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Sequence Variation in the T-Cell Epitopes of the *Plasmodium falciparum* Circumsporozoite Protein among Field Isolates Is Temporally Stable: a 5-Year Longitudinal Study in Southern Vietnam

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In an effort to decipher the nature and extent of antigen polymorphisms of malaria parasites in a setting where malaria is hypomesoendemic, we conducted a 5-year longitudinal study (1998 to 2003) by sequencing the Th2R and Th3R epitopes of the circumsporozoite protein (CSP) of 142 *Plasmodium falciparum* field isolates from Bao Loc, Vietnam. Samples were collected during the high-transmission season, September through December 1998 ($n = 43$), as well as from July 2000 to August 2001 ($n = 34$), September 2001 to July 2002 ($n = 33$), and August 2002 to July 2003 ($n = 32$). Marked sequence diversity was noted during the high-transmission season in 1998, but no significant variation in allele frequencies was observed over the years ($\chi^2 = 70.003$, degrees of freedom = 57, $P = 0.116$). The apparent temporal stability in allele frequency observed in this Bao Loc malaria setting may suggest that polymorphism in the Th2R and Th3R epitopes is not maintained by frequency-dependent immune selection. By including 36 isolates from Flores Island, Indonesia, and 19 isolates from Thaton, Myanmar, we investigated geographical patterns of sequence polymorphism for these epitopes in Southeast Asia; among the characterized isolates, a globally distributed variant appears to be predominant in Vietnam (75 of 142 isolates, or 52.8%) as well as in Myanmar (15 of 19 isolates, or 78.9%) and Indonesia (31 of 36 isolates, or 86.1%). Further analyses involving worldwide CSP sequences revealed distinct regional patterns, a finding which, together with the unique mutations observed here, may suggest a possible role for host or local factors in the generation of sequence diversity in the T-cell epitopes of CSP.

As one of the most threatening tropical diseases, malaria continues to maintain its toll in the developing world and poses a heavy economic burden on many countries. Estimates show that 300 million to 500 million clinical cases occur per year, with at least a million deaths, most of which are seen among children under 5 years of age (8). Resistance to antimalarial drugs and insecticides, coupled with the lack of availability of an effective vaccine, is the leading factor behind the success of the parasite's continuing burden. Apart from its complex life cycle, which alternates between the human and the mosquito host, the malaria parasite also exhibits stages characterized by extensive genetic and antigenic diversity which may present adverse obstacles to antimalarial control measures.

The *Plasmodium falciparum* circumsporozoite protein (CSP), a leading malaria vaccine candidate antigen, is predominantly distributed on the surface of the sporozoites (40) and has approximately 120 residues, with a molecular mass of about 58 kDa. The structure of CSP can be divided into a polymorphic central repeat region known to contain immunodominant B-cell epitopes (41) and flanked by a less variant 5′ and a highly polymorphic 3′ end where T-cell epitopes have been identified (19, 27). Clinical trials of *Plasmodium falciparum* CSP-based vaccines have shown that they induce both humoral and cellular immune responses (29, 39) and that the cellular immune responses are central in immunity against the exoerythrocytic stages of malaria.

In an attempt to understand the nature and the extent of the polymorphism patterns of malaria parasites in a setting of malaria hypomesoendemicity, we have conducted a 5-year longitudinal study of the most variable terminal 3′ region of the CSP gene among *Plasmodium falciparum* field isolates collected from Bao Loc, southern Vietnam, over 5 years (1998 to 2003). We compare our data with the reported CSP sequences and also discuss the geographical patterns of polymorphism at this locus in Southeast Asia and globally. Our findings show that the genetic diversity of the Th2R and Th3R epitopes in this Bao Loc malaria setting is less than that in areas of hyperendemicity and that the variation patterns tend to corre-

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late with malaria transmission intensity. Furthermore, the polymorphisms appear to be stable over time in this Bao Loc setting of relatively low malaria endemicity. Findings from our analyses involving worldwide CSP sequences seem to suggest that the sequence polymorphisms observed in the Th2R and Th3R epitopes of CSP not only appear to be geographically restricted but also may be a result of a balanced state(s) of interaction between the parasite and its hosts.

MATERIALS AND METHODS

Study population. Bao Loc lies about 180 km northeast of Ho Chi Minh City and is a district with a growing population of 151,000 inhabitants. The city belongs to Lam Dong Province, which is the southernmost of the four provinces belonging to the Tay Nguyen Highlands. Lam Dong is in an area in the southern highlands of Vietnam where malaria is mesoendemic and where many minority ethnic groups reside. Although malaria has disappeared from many areas in Vietnam, it is still endemic in the central and southern provinces of the Tay Nguyen Highlands (38), where all four *Plasmodium* species that cause human malaria are present year round, with a peak in transmission shortly after the rainy season (October to December). The prevalence of parasitemia in the general population typically fluctuates between 10% and 30%, of which approximately 75% of the cases are due to *P. falciparum* (unpublished observations). Local malaria vectors include *Anopheles dirus* sensu lato and *Anopheles minimus* sensu lato. After 10 years of intensive control efforts, the rates of malaria morbidity and mortality in Vietnam have decreased by 60% and 97%, respectively.

Sample collection. This study was approved by the ethical committees of the Nagoya University Graduate School of Medicine and the Bao Loc District General Hospital. Following the receipt of informed consent, venous blood samples were collected from malaria patients presenting at the Bao Loc General Hospital over the period from September 1998 to December 1998 and from July 2000 to August 2003. Bao Loc is a region for which no previous epidemiologic data exist for this malaria antigen gene, although epidemiologic data are available for other candidate genes (16, 33). The blood samples were categorized into four groups: (i) group I (BL1998), September 1998 to December 1998; (ii) group II (BL2001), July 2000 to August 2001; (iii) group III (BL2002), September 2001 to July 2002; and (iv) group IV (BL2003), August 2002 to July 2003. We further included field isolates collected from Thaton, Myanmar (n = 19), during a field survey in 1999 and another 36 collected from Flores Island, Indonesia, in 2003 (n = 14) and 2004 (n = 22).

DNA extraction, amplification, and sequencing. Slide-positive *P. falciparum* infections, as demonstrated by Giemsa staining, were analyzed. Genomic DNA was extracted from frozen venous blood samples by using a DNeasy kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions. The carboxy-terminal portion of the CSP gene was amplified by using primers PfCSF (TGT AGA TGA AAA TGC TAA TGC) and PfCSR (CGA CAT TAA ACA CAC TGG A) in a 25-μl PCR mixture containing 0.5 μM of each primer, 1 to 3 μl of template DNA, 2.5 μl of 10× buffer, 200 μM of each deoxynucleoside triphosphate, and 0.5 units of AmpliTaq Gold DNA polymerase (PE Applied Biosystems). Thermal conditions were incubation at 95°C for 5 min, followed by 35 cycles of 94°C for 30 s, 58°C for 50 s, and 72°C for 1 min. A final extension of 72°C for 5 min was also included. The 268-bp amplicons were resolved on 2.0% agarose gels following electrophoresis in the presence of ethidium bromide (0.5 mg/ml). Distilled water and genomic DNA from *P. vivax* were used as negative controls. Positive samples (i.e., PCR fragments) were purified with a High Pure PCR product purification kit (Boehringer Mannheim, Mannheim, Germany), as directed by the manufacturer, and a precipitation step was achieved with Etachinmate (Tokyo, Japan). DNA quantity was estimated by both electrophoresis and measurement of absorption values by determination of the optical density at 260 nm, after which a 1:10 dilution was prepared. Sequencing reactions were performed with the forward and reverse primers in separate reaction mixtures with the BigDye Terminator sequencing kit (version 1.1; PE Applied Biosystems). The sequences in both directions were then read with an ABI 310 DNA sequencer. Whenever a mutant or singleton was encountered, a third independent reaction mixture was made, and the product was resequenced to confirm the mutation.

Data analyses. To determine the concurrence of the findings of the present study with the findings of previous reports, the sequences were aligned and the amino residues were numbered with reference to the sequence of the 7G8 clone from Brazil (10). The alignment was done with the software Genetyx Mac (version 11.0), whereas the DNA sequence polymorphism software DnaSP (version 4.0) (32) was used to calculate genetic diversity parameters and MEGA2.1 (25) was used to reconstruct a phylogenetic tree. A chi-square test of independence was done by treating the CSP sequences as independent entities through the use of the statistical software package SPSS (version 10.0; SPSS, Inc., Chicago, IL). To examine spatial diversity patterns, the GenBank sequences of field isolates collected from different geographical areas were also included. Of these, 6 (GenBank accession numbers AB116602 to AB116607) were from Vanuatu (36); 44 (GenBank accession numbers AY878598 to AY878641) were from The Gambia (unpublished); 48 sequences (GenBank accession numbers AF540441 to AF540488) included 11 from India, 9 from Cameroon, 10 from Venezuela, and 18 from Kenya (14); 10 isolates (GenBank accession numbers AJ269961 to AJ269970) were from Senegal, 8 (GenBank accession numbers AJ269971 to AJ269978) were from Brazil; 6 (GenBank accession numbers AJ269955 to AJ269960) were from Kanbauk, Myanmar (12); and 23 (GenBank accession numbers M83171 to M83151) were from Thailand (24). All of these sequences, together with those sequenced here (from Vietnam, Indonesia, and Myanmar), were read by DnaSP (version 4.0) and grouped into their respective countries. We used a nonparametric statistical method that uses Monte Carlo simulations to estimate the levels of significance for geographic subdivision among the nucleotide sequences, i.e., by testing of the null hypothesis of no genetic differentiation between subpopulations at different localities (20). This test is incorporated in the gene flow and genetic differentiation (21) command in DnaSP (version 4.0), and 1,000 permutations were done. Among the genetic distances that resulted from this test, Nei's Dxy value (30), which is based on the average number of pairwise nucleotide substitutions per site between populations, was exported to MEGA2.1 for reconstruction of a neighbor-joining tree. Intrapopulation average pairwise genetic distances (d) and standard errors were also estimated by use of the Tamura-Nei (gamma) parameter at 1,000 replications by using MEGA2.1, and the results are shown in Table 1. Values of neutrality tests are also shown. Tajima's D test (35) is based on the differences between the number of segregating sites and the average number of nucleotide differences, whereas Fu and Li's (17) F^* statistic is based on the differences between the number of singletons (mutations appearing only once among the sequences) and the average number of nucleotide differences between pairs of sequences.

