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有末伸子、 賈黙稚、 Niriann Palacpac、 田邉和裕、 堀井俊宏	Group I. II 及び III SERA 遺伝子配列から推定したマラリア原虫の系統関係	第 6 回分子寄生虫・マラリア研究フォーラム	10/27-28、2007	松山
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Independent Evolution of Pyrimethamine Resistance in *Plasmodium falciparum* Isolates in Melanesia[▽]

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Pyrimethamine resistance in *Plasmodium falciparum* has previously been shown to have emerged once in Southeast Asia, from where it spread to Africa. Pyrimethamine resistance in this parasite is known to be conferred by mutations in the gene encoding dihydrofolate reductase (*dhfr*). We have analyzed polymorphisms in *dhfr* as well as microsatellite haplotypes flanking this gene in a total of 285 isolates from different regions of Melanesia (Papua New Guinea, Vanuatu, and the Solomon Islands) and Southeast Asia (Thailand and Cambodia). Nearly all isolates (92%) in Melanesia were shown to carry a *dhfr* double mutation (CNRNI [underlining indicates the mutation]) at positions 50, 51, 59, 108, and 164, whereas 98% of Southeast Asian isolates were either triple (CIRNI) or quadruple (CIRNL) mutants. Microsatellite analysis revealed two distinct lineages of *dhfr* double mutants in Melanesia. One lineage had the same microsatellite haplotype as that previously reported for Southeast Asia and Africa, suggesting the spread of this allele to Melanesia from Southeast Asia. The other lineage had a unique, previously undescribed microsatellite haplotype, indicative of the de novo emergence of pyrimethamine resistance in Melanesia.

Malaria is a major cause of morbidity and mortality in large areas of the tropical world. The antifolate drug sulfadoxine-pyrimethamine (SP) has been widely used to treat uncomplicated malaria, mainly as a monotherapy, but also in combination with other antimalarial drugs in most regions of endemicity for malaria in the world.

Pyrimethamine and sulfadoxine inhibit two separate enzymes in the folate synthesis pathway of *Plasmodium falciparum*: dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS), respectively. Point mutations at amino acid positions 16, 50, 51, 59, 108, and 164 in the DHFR gene (*dhfr*) are the major causes of resistance to pyrimethamine (3, 17, 18, 24). The mutation at position 108 (Ser→Asn) appears to be an initial prerequisite for a significant (10-fold) increase in in vitro resistance (24). Additional mutations at other amino acid positions within the gene are associated with stepwise increases in resistance. Isolates harboring four mutations at positions 51, 59, 108, and 164 (CIRNL at positions 50, 51, 59, 108, and 164 [mutations are indicated by underlining]) show the highest pyrimethamine resistance so far described.

Various *dhfr* alleles have been observed in regions of endemicity (30). A *dhfr* triple mutant (CIRNI) represents the most common type in Africa and Southeast Asia, while the *dhfr* quadruple mutant (CIRNL) is observed predominantly in Thailand and some other regions in Southeast Asia where SP resistance is very high (1, 12, 30). Two distinct triple *dhfr* mutant genotypes (RICNI and CICNL) are prevalent in South America (2, 18). A five-amino-acid insertion after position 30, termed the Bolivia repeat, is also exclusive to South America, suggesting two unique and different evolutionary origins of pyrimethamine resistance in South America (2).

The migration of drug-resistant alleles can be traced by the analysis of microsatellite markers closely linked to the gene conferring resistance. Microsatellite analysis flanking *pfcrt* has revealed that chloroquine resistance evolved independently in at least four different regions: Southeast Asia, two regions in South America, and New Guinea (31). Meanwhile, all *dhfr* triple (CIRNI) and quadruple (CIRNL) mutants from Southeast countries displayed the same or nearly identical microsatellite haplotypes flanking *dhfr* (12). Strikingly, pyrimethamine-resistant isolates in Africa also harbored microsatellite haplotypes identical to those found in Southeast Asia (21), suggesting a single origin of pyrimethamine resistance in Southeast Asia, which subsequently spread to Africa. However, whether the Melanesian *dhfr* mutants originated in Southeast Asia or arose independently remains unclear.

