. *P. falciparum* populations on islands are generally small and have few opportunities to encounter novel *msp1* alleles from other populations, compared with conditions on continents, where novel alleles is recurrently and easily introduced from outside a population because of geographical non-isolation. This suggests that there are differences in population genetic features of *P. falciparum msp1* allelic diversity between island populations and continental populations. We previously observed relatively low frequency of recombination events in *msp1* in populations from the southwestern Pacific islands, compared with populations from northwestern Thailand [16]. In the present study, we investigated recombination-based allelic diversity of *msp1* in a *P. falciparum* population from Palawan, the Philippines, where malaria prevalence is hypo- to mesoendemic. The present results indicate that there is relatively high diversity of *msp1* haplotypes in Palawan, compared with a population from the Solomon Islands, whereas the frequency of recombination events in Palawan is low, which is consistent with our hypothesis that the extent of *msp1* allelic diversity is not always dependent on the intensity of malaria transmission. We also observed a high prevalence of the chloroquine (CQ)-sensitive allele of *P. falciparum* CQ resistance transporter (*pfcrt*) [17] in Palawan. Contribution of this high prevalence of CQ-sensitive *pfcrt* to the relatively high *msp1* diversity in Palawan is discussed.

2. Materials and methods

2.1. Study area and sample collection

Field isolates of *P. falciparum* were collected between May 1997 and October 1997 on Palawan Island, the Philippines. Palawan Island is the largest and most populated island in Palawan Province, an island province composed of 1768 islands, located 600 km southwest of Manila (Fig. 1). This island is 425 km long and 8.5 to 40 km wide, with a population of 584,490 (according to the year 2000 census [18]). Although malaria in Palawan Province is reportedly hypo- to mesoendemic [19], with a slide positive rate of 5.4% in 1997 [20], the annual parasite incidence was 10.07 (slide-positives per 1000) in 1997, which was the sixth highest of the 78 provinces of the Philippines [20]. The total population of Palawan Province (755,412 in the year 2000 census [18]) has been growing rapidly and continuously due to migration from other areas of the Philippines. The majority of those migrants settle on Palawan Island and account for 63% of its residents. Migrants mainly come from Central Luzon, Southern Luzon and the Visayas, where malaria is less endemic than in Palawan. Malaria transmission is perennial, with a peak during the rainy season from May to December. The main malaria vectors in Palawan are *Anopheles flavirostris*, *A. balabacensis*, *A. litoraris*, and *A. maculatus*; the primary and secondary vectors are *A. flavirostris* and *A. balabacensis*, respectively [21]. Both *P. falciparum* and *P. vivax* are prevalent in Palawan; 71.5% of the confirmed cases in 1997 (3854/5386) were due to *P. falciparum*, in contrast to other areas in the Philippines where *P. vivax* predominates [20]. As of 1997, the first-, second- and third-line drugs used to treat malaria in the Philippines were chloroquine, sulfadoxine–pyrimethamine, and quinine, respectively [22].

A total of 114 *P. falciparum* isolates were collected at Palawan Provincial Hospital and Malaria Control Service Palawan Office located in Puerto Princesa, the capital city of Palawan Province (Fig. 1). The isolates were obtained from 81 out-patients and 33 in-patients with symptomatic uncomplicated malaria; the patients had a mean age of 29.8 years (range, 5–69 years). Most of the patients ($n=84$) were residents of Puerto Princesa city; 15 patients were residents of 6 municipalities outside the city; and 15 patients had unknown addresses. Malaria was diagnosed by microscopic examination of Giemsa-stained blood smears. Venous blood was collected from *P. falciparum*-positive patients and stored frozen at −20 °C. Parasite genomic DNA was isolated using the QIAamp DNA Mini Kit (QIAGEN, Germany), as described elsewhere [23,24]. Informed consent was obtained from the donors or their guardians. This study has been approved by the Palawan Provincial Health Office.

For comparison, we also included data previously obtained from *P. falciparum* isolates collected in the Solomon Islands [16] and northwestern Thailand [11]. These isolates are described in detail elsewhere [16]. The isolates from the Solomon Islands ($n=90$) were collected from individuals with clinical malaria or asymptomatic malaria in northeastern Guadalcanal in 1995 and 1996. The isolates from Thailand ($n=111$) were clinical isolates collected from patients attending a malaria clinic in Mae Sot near the Thailand–Myanmar border, in 1995.

In this study, we aimed to investigate recombination-based allelic diversity of *P. falciparum msp1* in Palawan Island and compared with that in the Solomon Islands (northern Guadalcanal) and northwestern Thailand (Fig. 1). For this comparison, the estimates of entomological inoculation rate (EIR) would be suitable for standardization of the intensity of malaria transmission across geographic areas. EIR in Palawan Island has been reported to be 9.7 bites per person per year (based on sampling by human bait catch and microscopic detection of sporozoites) [25]. EIRs in northern Guadalcanal were reportedly 584–1022 bites per person per year [16] (based on sampling by human bait catch and sporozoite detection by enzyme-linked immunosorbent assay (ELISA) [26]), a value comparable with those in high-transmission areas in Africa such as Tanzania [27]. Thus, malaria transmission intensity in Palawan Island seemed to be much lower than in the Solomon Islands. Consistently, the slide positive rate was lower in Palawan Island (5.4%) [20] than in northern

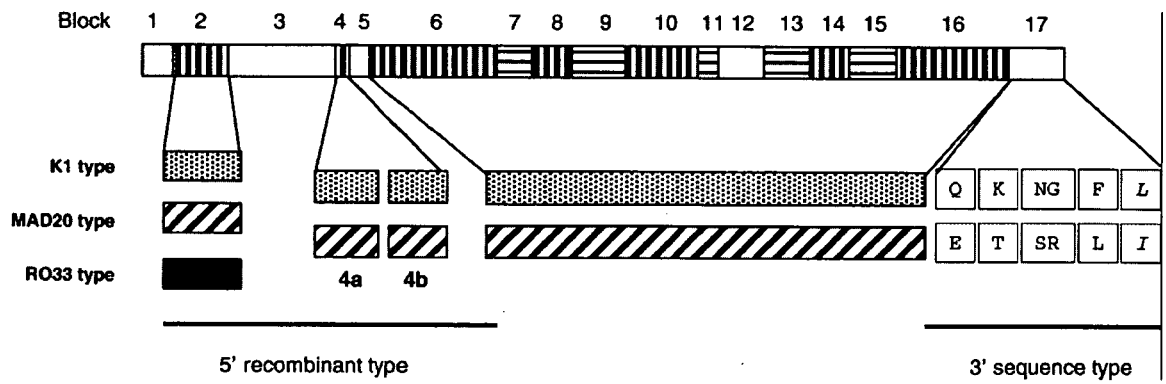

Fig. 2. Structure of *Plasmodium falciparum msp1* and strategy for determination of 5′ recombinant type and 3′ sequence type. Inter-allele conserved, semi-conserved, and variable blocks are indicated by open, horizontally hatched, and vertically hatched columns, respectively. For variable blocks, MAD20-type, K1-type, and RO33-type sequences are presented by half-tone, slashed, and closed bars, respectively. The 5′ recombinant type was determined by PCR amplification of blocks 2 to 6 using allelic type-specific primers for blocks 2 and 6, followed by nested PCR between blocks 4a and 4b using allelic-type-specific primers for blocks 4a and 4b. The six amino acid substitutions within block 17, indicated by one-letter codes, were determined by direct sequencing. A previously unidentified amino acid substitution is indicated in italics (see Results). The 3′ sequence type is a unique combination of these substitutions.