Nucleotide sequence accession numbers. The sequences obtained in this study have been submitted to GenBank with accession numbers DQ193573 to DQ193595.

TABLE 1. Analyses of the C-terminal region (231 bp) of the CSP sequences of *P. falciparum* field isolates from different countries

Country	No. of sequences	No. of haplotypes	Fu and Li's (17) F^*	Tajima's (35) D	Avg (SE) d^a
Brazil	8	2	−1.80	−1.53	0.005 (0.002)
Venezuela	10	7	0.86	0.65	0.030 (0.009)
Kenya	18	13	−0.16	−0.17	0.027 (0.007)
Cameroon	9	7	−0.81	−0.84	0.021 (0.006)
Gambia	44	21	1.36	0.77	0.027 (0.007)
Senegal	10	8	1.14	0.52	0.021 (0.007)
India	11	3	−0.63	−1.03	0.004 (0.003)
Vanuatu	6	2	−1.20	−1.13	0.003 (0.002)
Thailand	23	8	−0.62	−0.52	0.014 (0.004)
Kanbauk[b]	6	3	−1.40	−1.30	0.006 (0.003)
Thaton[b]	19	3	−0.58	−0.82	0.005 (0.002)
Indonesia	36	5	−1.78	−1.93[c]	0.003 (0.001)
Vietnam	142	20	0.43	−0.80	0.009 (0.003)
All	342	68	−1.23	−0.96	0.016 (0.004)

[a] Within-population average distances (d) and standard errors (SE) were estimated by MEGA2.1 by the bootstrap method with 1,000 replications, and the values are shown.
[b] Note that both Kanbauk and Thaton are located in Myanmar.
[c] A value statistically different from zero ($P < 0.05$).

RESULTS

We successfully obtained the DNA sequences of the 3′ region spanning the Th2R and Th3R epitopes at the C-terminal portion of the circumsporozoite protein of *Plasmodium falciparum* isolates from 142 symptomatic malaria patients present-

TABLE 2. Ages of patients and nucleotide diversity for Th2R and Th3R epitopes of Vietnamese isolates over the years 1998 to 2003

Group	No. of isolates	Age (yr) of patients		Sequence diversity parameters (π[Hd])[a]		
		Range	Mean ± SD	Th2R epitope	Th3R epitope	Whole 3′ end
BL1998	43	11–68	30.7 ± 14.1	0.03482 (0.758)	0.02566 (0.506)	0.01383 (0.793)
BL2001	34	11–71	29.2 ± 14.2	0.02063 (0.613)	0.00878 (0.114)	0.00699 (0.613)
BL2002	33	11–66	30.9 ± 13.8	0.01649 (0.578)	0.01953 (0.411)	0.00798 (0.587)
BL2003	32	6–60	29.8 ± 13.6	0.02143 (0.696)	0.01327 (0.339)	0.00808 (0.725)
All	142	6–71	30.2 ± 13.8	0.02391 (0.675)	0.01697 (0.359)	0.00960 (0.697)

[a] Nucleotide diversity (π) is calculated with the Jukes and Cantor correction (28), and haplotype diversity (Hd) is calculated by Nei's method (30).

ing at the Bao Loc General Hospital in southern Vietnam. The age distributions as well as the DNA polymorphism parameters for the respective groups in Bao Loc are depicted in Table 2. Among the 142 isolates sequenced from Bao Loc, we characterized 20 variants (Fig. 1) of the 3′ region of the CSP gene of *P. falciparum*, of which 16 are newly identified. Fifteen haplotypes were found in BL1998 alone, during the peak transmission period, whereas six, eight, and nine alleles were found in BL2001, BL2002, and BL2003, respectively. Three variants, i.e., alleles 1 to 3 in Fig. 1, accounted for the majority of the sequences found in Bao Loc, representing 52.8%, 10.6%, and 10.6% of the Vietnamese sequences, respectively, and they appeared to be stable over our study period, as no significant difference in allele frequencies was seen between the years (χ^2 = 70.003, degrees of freedom = 57, P = 0.116). Of note also is that most alleles (75%) identified in Bao Loc were predominant over the years, as evidenced by the fact that only five actually "new" variants were noted. Furthermore, most of the variants are novel and have not been reported previously. These results prompted us to investigate samples from Myanmar and Indonesia, and a similar trend of allele predominance was noted, with three more variants, i.e., alleles 21 to 23, detected (Fig. 1).

In reference to the 7G8 sequence, we observed eight mutation points at residues 332, 333, 336 to 340, and 342 in the Th2R epitope and four mutation points at residues 367, 369, 372, and 374 in the Th3R epitope (Fig. 2a and b, respectively). All polymorphisms were nonsynonymous point mutations, and at the nucleotide level, most mutations were at the first or second position of the codons. However, two third-position substitutions at residues 337 and 338 that led to K→N and I→M, respectively, were also seen, and both were within the Th2R epitope region. It is also of interest that the I→M mutation seems to have been described only in Southeast Asia (26; this study). Bhattacharya (5) reported unique substitutions at residues 331 (I→K) and 368 (K→Q) common to many clones and isolates from India, but we did not find these substitutions.

To evaluate the nature and the extent of the polymorphism patterns for parasites over the study period, we compared the sequences of the respective epitopes for each group of samples. Overall, 15 variants for the Th2R epitope (Fig. 2a) were identified, of which 11 were recorded in BL1998 alone, followed by 6 each for the BL2001 and BL2002 samples and 8 for the BL2003 samples. Excluding the 11 alleles found at the study start point (BL1998), one realizes that, in essence, only four variants were actually "new" or introduced into the population over the study period. A similar pattern was seen in the Th3R epitope (Fig. 2); again, BL1998 had the highest number of variants, i.e., seven, whereas two, four, and five alleles were

Id	PSDKHIEQYLKKIKNSISTEWSP CSVTCGNGIQVRIKP GSANKPKDELDYENDIEKKICKMEK	BL 1998	BL 2001	BL 2002	BL 2003	FL 2003	FL 2004	TT 1999	Total
7G8									
1	.....Q..L.....	19 (44.2%)	20 (58.8%)	21 (63.6%)	15 (46.9%)	10 (71.4%)	21 (95.5%)	15 (78.9%)	121 (61.4%)
2	.....MQ..L.....	1 (2.3%)	4 (11.8%)	2 (6.1%)	8 (25.0%)	1 (7.1%)			16 (8.1%)
3	.....N.Q..L.....	4 (9.3%)	7 (20.6%)	1 (3.0%)	3 (9.4%)				15 (7.6%)
4	.....QY.L.....	2 (4.7%)		4 (12.1%)	1 (3.1%)			3 (15.8%)	10 (5.1%)
5	.....TE.....Q..L.....	4 (9.3%)	1 (2.9%)			2 (14.3%)			7 (3.6%)
6	.....KE..T..Q.....	2 (4.7%)	1 (2.9%)	1 (3.0%)	1 (3.1%)				5 (2.5%)
7	.....N.Q..L.....	1 (2.3%)		2 (6.1%)					3 (1.5%)
8	.....E.Q..L.....	2 (4.7%)			1 (3.1%)				3 (1.5%)
9	.....TE.....Q..L.....	2 (4.7%)							2 (1.0%)
10	.....Q..L.....	1 (2.3%)			1 (3.1%)				2 (1.0%)
11	.....K...T..Q.....		1 (2.9%)						1 (0.5%)
12	.....T.....Q..L.....	1 (2.3%)							1 (0.5%)
13	.....T....N.Q..L.....	1 (2.3%)							1 (0.5%)
14	.....K...E.Q..L.....				1 (3.1%)				1 (0.5%)
15	.....N.Q..L.....			1 (3.0%)					1 (0.5%)
16	.....NMQ..L.....			1 (3.0%)					1 (0.5%)
17	.....TN.Q..L.....				1 (3.1%)				1 (0.5%)
18	.....R.MQ..L.....	1 (2.3%)							1 (0.5%)
19	.....RN.Q..L.....	1 (2.3%)							1 (0.5%)
20	.....RN.Q..L.....	1 (2.3%)							1 (0.5%)
21	.....Q..L.....					1 (7.1%)			1 (0.5%)
22	.....Q..L.....						1 (4.5%)		1 (0.5%)
23	.....E.....Q..L.....							1 (5.3%)	1 (0.5%)
Total		43	34	33	32	14	22	19	197

FIG. 1. Proportions and nonsilent mutations observed in CSP sequences of alleles found in Bao Loc, Vietnam (1998 to 2003), as well as in Flores, Indonesia, and Thaton, Myanmar. No significant difference in allele frequencies was noted over the years (χ^2 = 70.003, degrees of freedom = 57, P = 0.116). The 7G8 strain (10) is used as the reference; BL, FL, and TT, Bao Loc, Flores, and Thaton, respectively. The figures adjacent to these letters representing the cities indicate the year of sample collection. The (I→M) mutation appears to be unique to Southeast Asian populations (26; this study).