In the present study, we determined *dhfr* and microsatellite haplotypes flanking the gene in *P. falciparum* isolates from Melanesia (Papua New Guinea, Vanuatu, and the Solomon Islands) and Southeast Asia (Thailand and Cambodia). Our

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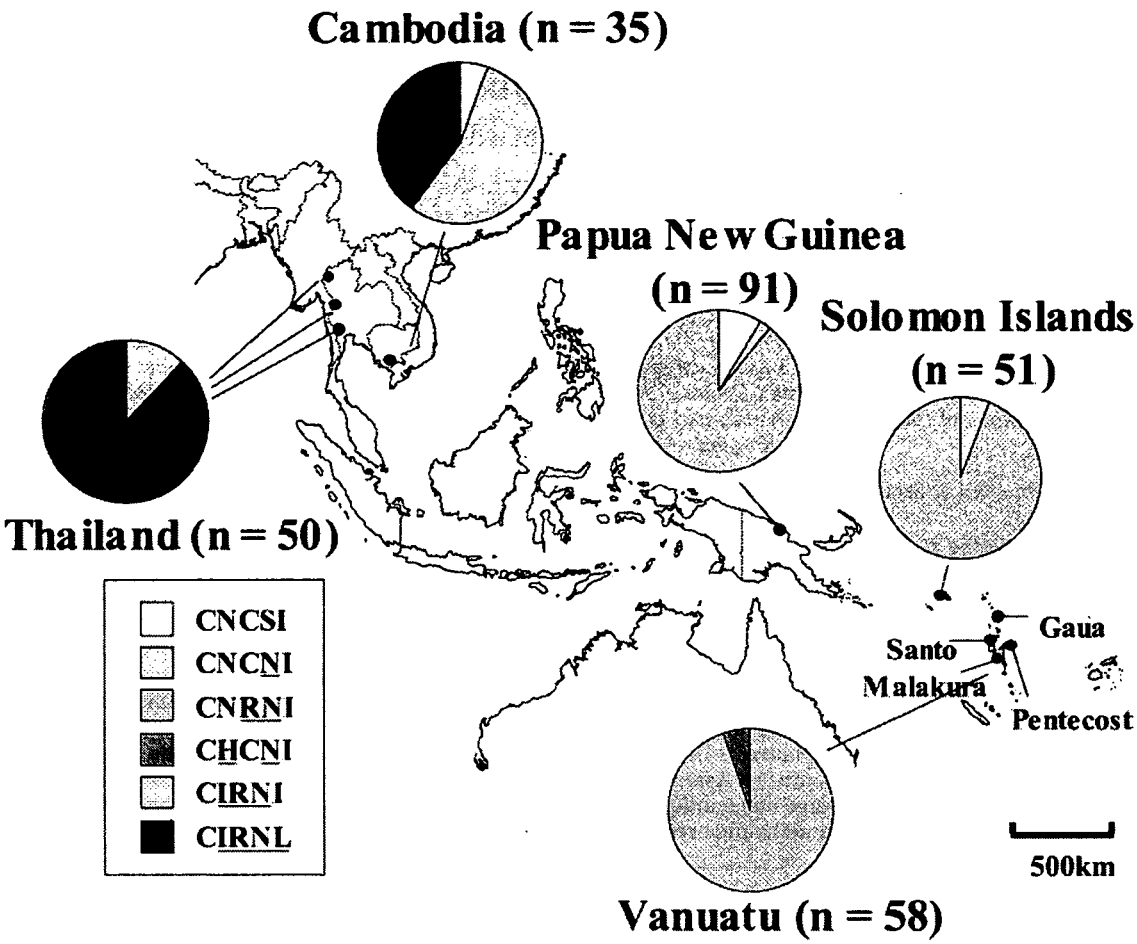

FIG. 1. Frequency of *dhfr* genotypes in *P. falciparum* isolates from Papua New Guinea, the Solomon Islands, Vanuatu, Cambodia, and Thailand.

results show two major lineages of pyrimethamine resistance in Melanesia. One has apparently originated in Melanesia, while the other originated in Southeast Asia and spread to Africa and Melanesia. This is the first unambiguous demonstration of the unique evolution of *P. falciparum* pyrimethamine resistance in Melanesia.