(a)

Allele	326 P	S	D	K	H	I	332 E	333 Q	Y	L	336 K	337 K	338 I	339 Q	340 N	341 S	342 L	Vietnam	Indonesia	Myanmar	Total
7G8	CCA	AGT	GAT	AAG	CAC	ATA	GAA	CAA	TAT	TTA	AAG	AAA	ATA	AAA	AAT	TCT	ATT				
1	...	...	...	...	...	...	...	...	...	...	...	...	...	C..	...	...	C..	77 (54.2%)	33 (91.7%)	15 (78.9%)	125 (63.5%)
2	...	...	...	...	...	...	...	...	...	...	...	..T	...	C..	...	...	C..	19 (13.4%)			19 (9.6%)
3	...	...	...	...	...	...	...	...	...	...	...	...	..G	C..	...	...	C..	15 (10.6%)	1 (2.8%)		16 (8.1%)
4	...	...	...	...	...	...	...	...	...	...	...	...	...	C..	T..	...	C..	7 (4.9%)		3 (15.8%)	10 (5.1%)
5	...	...	...	...	...	...	AC.	G..	...	...	...	...	...	C..	...	...	C..	7 (4.9%)	2 (5.6%)		9 (4.6%)
6	...	...	...	...	...	...	A..	G..	...	...	.C.	...	...	C..	...	...	...	5 (3.5%)			5 (2.5%)
7	...	...	...	...	...	...	...	...	...	...	...	G..	...	C..	...	...	C..	3 (2.1%)			3 (1.5%)
8	...	...	...	...	...	...	...	...	...	...	.G.	..T	...	C..	...	...	C..	2 (1.4%)			2 (1.0%)
9	...	...	...	...	...	...	A..	...	...	...	.C.	...	...	C..	...	...	...	1 (0.7%)			1 (0.5%)
10	...	...	...	...	...	...	AC.	...	...	...	...	...	...	C..	...	...	C..	1 (0.7%)			1 (0.5%)
11	...	...	...	...	...	...	AC.	...	...	...	...	..T	...	C..	...	...	C..	1 (0.7%)			1 (0.5%)
12	...	...	...	...	...	...	...	A..	...	...	...	G..	...	C..	...	...	C..	1 (0.7%)			1 (0.5%)
13	...	...	...	...	...	...	...	...	...	...	...	..T	..G	C..	...	...	C..	1 (0.7%)			1 (0.5%)
14	...	...	...	...	...	...	...	...	...	...	.C.	..T	...	C..	...	...	C..	1 (0.7%)			1 (0.5%)
15	...	...	...	...	...	...	...	...	...	...	.G.	...	..G	C..	...	...	C..	1 (0.7%)			1 (0.5%)
16	...	...	...	...	...	...	...	G..	...	...	...	...	...	C..	...	...	C..			1 (5.3%)	1 (0.5%)

(b)

Allele	366 A	367 N	K	369 P	K	D	372 E	L	374 D	Y	E	N	D	I	380 E	Vietnam	Indonesia	Myanmar	Total
7G8	GCT	AAT	AAA	CCT	AAA	GAC	GAA	TTA	GAT	TAT	GAA	AAT	GAT	ATT	GAA				
1	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	113 (79.6%)	34 (94.4%)	15 (78.9%)	162 (82.2%)
2	...	G..	...	...	...	...	C..	...	...	...	...	...	...	...	...	9 (6.3%)	1 (2.8%)	3 (15.8%)	13 (6.6%)
3	...	...	...	...	...	...	C..	...	...	...	...	...	...	...	...	9 (6.3%)			9 (4.6%)
4	...	GG.	...	...	...	...	...	...	A..	...	...	...	...	...	...	6 (4.2%)			6 (3.0%)
5	...	GG.	...	T..	...	...	...	...	...	...	...	...	...	...	...	2 (1.4%)	1 (2.8%)		3 (1.5%)
6	...	G..	...	...	...	...	...	...	...	...	...	...	...	...	...	2 (1.4%)			2 (1.0%)
7	...	...	...	...	...	...	...	...	A..	...	...	...	...	...	...			1 (5.3%)	1 (0.5%)
8	...	GG.	...	...	...	...	...	...	...	...	...	...	...	...	...	1(0.7%)			1 (0.5%)

FIG. 2. Nucleotide sequence variation in the Th2R (a) and Th3R (b) epitope regions of the circumsporozoite gene among *P. falciparum* field isolates collected from Southeast Asia. The numbers above indicate amino residue positions in reference to the sequence of the 7G8 strain (10).

found in BL2001, BL2002, and BL2003, respectively. However, unlike for the Th2R epitope, no new variant was noted. As shown in Fig. 2, the most common variants in Bao Loc for both epitopes appear to be the "universal" variants described by Escalante et al. (14), i.e., PSDKHIEQYLKKIQNSL for the Th2R epitope (54.2%) and GSANKPKDELDYENDI for the Th3R epitope (79.6%). These same sequences were recently reported to be predominant alleles in western Thailand (26); however, they contrast with the sequences described in The Gambia (1). Consistent with most previous reports, we noted that the Th2R epitope appears to be more polymorphic than the Th3R epitope, a finding that is reflected both in the number of allelic types and in the diversity parameters (Table 2; Fig. 2). For instance, in each group of isolates, the diversity in Th2R is almost twice that in Th3R except for BL2001. With the exception of the Th2R and Th3R epitopes, no mutation was found in the other regions of the gene studied (Fig. 1).

DISCUSSION

Because CSP is one of the most widely characterized malaria vaccine candidate antigens, and the only one whose components have gone so far as completion of a successful phase IIb clinical trial (3, 6), the generation of relevant epidemiologic and immunologic data for the CSP gene is of crucial significance, but such data are lacking, particularly for regions of low malaria endemicity. The first longitudinal study on the sequence polymorphisms of the T-cell epitopes of CSP for a setting of low to moderate malaria endemicity is presented here for a 5-year observation period in southern Vietnam. The region of the gene was chosen since it has been established that most sequence variation within the CSP gene has largely been restricted to the B- and T-cell epitopes, of which the 3′ portion is the most polymorphic (14, 22, 34). Like Doolan et al. (13) and Jongwutiwes et al. (24), we have noted that the genetic diversity within the circumsporozoite protein of *P. falciparum* isolates appears to be regionally restricted, as portrayed by both the many unique, previously undescribed sequences found in this study and the apparent temporal stability of the predominant alleles noted here. For regions of low to moderate malaria endemicity (like Bao Loc), there seems to be a major allelic type representative of the population, together with minor variants present in different proportions (26, 36; this study). This is in contrast to the situation in regions of high endemicity, like Africa, where genetic polymorphisms are reportedly high (1, 4, 27). For instance, the most common allele in Vietnam (allele 1 in Fig. 1) appears to be the major sequence reported among isolates from India (14), Thailand (24), Myanmar (12), and Vanuatu (36) and has a sequence identical to those of the T4 and T9-101 strains, both of which originated from Thailand. Most of the Brazilian sequences reported by De Stricker et al. (12), including the B1 strain, were identical to those of the 7G8 strain and three Venezuelan isolates reported by Escalante et al. (14). Our results thus seem to suggest that variation in the Th2R and Th3R epitopes of CSP in Vietnam (or presumably in Southeast Asia) may not be due to frequency-dependent selection, since the frequencies of predominant alleles were stable over the period of study, while rare variants remained at a lower frequency. Similar findings have been documented for the blood-stage malaria merozoite surface proteins in Vietnam (15) and The Gambia (9). However, the absence of variation in allele frequencies over this period of a few years alone may not completely rule out the possibility of balancing selection operating over a different