MATERIALS AND METHODS

Study site and patients. Blood samples were obtained from *P. falciparum*-infected patients living in five countries where malaria is endemic: (i) Papua New Guinea, where isolates were from finger-prick blood samples taken during in vitro studies at a town clinic in Wewak, East Sepik Province, in November of both 2002 and 2003 (9, 10); (ii) Solomon Islands, where isolates from venous-blood samples were taken as part of a cross-sectional survey of three villages located in the northwestern part of Guadalcanal in 1995 and 1996 (23); (iii) Vanuatu, where isolates were from finger-prick blood samples obtained during a cross-sectional survey of rural villages and primary schools from February to March 1996 to 1998 in four islands, Gaua, Santo, Pentecost, and Malakula (22); (iv) Cambodia, where isolates were obtained from finger-prick blood samples taken during a cross-sectional survey of rural villages in Chumkiri, Kampot Province, in December 2004; (v) Thailand, where isolates were obtained from pretreatment venous blood samples taken during in vitro studies at a town clinic located at the western border of Tak, Kanchanaburi and Ratchaburi provinces, from 2001 to 2002.

DNA preparation. Finger-prick blood (75 μl) was spotted onto chromatography filter paper ET31CHR (Whatman Limited, Kent, United Kingdom). Venous blood was transferred into heparin-containing test tubes. Parasite DNA was purified using a QIAamp DNA blood mini kit (QIAGEN, Germany) according to the manufacturer's instructions with some modifications (22).

***dhfr* genotyping.** *dhfr* was amplified by PCR, and amplified products were directly sequenced with a BigDye Terminator 1.1 cycle sequencing kit in the ABI 377 DNA sequencer (GE Healthcare UK Ltd., Buckinghamshire, England) as previously reported (10, 19).

Microsatellite haplotyping. In order to determine the evolutionary history of pyrimethamine-resistant alleles of *dhfr*, we measured variation in the number of TA repeats at six microsatellite loci closely linked to the gene. These were located on chromosome 4, 0.1, 3.87, and 4.49 kb upstream and 0.52, 1.48, and 5.87 kb downstream of *dhfr*. In some cases, in order to estimate the limit of genetic hitchhiking, which is defined as a valley of reduced variation around *dhfr*, an additional six-microsatellite markers were analyzed at 7.55, 29.61, 57.68, and 363.33 kb upstream and 30.31 and 299.72 kb downstream of *dhfr*. Polymorphisms in these microsatellite markers were determined as previously described (12). Briefly, seminested PCR was performed using fluorescent end-labeled primers. Size variations in the amplified products were determined by electrophoresis on an ABI 377 sequencer and analyzed with GeneScan software (GE Healthcare UK Ltd.). Samples with two or more peaks at the same locus in the electropherogram were considered to be mixed infections and were excluded from further analysis.

Polymorphism between microsatellite markers is measured as variation in nucleotide length derived from various numbers of TA repeats. Microsatellite haplotypes harboring an association of bp 200-194-176-106-203-111 at microsatellite positions 4.49, 3.87, and 0.1 kb upstream and 0.52, 1.48, and 5.87 kb downstream of *dhfr* were designated "SEA" haplotypes, and those harboring an association of bp 220-202-156-100-205-111 were designated "Melanesia" haplotypes. Microsatellite haplotypes showing slight differences at one or two microsatellite markers from the SEA haplotype, e.g., at bp 204- 200-176-106-203-111

TABLE 1. Microsatellite polymorphisms in 15 *P. falciparum* isolates with wild-type *dhfr* or single-mutant *dhfr*

Isolate	Country[a]	Size (bp) of microsatellite marker at indicated position (kb)					
		−4.49	−3.87	−0.1	+0.52	+1.48	+5.87
CNCSI	Cambodia	198	206	156	94	203	105
(*n* = 10)	Cambodia	198	206	156	94	203	105
	PNG	202	196	156	94	203	121
	PNG	214	198	156	94	203	123
	PNG	202	192	156	96	203	115
	PNG	204	194	172	96	203	103
	PNG	204	194	172	92	203	103
	PNG	204	206	172	100	203	111
	PNG	202	192	176	96	203	115
	PNG	202	192	176	96	203	115
CNCNI	Solomon	210	194	172	96	203	113
(*n* = 5)	Solomon	204	208	176	94	203	120
	Solomon	204	208	176	94	203	120
	PNG	210	194	178	102	203	113
	PNG	210	194	178	102	203	113

[a] PNG, Papua New Guinea; Solomon, Solomon Islands.

(underlining indicates the differences), were considered SEA variation haplotypes. Similarly, haplotypes displaying minor variation at one or two markers from the Melanesia haplotype, e.g., bp 220-202-156-100-205-113, were considered Melanesia variation haplotypes. Isolates showing mixed *dhfr* genotypes and/or microsatellite haplotypes were excluded from analysis.

Statistical analysis. We calculated the expected heterozygosity (h) at each microsatellite locus as $h = [n/(n-1)][1 - \Sigma p_i^2]$, where n is the number of infections sampled and p_i^2 is the frequency of the i^{th} allele. The sampling variance of h was calculated according to the following formula (23), a slight modification of the standard diploid variance (13), $[2/n(n-1)]\{2(n-2)[\Sigma pi^3 - (\Sigma pi^2)^2] + \Sigma pi^2 - (\Sigma pi^2)^2\}$. A P value of <0.05 was considered statistically significant.

Nucleotide sequence accession number. The complete sequence of the allele identified has been submitted to the DDBJ and assigned accession number AB271908.

RESULTS

***dhfr* genotypes.** Among a total of 314 samples, 29 (9%) had multiple *dhfr* alleles and/or were of mixed microsatellite haplotypes and so were excluded from this analysis. *dhfr* allele types and flanking microsatellite haplotypes were thus determined for 285 isolates (91 from Papua New Guinea, 51 from the Solomon Islands, 58 from Vanuatu, 35 from Cambodia, and 50 from Thailand). The frequencies of *dhfr* genotypes differed considerably between Southeast Asia and Melanesia (Fig. 1). In Southeast Asia, nearly all parasites (98%) carried either triple (CIRNI at positions 50, 51, 59, 108, and 164) or quadruple (CIRNL) mutations at *dhfr*. In Cambodia, triple and quadruple mutants were near equally prevalent. In Thailand, 85% of isolates were quadruple mutants. In Melanesia, nearly all isolates (92%) harbored a *dhfr* double mutation (CNRNI). The pyrimethamine-sensitive, wild-type allele (CNCSI) was found in only Papua New Guinea and at relatively low prevalence (8%). Neither the triple (CIRNI) nor the quadruple (CIRNL) mutant was found in Melanesia. A unique CHCNI allele was observed in three isolates from Gaua Island, Vanuatu.

Polymorphism in microsatellite markers flanking *dhfr*. The polymorphisms in six microsatellite markers flanking *dhfr* (−4.49 to 5.87 kb) from wild-type (n = 10) or single-mutant (n = 5) isolates are shown in Table 1. For those parasites carrying pyrimethamine-sensitive, wild-type alleles of *dhfr*, microsatellite markers were highly polymorphic. In contrast, *dhfr* double-mutant isolates (n = 184) showed remarkably little diversity at all loci (Fig. 2). Similarly, nearly all triple (n = 25) and quadruple (n = 58) mutants displayed limited microsatellite polymorphism at each locus (Fig. 2). The expected heterozygosity (h) at each microsatellite marker is given in Table 2. In isolates carrying wild-type or single-mutant *dhfr* alleles, h was high (0.60 to 0.89) at all six loci located between 4.49 kb upstream and 5.87 kb down-