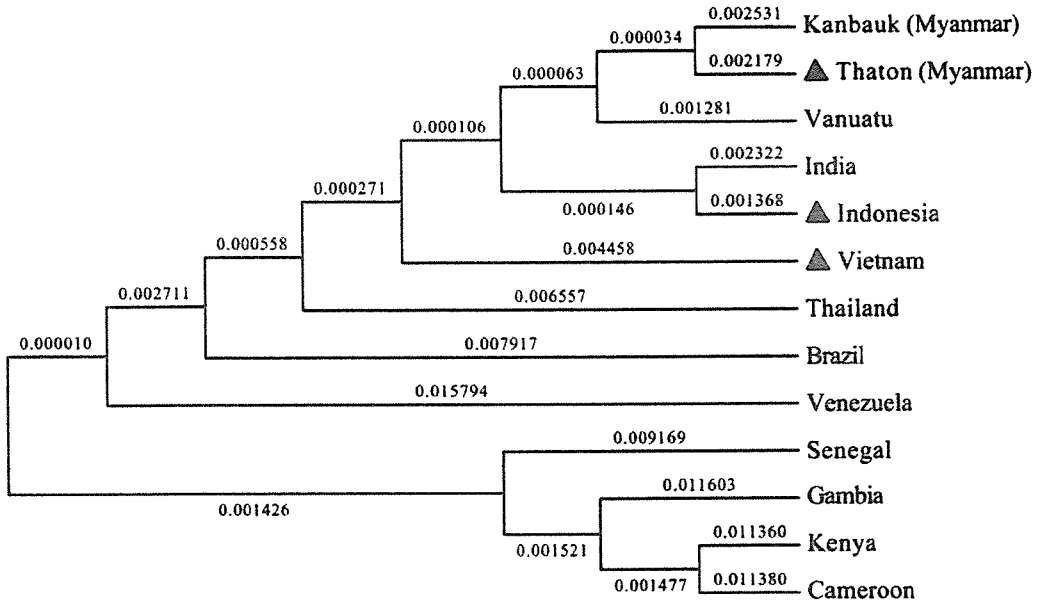

FIG. 3. A neighbor-joining tree based on population pairwise distances. Dxy is based on the average number of pairwise nucleotide substitutions per site between populations (30). Calculations were done with DnaSP (version 4.0) by using the partial *P. falciparum* CSP sequences from this study (shaded triangles) as well as from GenBank, and values were exported to MEGA2.1 for reconstruction of this tree. Note the distinct clusters among African and Southeast Asian sequences. Kanbauk and Thaton are both in Myanmar.

time scale. Moreover, it would be interesting to see temporal patterns of genetic diversity for CSP in an African setting.

Numerous reports have documented that in areas with higher rates of malaria transmission intensity the malaria isolates generally exhibit a greater amount of genetic diversity and that residents of these high-transmission areas tend to acquire antimalarial immunity at an earlier age than in areas where transmission is less intense. Also, transmission intensity is correlated with the prevalence of malaria and is a direct measure of parasite reproduction. This scenario is somewhat exemplified in our study, since most of the malaria patients presenting at the hospital were adults (Table 2), and the sequence heterogeneity observed in BL1998 alone (within a period of 4 months of the high-transmission season) was marked compared to that in the following years (Fig. 1). Although the effects of sampling or chance cannot be completely ruled out, this finding is consistent with the notion that the transmission dynamics together with other factors may be crucial in the generation and maintenance of genetic diversity within this antigen.

From this analysis of the most polymorphic part of the CSP gene, we have noted that the observed polymorphisms could be a result of host selective pressures, possibly the immune system (11, 22, 23), and importantly, there appear to be constraints on the parasite's ability to change. This probably reflects a balance between the parasite's biological and survival requirements. Within the Th2R epitope, mutations appear to be restricted both to certain positions, e.g., at residues 332 to 333 and 336 to 340, and to the nature of the replacing residue, as evidenced by the fact that all but one of the replacements were hydrophilic. The exception is the replacement with a neutral residue (I→M), which is possibly a physiologically acceptable substitution within the protein's conformational context. To date, this residue change appears to be restricted solely to Southeast Asian populations (26; this study), suggesting a possible role for host factors. A similar pattern was noted in Th3R, although both hydrophobic and hydrophilic replacements were present. Rathore and McCutchan (31) demonstrated that the region of CSP containing the cytotoxic T-lymphocyte epitopes plays a significant role in hepatocyte binding and that an intact carboxyl end is essential for this binding process. Thus, the host immune system may recognize a sequence motif that is essential for parasite survival, implying that only mutations that could at least satisfy the dual requirements of immune evasion and parasite survival would be favored. Some studies (7, 18, 37) have indicated that amino acid variations in T-cell epitope sequences affected HLA binding, whereas others more directly affected T-cell receptor residues by inducing antagonistic peptides. Taken together, these findings suggest that polymorphisms in CSP are not merely an arms race but are part of a complex process that may be driven and maintained by a balance of opposing forces resulting from interaction between the parasite, the host, and/or other environmental factors. It would be interesting to investigate the distribution and frequency of MHC alleles and their association with local epitope variants in these populations to better understand some of these mechanisms.

Table 1 shows the sequence diversity parameters for partial sequences of the CSP gene of *Plasmodium falciparum* field isolates from different countries. Apart from the sequences from Venezuela, there seem to be distinct differences among isolates from different regions, namely, Africa, Latin America, and Asia. This is reflected in the number of haplotypes as well as average genetic distances. Grouping of the sequences into the regional categories described above (Brazil and Venezuela as Latin American; Kenya, Cameroon, The Gambia, and Sene-

gal as African; and Vietnam, Myanmar, Indonesia, Thailand, Vanuatu, and India as Asian) and independent estimates of average Wright's fixation index (F_{ST}) values using the method of Hudson et al. (21) for each group revealed values of 0.428, 0.023, and 0.025 for the Latin American, African, and Asian parasite populations, respectively. Again, the high F_{ST} value noted for the American isolates is because of the marked diversity of the Venezuelan isolate sequences relative to that of isolates from Brazil. The computation of F_{ST} for, say, African versus Asian isolates or Asian versus Latin American isolates resulted in values up to 10 times higher than those shown above (data not shown). The average F_{ST} value for all populations combined was 0.257, a value suggestive of distinct differentiation among the subgroups. A similar result was obtained by phylogenetic analyses based on exported Dxy genetic distances (Fig. 3). Furthermore, tests of neutrality (Tajima's [35] D as well as Fu and Li's [17] F^*) also reveal distinct patterns among African and Asian parasite populations, with the implication that the former are under balancing selection, whereas the Asian sequences all had negative values. However, caution in the interpretation of the results of these tests is necessary, since most lack statistical significance (Table 1), and because the power of these tests is affected by the number of mutations in the sample (more segregating sites have more power), the results could have also been influenced by the length of the DNA region sequenced.

In conclusion, it is apparent from this study that diversity in the *Plasmodium falciparum* CSP gene appears to be regionally restricted, as shown by the distinct differences in Asian and African parasite populations. Moreover, within different countries there seem to be unique mutation patterns. Taken together, these findings seem to suggest that regional and environmental factors, together with host genetic factors, could be crucial in generating polymorphisms, at least in the carboxy-terminal T-cell epitopes of *P. falciparum* CSP. Irrespective of what governs these polymorphisms, the absence of variation in T-cell epitope haplotype frequencies of CSP over relatively short periods of time is a very interesting finding, with major implications for malaria vaccine development (since no new variants seem to emerge within a few years of exposure to locally prevalent variants). This is probably reflected in the recent apparent success in clinical trials of a CSP-based vaccine, RTS,S/AS02 (3, 6), and the observation that protection was not strain specific (2).

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2-Cys Peroxiredoxin TPx-1 is involved in gametocyte development in *Plasmodium berghei*

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Abstract

Peroxiredoxins (Prxs) constitute a ubiquitous family of antioxidant enzymes involved in diverse cellular functions including cell proliferation and differentiation. To investigate the physiologic role of typical 2-Cys Prx in malaria parasites (TPx-1), we disrupted this gene in the rodent malaria parasite *Plasmodium berghei* (*pbtpx-1*). The gene-disrupted parasite (Prx KO) developed normally in mouse erythrocytes and multiplied at a rate similar to that of the parent strain (WT) during the experimental period. The normal growth rate was not altered after 10 passages, and the level of 8-hydroxy-2′-deoxyguanosine, which accumulates in the parasite genome during the cell cycle, was similar between Prx KO and WT. These results suggest that TPx-1 does not prevent parasite DNA oxidation, in contrast to mammalian Prx, and that it is not essential for asexual parasite growth in mouse erythrocytes. However, Prx KO produced up to 60% fewer gametocytes, sexual-stage parasites involved in the transition between the mammalian host and the mosquito, than WT did. The peak of gametocytemia was also delayed; however, the male/female ratio of gametocytes and the exflagellation activity of male gametocytes were normal. These results suggest that TPx-1 is required for normal gametocyte development but does not affect the male/female gametocyte ratio or male gametogenesis. Although the mechanism by which PbTPx-1 contributes to gametocyte development remains unknown, these findings suggest, for the first time, the involvement of Prx in the sexual development of the malaria parasite.

Keywords: Gametocyte; Peroxiredoxin; *Plasmodium berghei*; Thioredoxin peroxidase

1. Introduction

As *Plasmodium* spp. actively proliferate within erythrocytes of their vertebrate hosts, large quantities of reactive oxygen species (ROS), which damage biologic macromolecules, are generated [1,2]. A major source of ROS in parasite cells is heme, a byproduct of hemoglobin digestion for amino acid procurement [3,4]. ROS are also generated when the organism is exposed to various stress conditions such as that induced by the host immune system [5]. *Plasmodium* spp. (the malaria parasites) are highly susceptible to oxidative stress, and their antioxidant defenses are considered to play essential roles in their asexual development. They are thus potential targets for chemotherapy [6,7].