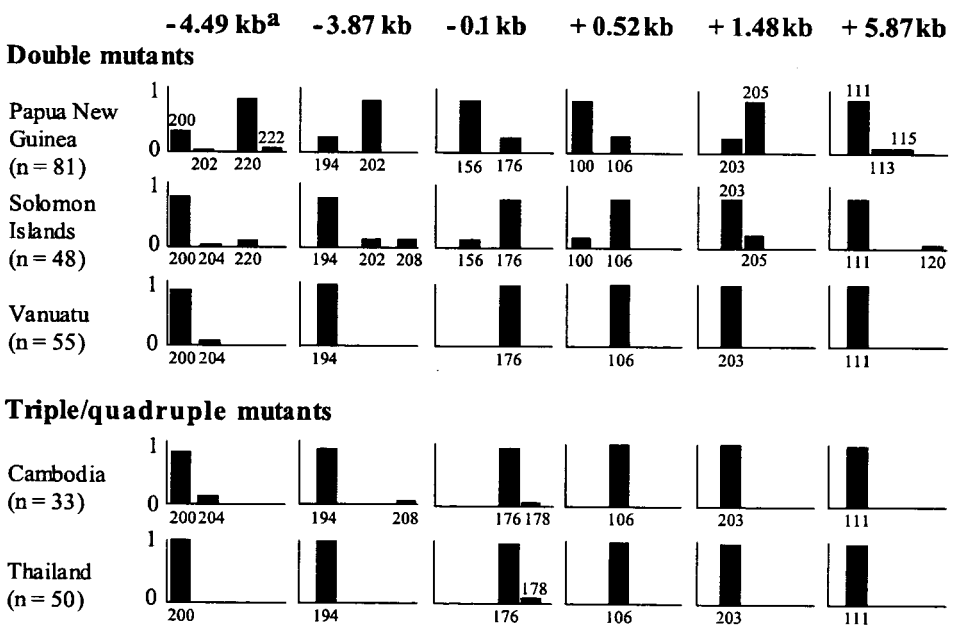

FIG. 2. Repeat length variations of six microsatellite markers flanking *dhfr* in *P. falciparum* isolates with *dhfr* double (CNRNI) and triple (CIRNI)/quadruple (CIRNL) mutants. x axes, size (bp) of microsatellite markers. y axes, frequency of microsatellite alleles. [a], location of microsatellite marker (distance from *dhfr*).

TABLE 2. Expected heterozygosity of microsatellite markers in *P. falciparum* isolates

Isolate	No. of isolates	*h* of microsatellite marker at indicated position (kb)						No. of haplotypes
		−4.49	−3.87	−0.1	+0.52	+1.48	+5.87	
CNCSI	10	0.78	0.84	0.69	0.71	0	0.89	8
CNCNI	5	0.60	0.60	0.80	0.80	0	0.60	5
CNRNI	184	0.54	0.49	0.48	0.49	0.48	0.17	2[a]
CIRNI	25	0.28	0.22	0.08	0	0	0	1[a]
CIRNL	58	0	0	0.10	0	0	0	1[a]

[a] Number of major haplotypes.

stream of *dhfr*, except at the monomorphic +1.48-kb locus. In contrast, those isolates carrying the triple or quadruple mutations at *dhfr* had very low *h* values (0 to 0.28) at all microsatellite loci, indicating limited diversity in those isolates. Isolates carrying double mutations at *dhfr* had intermediate values of *h* (0.48 to 0.54) lying somewhere between those of the wild-type/single mutants and triple/quadruple mutants.

Microsatellite haplotypes. Different microsatellite haplotypes were found in isolates carrying wild-type *dhfr* and in those carrying single mutations; 8 haplotypes were found in 10 wild-type *dhfr* isolates, and 3 haplotypes were found in 5 single mutants (Table 1). In contrast, only two distinct microsatellite haplotypes (SEA/SEA variation and Melanesia/Melanesia variation) were observed in a total of 184 *dhfr* double-mutant isolates (Fig. 3). Identical or very similar haplotypes (SEA/SEA variation) were found in all *dhfr* triple or quadruple mutation-carrying isolates (n = 83), suggesting that *dhfr* triple and quadruple mutants evolved directly from the *dhfr* double mutant.

In Southeast Asia, only SEA/SEA variation haplotypes were observed. These haplotypes were also predominant in Melanesian countries, except Papua New Guinea, where 78% of isolates were of the Melanesia/Melanesia variation haplotypes. In Vanuatu, all isolates showed SEA/SEA variation haplotypes. One isolate carrying a hybrid of the SEA and Melanesia haplotypes (it was of SEA haplotype upstream and Melanesia haplotype downstream of *dhfr*, bp 200-194-176-100-205-111) was observed in the Solomon Islands.