To protect biologic macromolecules from the effects of ROS, aerobes have evolved efficient defense systems composed of nonenzymatic and enzymatic antioxidants [8]. The four major cellular antioxidant enzymes are superoxide dismutase, catalase, glutathione (GSH) peroxidase, and peroxiredoxin [9]. Peroxiredoxins (Prxs) constitute a family of proteins structurally homologous to the thiol-specific antioxidant of yeast [10] and have been identified in all living organisms, from bacteria to human [11,12]. There are three subtypes of Prxs, 1-Cys Prx, typical 2-Cys Prx, and atypical 2-Cys Prx. Although the cellular function of 1-Cys Prx and electron donor for the molecule remain controversial [12,13], 2-Cys Prxs have been found to act

Abbreviations: DHFR-TS, dihydrofolate reductase-thymidylate synthase; GSH, glutathione; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; Prx, peroxiredoxin; ROS, reactive oxygen species

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In conclusion, it is apparent from this study that diversity in the *Plasmodium falciparum* CSP gene appears to be regionally restricted, as shown by the distinct differences in Asian and African parasite populations. Moreover, within different countries there seem to be unique mutation patterns. Taken together, these findings seem to suggest that regional and environmental factors, together with host genetic factors, could be crucial in generating polymorphisms, at least in the carboxy-terminal T-cell epitopes of *P. falciparum* CSP. Irrespective of what governs these polymorphisms, the absence of variation in T-cell epitope haplotype frequencies of CSP over relatively short periods of time is a very interesting finding, with major implications for malaria vaccine development (since no new variants seem to emerge within a few years of exposure to locally prevalent variants). This is probably reflected in the recent apparent success in clinical trials of a CSP-based vaccine, RTS,S/AS02 (3, 6), and the observation that protection was not strain specific (2).

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each rat was separated into wild-type and disruptant by limiting dilution. Both parasite genomes were determined by PCR and Southern blot analysis.

2.4. Nucleic acid techniques

Parasite-infected blood was passed through a CF11 (Whatman, Maidstone, Kent, UK) column and span with Lymphoprep™ density gradient medium (Axis-Shield, Oslo, Norway) to remove leukocytes. Parasite-infected erythrocytes were then washed with PBS and lysed with PBS containing 0.05% saponin. Parasite pellets were washed several times with PBS, snap-frozen in liquid nitrogen, and stored at −80 °C until use. Nucleic acids were extracted from parasite pellets. For Southern blot, genomic DNA was extracted by the standard phenol–chloroform method and incubated with *Spe*I. For Northern blot, total RNA was extracted with TRIZOL reagent (Invitrogen) according to the manufacturer's protocol. Nucleic acids were separated by agarose gel electrophoresis and transferred onto HyBond N^+ membranes (Amersham Biosciences UK Ltd., Little Chalfont, UK). Membranes were probed with a 368-bp fragment spanning the 5′ sequence of *pbtpx-1* for Southern blot (Fig. 1B) and the entire coding sequence for Northern blot (Fig. 2B). The probes were labeled, hybridized, and detected with the AlkPhos Direct Labeling and Detection System (Amersham Biosciences).

2.5. Western blot analysis and indirect immunofluorescence assay

For Western blot analysis, parasite pellets were solubilized in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer containing 5% 2-mercaptoethanol [22]. After separation by SDS-PAGE (12.5%), parasite proteins were transferred electrophoretically to polyvinylidene difluoride sheets (Immobilon; Millipore, Bedford, MA, USA) and incubated with anti-recombinant PfTPx-1 (rPfTPx-1) rabbit IgG (25 μg/ml). Interspecific crossreactivity between PbTPx-1 and anti-rPfTPx-1 rabbit IgG has been reported [18]. Immune complexes were visualized with horseradish peroxidase-conjugated goat anti-rabbit IgG (Cappel, Aurora, OH, USA). Protein concentrations were determined with a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Indirect immunofluorescence assay was performed as described previously [15]. The IgG fraction of rabbit antisera to rPfTPx-1 was used at 20 μg/ml. Parasite cells on slides were mounted with Prolong AntiFade Solution (Molecular Probes, Eugene, OR, USA) and observed with a confocal laser scanning microscope (LSM510, Carl Zeiss, Jena, Germany).

2.6. Analysis of parasite development in mice

Five-week-old female Balb/c and C57B6 mice (Clea Japan) were infected with *P. berghei* (10^6 parasitized cells per mouse) by i.p. injection. Parasitemia and gametocytemia were determined by microscopic examination of Giemsa-stained thin blood films. Male and female gametocytes were distinguished by size and coloration. Numbers of exflagellation centers were counted according to a previously described method [23]. Accumulation of 8-hydroxy-2′-deoxyguanosine (8-OHdG) in the parasite genome was evaluated with a competitive enzyme-linked immunosorbent assay (ELISA) kit (8-OHdG Check, Japan Institute for the Control of Aging, Shizuoka, Japan) [24]. DNA for ELISA was extracted from parasites collected from infected mice (blood from 10 infected mice was pooled) when

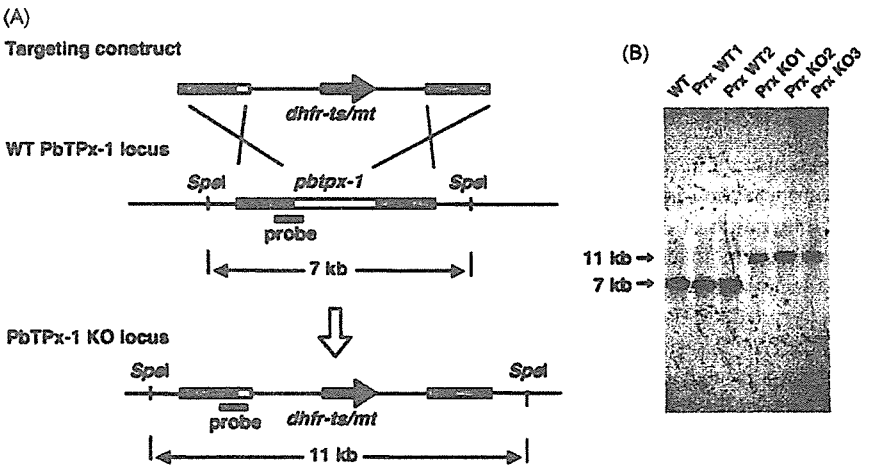

Fig. 1. Targeted disruption of the TPx-1 gene in *P. berghei*. (A) The targeting construct, composed of the 5′ and 3′ regions (hatched boxes) and partial open reading frame (open box) of the PbTPx-1 gene (*pbtpx-1*) and the pyrimethamine-resistant variant of DHFR-TS gene of *P. berghei* (*dhfr-ts/mt*) as a selectable marker (filled arrow), was integrated into the wild-type (WT) PbTPx-1 locus by double-crossover homologous recombination. Recombination disrupts *pbtpx-1* and creates the locus containing *dhfr-ts/mt* (PbTPx-1 KO locus), which confers pyrimethamine resistance to disruptants. The *Spe*I restriction sites and the position of the probe used for Southern blot analysis in B are indicated. (B) Southern blot analysis of genomic DNA samples from the parent parasite (WT), two wild-type parasite populations with pyrimethamine resistance (*dhfr-ts/mt* at the DHFR-TS locus) (Prx WT), and three *pbtpx-1* disruptant populations (Prx KO). DNA samples were digested with *Spe*I, separated on 0.7% agarose gels, transferred to nylon membranes, and hybridized with probe. The single 11-kb band in the Prx KO populations indicates *pbtpx-1* disruption, whereas the single 7-kb band in the WT and Prx WT populations indicates an intact PbTPx-1 locus.

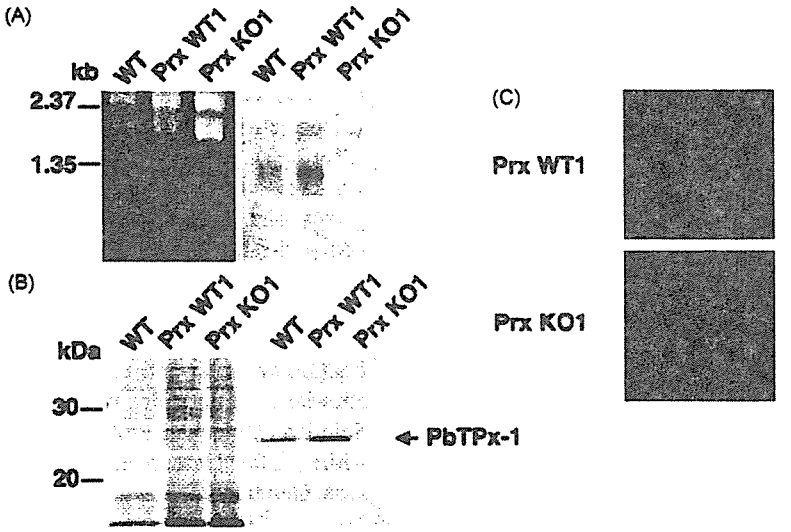

Fig. 2. Verification of the PbTPx-1 null phenotype in gene-disrupted *P. berghei*. Parasite populations, including the parent parasite (WT), Prx WT1 (*dhfr-ts/mt* at the DHFR-TS locus and intact *pbtpx-1*), and Prx KO1 (*pbtpx-1* disruptant) were inoculated into mice, and parasite-infected erythrocytes, total protein, and total RNA of the parasite cells were prepared. (A) The absence of TPx-1-specific mRNA expression in Prx KO was examined by Northern blot analysis. Total RNA samples (20 μg) were separated on 1.5% agarose-formaldehyde gel (left panel), transferred to nylon membranes, and hybridized with probe (right panel). Ethidium bromide-stained gel showed equal loading. Molecular size markers in kb are indicated on the left. (B) The absence of TPx-1 protein in Prx KO was examined by Western blot analysis. Total protein samples (20 μg) were separated by SDS-PAGE (left panel) and probed with anti-TPx-1 antibody (right panel). Coomassie brilliant blue-stained gel showed equal loading. Protein size markers in kDa are indicated on the left. (C) The absence of TPx-1 protein in Prx KO was confirmed by indirect immunofluorescence assay. TPx-1 protein is stained in green, and parasite nuclei are stained in blue.

parasitemia reached approximately 4% (4–5 days after infection). Levels of 8-OHdG (ng/mg DNA) are expressed as the mean of triplicate measurements.