Genetic hitchhiking in *dhfr* double-mutant parasites from Papua New Guinea. These results suggest that the *dhfr* double mutants present today in Melanesia emerged independently in Southeast Asia and Melanesia. To determine the history of these two lineages, we measured the extent of genetic hitchhiking, which is determined by the distance of reduced microsatellite variation around *dhfr*. For this purpose, the variance

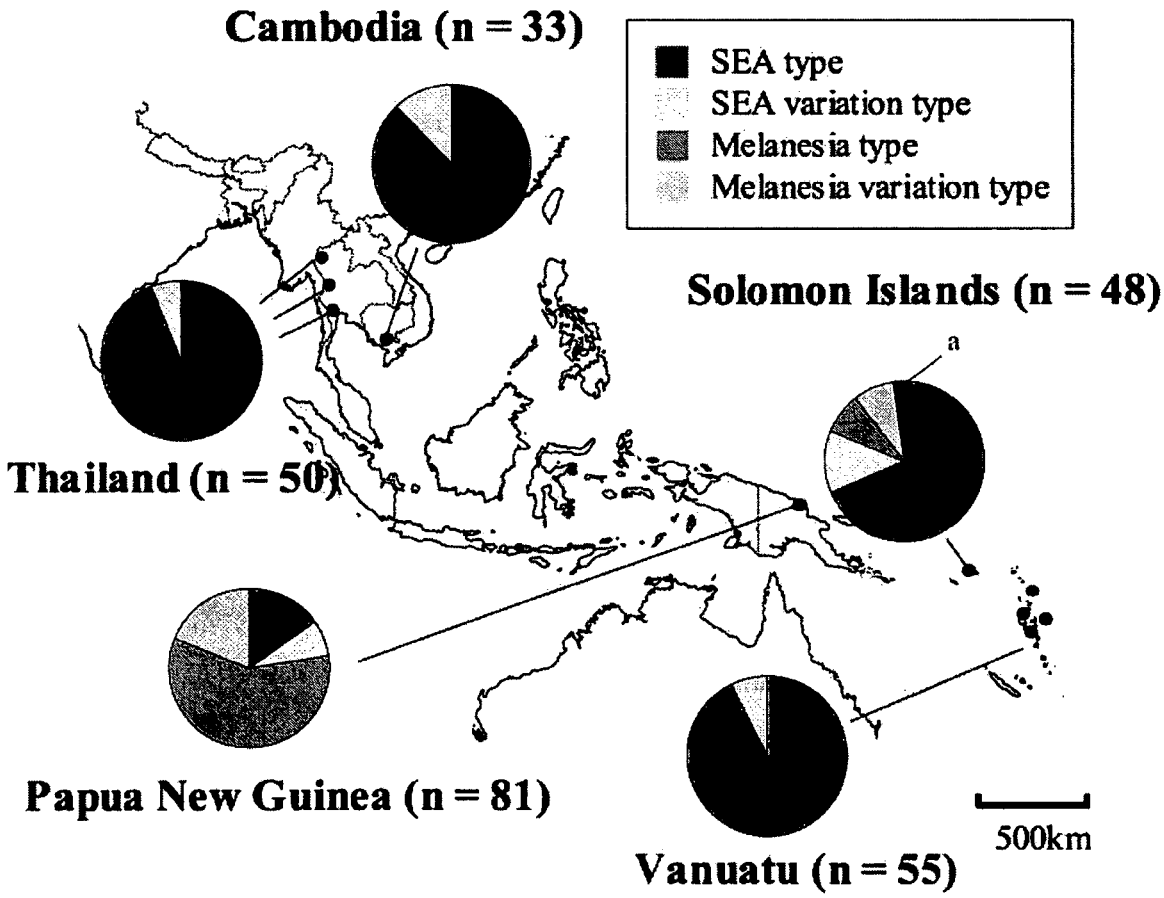

FIG. 3. Frequency of microsatellite haplotype flanking *dhfr* in *P. falciparum* isolates with *dhfr* double, triple, or quadruple mutants from Papua New Guinea, the Solomon Islands, Vanuatu, Cambodia, and Thailand. [a], isolate (n = 1) carrying a hybrid of the SEA and Melanesia haplotypes (it was of SEA haplotype upstream and Melanesia haplotype downstream of *dhfr*, bp 200-194-176-100-205-111).