2.7. Statistical analysis

Differences were evaluated with Student's *t*-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Targeted disruption of the PbTPx-1 gene

Merozoites in segmented schizonts were transfected with the targeting construct by electroporation and were subsequently inoculated into naïve rats. Integration of the construct into the PbTPx-1 locus by homologous recombination resulted in disruption of the single-copy gene and insertion of a selectable marker, the DHFR-TS gene with a pyrimethamine-resistance mutation (*dhfr-ts/mt*), at the locus (Fig. 1A). Parasites with *pbtpx-1* disruption were selected by treatment with pyrimethamine. PCR and Southern blot analyses showed that parasites selected with pyrimethamine were a mixture of wild-type parasites and *pbtpx-1* disruptants (data not shown). Parasite populations were separated into two groups by limiting dilution and subsequent inoculation into 18–22 rats. In two independent electroporation experiments, six disruptant populations (Prx KO) were obtained and cloned. In the first experiment, three Prx KO populations (Prx KO1–3) and two wild-type parasite populations (Prx WT1 and 2) were obtained (Fig. 1B). In the second experiment, two Prx KO populations (Prx KO4 and 5) were obtained (data not shown). The *dhfr-ts* locus of Prx WT parasites was amplified by PCR and sequenced, and replacement with the pyrimethamine-resistance mutation was confirmed (data not shown). Northern blotting, Western blotting, and immunofluorescence assay confirmed the TPx-1-null phenotype of Prx KO1 (Fig. 2). This phenotype was also confirmed for all Prx KO populations (data not shown).

3.2. PbTPx-1 gene disruption does not affect asexual development

Prx KO1–3 and Prx WT1 and 2 were used to infect Balb/c mice, and the course of parasite development within erythrocytes was compared to that of the parent strain (WT) (Fig. 3A). Two Prx WT populations showed equal development and multiplication within erythrocytes, similar to WT, and they showed a high level of parasitemia (>5%) 5 days after infection. The courses of parasitemia observed in Prx KO1-infected mice were also similar to that in WT-infected mice. This phenotype was confirmed in Prx KO2 and 3 and in Prx KO4 and 5, which were obtained from an independent electroporation experiment (Fig. 3B). There was no difference in the morphology of parasite cells between five Prx KO, two Prx WT, and WT populations (data not shown). These eight parasite populations did not kill mice until day 8 of observation. Prx KO1, Prx WT2, and WT populations each killed one of three mice, respectively, on day 9 of observation. It is known that Balb/c mice in some laboratories are resistant to the development of cerebral malaria (CM) induced by the *P. berghei* ANKA strain and do not develop lethal infection [25]. To determine the phenotype of *pbtpx-1* disruption in strains of mice with differing susceptibility to the *P. berghei*

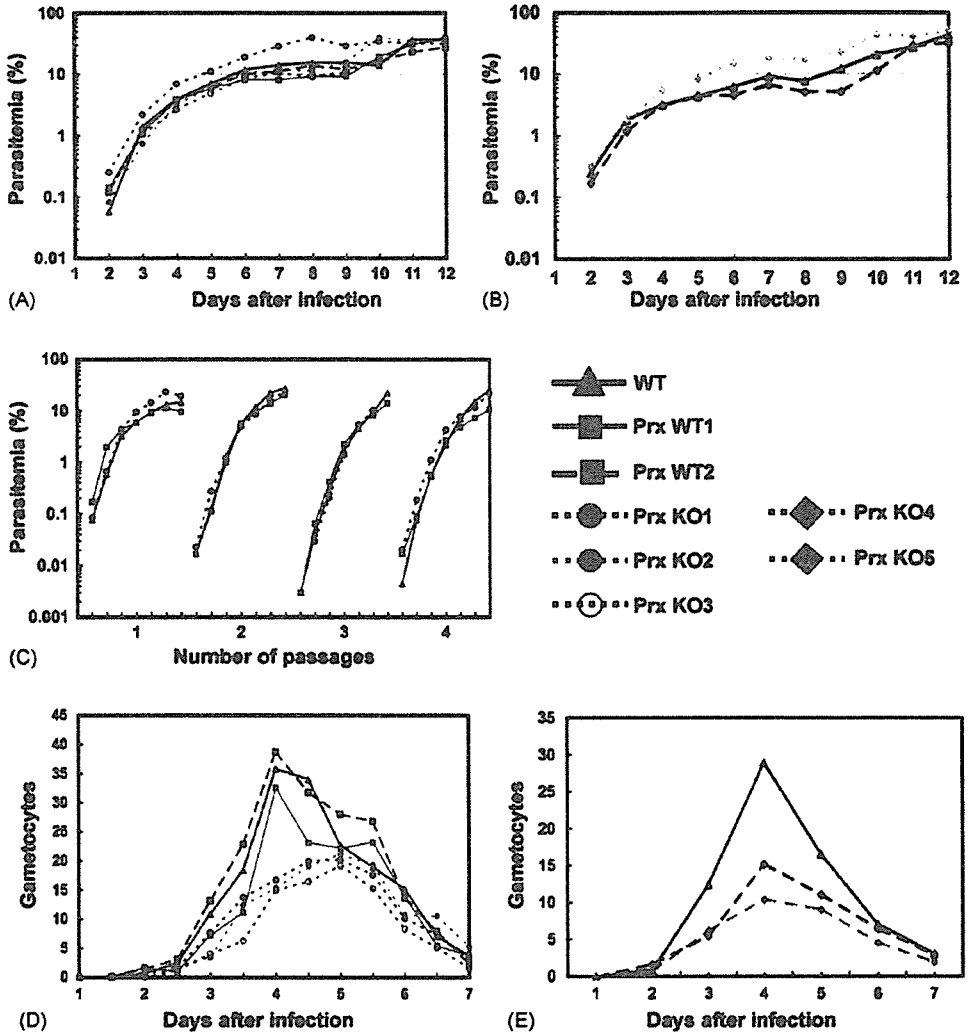

Fig. 3. Infection of mice with TPx-1 gene-disrupted *P. berghei*. Parasite populations, including the parent parasite (WT), two Prx WT (*dhfr-ts/mt* at the DHFR-TS locus and intact *pbtpx-1*) and five Prx KO (*pbtpx-1* disruptant), were inoculated into mice, and the courses of parasite development in erythrocytes were observed. (A and B) Changes in parasitemia 12 days after infection (three mice per group: six groups in (A) WT, Prx WT1 and 2, and Prx KO1–3; three groups in (B) WT and Prx KO4 and 5). (C) Changes in the course of parasitemia during four blood passages (two mice per group: three groups, WT, Prx WT1, and Prx KO1). (D and E) Changes in gametocytemia 7 days after infection (three mice per group: six groups in (D) WT, Prx WT1 and 2, and Prx KO1–3; three groups in (E) WT and Prx KO4 and 5). Data are mean values of parasitemia percentage (A–C) and gametocyte number (gametocytes/10^4 erythrocytes) (D and E) in each group.

ANKA strain, C57B6 mice, which are known to develop CM and lethal infection [25], were infected with Prx KO1, and the course of parasite development within erythrocytes was compared to that of WT. Prx KO1 and WT multiplied equally and killed mice 7–8 days after infection. The morphology of parasite cells and the numbers of animals killed during 8 days of observation between the two parasite populations did not differ (data not shown).

A function of 2-Cys Prx in the prevention of damage to DNA by oxidative stress has been suggested in mammals [26]. Therefore, the cumulative effect of oxidative stress on parasite growth in vivo in the *pbtpx-1* disruptant was evaluated. For this purpose, Prx KO1, Prx WT1, and WT were continuously maintained in Balb/c mice by inoculating parasite-infected blood and comparing the course of parasitemia between passages. Blood passage was performed on the 7 day of infection and was repeated four times. The courses of parasitemia observed in Prx KO1-, Prx WT1-, and WT-infected mice from the first to the fourth passage are shown in Fig. 3C. The course of parasitemia after four passages compared to that after the first inoculation did not differ between the three parasite populations. Passage was repeated six more times, and the course of parasitemia observed for the three parasite populations at the 10th passage was similar to that observed at the fourth passage (data not shown). To determine whether *pbtpx-1* disruption enhances DNA damage in the parasite genome, the level of 8-OHdG, a marker of oxidative DNA damage, was compared between Prx KO1 and WT. The level of 8-OHdG in the parasite genome was similar between Prx KO1 (11.7 ng/mg DNA) and WT (9.1 ng/mg DNA), suggesting that *pbtpx-1* disruption does not affect the physiologic level of DNA oxidation. This result was confirmed in two independent experiments (data not shown).

3.3. PbTPx-1 gene disruption affects gametocytogenesis

To evaluate the effect of *pbtpx-1* disruption on development of sexual-stage parasites, the numbers of gametocytes in parasite-infected blood were counted and compared among Prx KO1–3, Prx WT1 and 2, and WT (Fig. 3D). In Prx WT1- and WT-infected mice, gametocytes were observed from 2 days after infection; they increased in number with parasitemia progression and peaked 4 days after infection. Gametocytemia, recorded as the number of gametocytes/10^4 erythrocytes in Prx WT1- and WT-infected mice at the day 4 of infection, was 32.5 ± 5.5 and 35.8 ± 11.6, respectively ($P = 0.34$). The course of gametocytemia between Prx WT1- and WT-infected mice did not differ. This phenotype was confirmed in two populations of Prx WT. In Prx KO1-infected mice, gametocytes were also observed from 2 days after infection and peaked 5 days after infection. The peak of gametocytemia in Prx KO1-infected mice was thus delayed 1 day from that of Prx WT1- and WT-infected mice. Gametocytemia, recorded in Prx KO1-infected mice as the number of gametocytes/10^4 erythrocytes on day 5 of infection, was 20.2 ± 0.9. The highest number of gametocytes was thus significantly lower ($P < 0.01$) in Prx KO1-infected mice than in WT-infected mice. The *pbtpx-1* disruption phenotype, in addition to the delay in gametocytemia peak, showed a significant decrease in the numbers of gametocytes ($P < 0.02$) in Prx KO1–3 and in Prx KO4 and 5, which were obtained from an independent electroporation experiment (Fig. 3E).

To determine whether *pbtpx-1* disruption affects the male/female ratio of gametocytes or exflagellation activity of male gametocytes, Prx KO1, Prx KO4, Prx WT1, and WT were used to infect mice (Table 1). The male/female ratios of gametocytes in the three parasite populations at the day of peak gametocytemia were similar: 0.44, 0.53, and 0.64 for Prx KO1-, Prx WT1-, and WT-infected mice in experiment 1; 0.62 and 0.50 for Prx KO4- and WT-infected mice in experiment 2. This phenotype was confirmed in five populations of Prx KO (data not shown). The percentages of exflagellation, calculated according to the numbers of exflagellation centers and the numbers of male gametocytes in the blood at the day of peak gametocytemia, were 70, 64, and 76% for Prx KO1-, Prx WT1-, and WT-infected mice in experiment 1; 59 and 60% for Prx KO4- and WT-infected mice in experiment 2. Thus, the majority of male gametocytes observed in blood were equally viable in terms of exflagellation activity among the three parasite populations. This finding suggests that low gametocytemia in Prx KO is attributed to impaired development of gametocytes rather than to their decreased survival.

4. Discussion

The results presented here suggest that *pbtpx-1* disruption does not affect asexual intraerythrocytic growth of parasites. The results from passage experiments and evaluation of 8-OHdG suggest that TPx-1 does not prevent parasite DNA oxidation, in contrast to mammalian Prx [26], and that it is not essential for asexual parasite growth in mouse erythrocytes. However, the fact that the parasite lacks catalase and genuine GSH peroxidase and that the major cytosolic peroxide-detoxifying capacity appears to be provided by Prx may contradict the present findings [1,2]. Microarray analysis of the disruptant and the parent parasite may provide insights with respect to redundancy in parasite antioxidant defenses and compensation for the lack of Prx. Our results may not indicate that the gene is inessential for asexual growth in *P. falciparum* because there are differences in the life cycle of the mammalian stage between *P. falciparum* and rodent malaria parasites. *P. falciparum* develops in erythrocytes sequestered in the microvasculature, where the parasite may experience more severe oxidative stress than do parasites in the blood circulation [5]. The parasite may require Prx for development under such stressful conditions. Such a possibility should be investigated in a monkey model of *P. falciparum* infection [27]. The relatively high level of 8-OHdG formation in the parasite genome compared to that in mammalian cells [24] may reflect the absence of nuclear Prx in this organism [1,2,9]. How malaria parasites deal with the accumulation of oxidative DNA damage is of interest; a hypothetical protein with putative excision DNA repair function or DNA glycosylase function (PF10835c) has been identified in the genome of the malaria parasite.

Table 1
Male/female ratios of gametocytes and exflagellation activity of male gametocytes in TPx-1gene-disrupted *P. beghei*[a]

	Experiment 1			Experiment 2	
	WT	Prx WT1	Prx KO1	WT	Prx KO4
Peak of gametocytemia					
Gametocyte number[b]	30 ± 5.5	37 ± 5.5	18 ± 2.5	31 ± 3.2	14 ± 1.6
Days after inoculation	4.0	4.0	4.5	4.0	4.5
Male/female ratio					
Day of peak gametocytemia	0.64	0.53	0.44	0.50	0.62
Day 5.5	1.06	1.07	0.92	1.01	1.15
Exflagellation (%)[c]					
Day of peak gametocytemia	76 (52–88)	64 (51–81)	70 (53–81)	60 (54–64)	59 (55–63)
Day 5.5	16 (10–20)	13 (7–27)	24 (9–54)	14 (12–17)	4.1 (0.6–10)

[a] Values are means ± standard deviations (gametocyte number), mean (male/femal ratio), and mean with range (exflagellation, %) calculated from data obtained from four mice.
[b] Gametocytes per 10^4 erythrocytes.
[c] Exflagellation (%) = (number of exflagellation centers in 10^4 erythrocytes/number of male gametocytes in 10^4 erythrocytes) × 100.

Nonetheless, the results presented here suggest that PbTPx-1 is required for normal gametocyte development; however, it does not affect the male/female ratio of gametocytes. We could not observe the defect in gametocyte development in *P. falciparum*, since we disrupted the gene in the FCR-3 strain, which produces only a little numbers of gametocyte [16]. Gametocytes are sexual-stage parasites involved in the transition between the mammalian host and the mosquito. This stage arises during asexual cycling, and it has generally been accepted that the trophozoites of the preceding asexual generation are already committed to either develop into gametocytes or continue asexual cycling [28]. Although the mechanism by which PbTPx-1 contributes to gametocyte development is unknown, ROS and antioxidants, including Prx, are known to influence the expression of a number of genes and to influence signal transduction pathways during cell differentiation and during organismal development [29,30]. A model in which Prx regulates peroxide-mediated signaling cascades by acting as a floodgate for H_2O_2 in mammalian cells has been proposed [12,14]. Homology modeling of PbTPx-1 based on structural alignment with human homologues showed that it possesses a redox-sensitive type of peroxidatic active site structure, which enables the enzyme to act according to the floodgate model (data not shown). A model in which 2-Cys Prx regulates activation of stress-activated mitogen-activated protein kinase (MAPK) (Sty1) by forming peroxide-induced disulphide complexes in yeast has also been proposed [31]. A molecule homologous to Sty1 (MAPK1) has been identified in the *P. falciparum* genome, although its function is unknown. Gametocyte development can be induced by environmental factors such as host factors or drug treatments, and there is consistent evidence for the involvement of signal transduction pathways in this process [28]. PbTPx-1 may be involved in transducing extracellular signals for gene expression and in initiation of gametocyte development. If this is the case, gametocyte development and its sexual determination may occur independently, with PbTPx-1 participating only in the former process. Alternatively, PbTPx-1 may contribute to protein synthesis in both male and female gametocyte development as a molecular chaperone. Evidence that Prx acts as a molecular chaperone in yeast and in human cells has recently been reported [32,33]. PbTPx-1 may not take part in the process of male gamete formation, including gender-specific signaling, because the disruptant retained normal exflagellation activity [34,35]. Whether it participates in female gametogenesis will require fertilization experiments in vitro and in the mosquitos [35,36]. Although the mechanism by which PbTPx-1 contributes to gametocyte development remains unknown, the present study suggests the involvement of this moleclue in the sexual development of the malaria parasite. Further studies to elucidate the role of TPx-1 in gametocyte development will provide further insight into the involvement of this antioxidant protein in the sexual development of malaria parasites and may provide novel transmission-blocking strategies.

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はじめに

1951年に検疫法が公布されて以来，数次の改正を経て検疫対象疾患は減少し，1996年の第6次改正では，コレラ・ペスト・黄熱病の3疾患となった。しかし，1998年，新感染症法の制定を受け，検疫感染症にエボラ出血熱やラッサ熱などの1類感染症が加えられることになった。さらに2003年には，感染症法の改定を受けてSARSや痘瘡といった新1類感染症が加えられる一方，マラリアやデング熱といった一部の4類感染症も検疫感染症として指定されて，あわせて11疾患となるに至った。これらの改正は，病原体を使ったバイオテロリズムの危険性への対応という側面もあるが，やはり大きな理由は，SARSや高病性鳥インフルエンザといった新しい感染症が，文字通り地球規模で問題となっていることにある。これらの感染症のわが国への侵入を水際で食い止めようという試みが，検疫感染症の拡大につながっているわけだが，その点，2003年の改正で，日本で今後再興感染症として問題化が懸念される蚊媒介性疾患から，マラリアやデング熱が検疫感染症に加わったのは，従来とは質的に異なる改

Malria : An Infectious Disease for Quarantine in Japan

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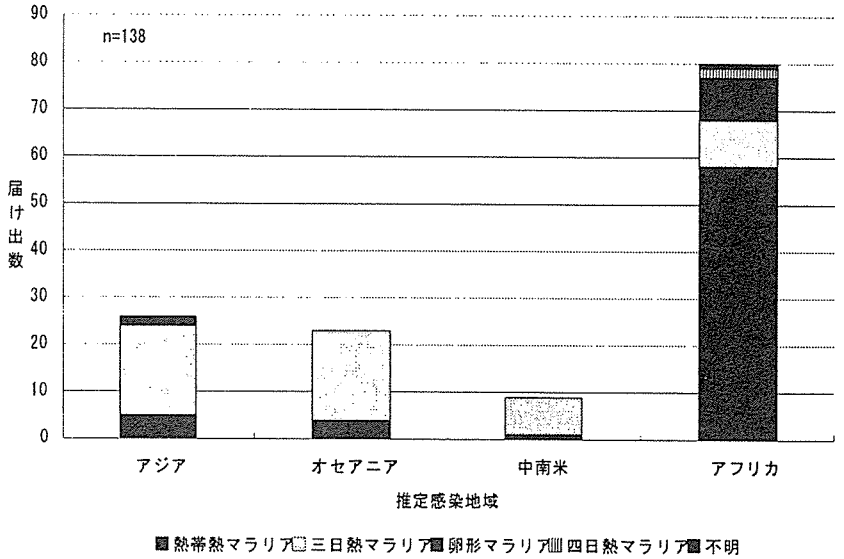

図1　わが国における輸入マラリアの傾向（2004～2005年）[6] 原図　木村幹夫ら

表1　成田国際空港健康相談室におけるマラリア検出状況（1997～2005年）

年度	陽性者数／検査数	マラリアの種別と推定感染地
1997	0／7	
1998	4／61	熱帯熱4（アフリカ3，アジア1）
1999	2／51	熱帯熱1（アフリカ）　三日熱1（アジア）
2000	2／45	熱帯熱2（アフリカ1，アジア1）
2001	1／84	熱帯熱1（アジア）
2002	5／143	熱帯熱4（アフリカ2，アジア2）　三日熱1（アジア）
2003	1／147	三日熱1（南米）
2004	1／121	熱帯熱1（アフリカ）
2005	0／101	
総計	16／760	熱帯熱14（アフリカ9，アジア5）　三日熱4（アジア2，南米1）

正と言えよう。本稿においては，地球温暖化の問題とも絡んで，検疫感染症としてのマラリアへの対応の現状と今後について論じたい。

最近のわが国におけるマラリアの動向

感染症発生動向調査（期間：1999年4月～2004年12月）によると，この間のマラリアの報告症例は約600例だが，年度別では2000年をピークとして，2002年以降は年間100例以下となって減少する傾向を示している。種別では，熱帯熱マラリアが最も多く，次いで三日熱マラリアであったが，卵形マラリア，四日熱マラリアは少数例にとどまった（図1）。地域別にみると，推定感染地域ではアフリカが最も多く，アジア，オセアニアがそれに続く。また，種別の動向を地域毎にみると，アフリカでの感染では70%が熱帯熱マラリアであったのに対し，アジアでの感染では逆に70%が三日熱マラリアであった。近年，アジア諸国ではマラリア対策が成功し，マラリアの死亡者数や罹病者数が減少するとともに，特に熱帯熱マラリアの感染者数が減少しているとの報告が多い[1]。国内での輸入マラリアの感染地をみても，その状況を反映してか，アジアが感染地として推定される例は減少する傾向を示している。もっとも，アジアといっても地域差が見られ，東南アジアを推定感染地とする例に比して，インドを中心とした南アジアを推定感染地とする例はあま

表2　アジア地域から成田国際空港への直行便おいて採取された蚊族の状況

採集された蚊の種類	採集できた飛行機数と起点及び経由地
Culex pipiens pallens group	6　バンコク2, ムンバイーデリーーバンコク3, シンガポールーマニラ
Culex tritaeniortynchus	1　デリー
Other Culex spp.	1　シンガポールーバンコク
Other mosquitos	3　ムンバイーデリー2,　ムンバイーデリーーバンコク
総　計	11　(総機内検査数：970)

り減少していない。

一方，国内感染患者としては，1961年に国内でのマラリア生活環による流行は終結したとされるが，1971年以降少なくとも11例が報告されている[2]。7例は輸血や針刺し事故であるが，マラリア流行地から飛来した航空機によって運ばれてきた感染蚊による伝播が示唆された空港マラリアの1例，感染経路が確定されなかった2例があり，これらはいずれも三日熱マラリアであった[2)3)]。

検疫におけるマラリアへの対応

感染症防疫における検疫の大きな目的は，国内における2次感染を防ぐことにある。検疫感染症としてマラリアをみた場合，海外でマラリアに感染しマラリア原虫を保有したヒトが帰国して感染源となる場合と，航空機や船舶によって運び込まれたマラリア感染蚊が国内で吸血する場合の2つを分けて考える必要がある。前者に対しては，国際空港における健康相談室が第1線機関としての役割を果たしているが，成田空港健康相談室におけるマラリアの検出数は，近年，国内全体の輸入マラリア報告数が示す傾向以上に，減少する傾向を示している（表1)。前述したように，わが国に近く，渡航者も多いアジアや南太平洋地域のマラリア流行地では，症状に乏しく潜伏期間の特定が難しい三日熱マラリアの例が相対的に増加している。また，わが国でもマラリア予防内服に利用できるメフロキンが市販され入手しやすくなった結果，たとえマラリアに感染していても帰国時点で発症していない例が増えているのかもしれない。しかし，これらのことが，健康相談室におけるマラリア検出数の減少と実際どの程度関係しているかについては，推測の域を出ない。

一方，感染蚊の流入について考えると，航空機内の昆虫に関して，着陸した飛行機に対する機内調査が継続して行われている。現在，採集した蚊について種の鑑別は行われているが，マラリア原虫保有の有無については正確に調査する体制は整っていない。現在，わが国の国際路線航空機の約60%は成田国際空港に到着するが，その中にはアジア・南太平洋地域のマラリア浸淫地13カ国からの直行便も含まれている。ただし，2001年から2005年までの調査でみると，ハマダラカは北米からの便で採取されたことがあるだけで，アジア・南太平洋地域からの便で採取されたのはイエカが中心であった（表2)。アフリカの熱帯熱マラリアからの直行便が多く飛来するヨーロッパでは，いわゆる空港マラリアが大きな問題となっているが，わが国では，1970年代に1例報告されたにとどまっている[2)3)]。日本への直行便の起点となるアジア諸国の都市部では，急速な都市化が進んだ結果，空港周辺も含めて，事実上マラリアの生活環が維持できないところも多いと推測される。

今後の望まれる体制

温度条件だけを考えると，年平均気温14℃と20℃が三日熱マラリアと熱帯熱マラリアの各々の分布限界と言われており，今後わが国において温暖化が進み年平均気温が3℃上昇すると，東北地方南部までがマラリア流行地になる可能性があると言われている[4]。マラリア媒介蚊の分布を規定する因子は温度だけではなく，都市化が進んだ現在の日本の状況からみて大きな流行は疑問視されるが，現在も成田空港周辺には，かつてわが国で三日熱マラリアの主要媒介蚊であったとされるシナハマダラカの生息が確認されている[5]。温暖化に伴ってハマダラカの生息域が拡がり，成田国際空港周辺での生息数も増加するとは，早計に考えることはできないが，今後は他の国際空港周辺を含めた定点観測を全国で

行い，ハマダラカ属の生息調査を継続していく必要があろう。また，前述したように，アジアからの輸入マラリア例は近年減少しているのに対し，インド亜大陸からの輸入マラリア例はあまり減少していない。インドでは，都市部でも三日熱マラリアの流行が多く報告されており，わが国では，ヨーロッパ諸国とは異なった形で空港マラリアが問題になる可能性も否定できない[6)7)]。空港マラリアの発生を防ぐためには，機内調査で捕獲される蚊も含めて，蚊からのマラリア原虫の検出についても体制を整備していく必要があると思われる。

三日熱マラリア感染者に関しては，初感染であったとしても潜伏期がはっきりせず典型的な症状を示さないことがあるので，熱帯熱マラリアよりも検疫でのチェックが難しい。さらに，診療面では，再発の原因となる肝臓内休眠型に対する治療薬：プリマキンの入手経路が，わが国では厚生省研究班からに限られるなど，致死的ともなる熱帯熱マラリアの診療とは異なった問題点も指摘できる。1990年代から始まった韓国の三日熱マラリアの再流行をみてもわかるように，三日熱マラリアは，高緯度地方であっても一定の条件が重なれば再流行を起こし，また，一度再流行を起こすとなかなか制圧できないと考えられる[1)]。国内における2次感染や感染拡大を防ぐため，今後わが国においても，感染者の検出と治療を徹底するべく，検疫の強化だけではなく，診療にあたる医療機関も参加した活動が重要になってくるかもしれない。

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