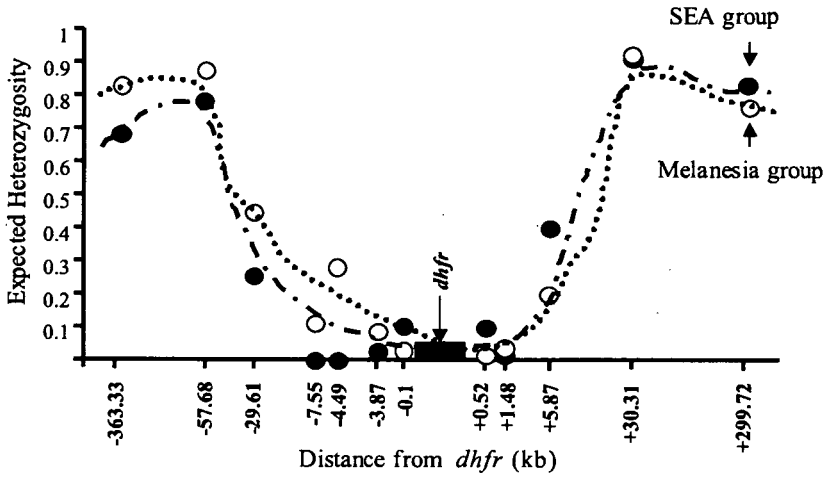

FIG. 4. Reduced microsatellite polymorphism near *dhfr* in *P. falciparum* isolates with CNRNI genotype from Papua New Guinea.

of *h* at 12 microsatellite markers spanning 363.33 kb upstream to 299.72 kb downstream of *dhfr* was measured for Papua New Guinean isolates with a *dhfr* double mutant displaying a SEA/SEA variation haplotype (n = 17) and those displaying the Melanesia/Melanesia variation haplotype (n = 64) (Fig. 4). The patterns of genetic hitchhiking in both haplotypes were similar within a distance of 58 kb upstream and 30 kb downstream of *dhfr*. These results suggest that these two lineages, both carrying the same point mutations (CNRNI), appeared coincidentally in Papua New Guinea.

DISCUSSION

This study clearly shows that pyrimethamine-resistant *P. falciparum* evolved independently in Melanesia. It has previously been shown that a single lineage of pyrimethamine-resistant parasites arose in Southeast Asia, and subsequently spread to Africa (21). Pyrimethamine-resistant parasites from South America, which show *dhfr* genotypes different from those of other geographic areas, independently evolved in two foci within South America (2). Thus, there are at least four distinct independent origins of *dhfr* resistance presently identified. This is similar to the situation with chloroquine resistance, which has also been reported to have arisen independently a total of four times, once in Southeast Asia, twice in South America, and once in Melanesia (31).

A recent study has reported multiple origins of *dhfr* resistance within Kenya (6). However, care must be taken when basing conclusions about the origins of drug resistance on microsatellite variation from areas of high endemicity, such as Kenya. Two factors are likely to affect microsatellite polymorphism in areas of intense transmission. First, new microsatellite haplotypes are easily generated by meiotic recombination because of a high recombination rate and high prevalence of mixed-haplotype infections. Second, interallelic recombination within a microsatellite may generate new microsatellite alleles by an unequal crossing-over mechanism. Indeed, in a study by Roper et al. (21), nonuniform microsatellite haplotypes were noticeable around *dhfr* in pyrimethamine-resistant African isolates. These factors may be less important in areas of low transmission, such as Southeast Asia, and so do not compromise the conclusions of the present study.

Genetic hitchhiking reduces the expected heterozygosity of microsatellite markers around a selected gene, resulting in a valley of reduced variation. However, this association is easily broken down by recombination, resulting in a narrowing of the selection valley as the number of generations increases. In this study, we compared the selection valleys around the *dhfr* gene in two *dhfr* double mutants carrying SEA and Melanesia microsatellite haplotypes from Papua New Guinea. In both haplotypes, the microsatellite patterns within the valley were very similar from a distance of 58 kb upstream to 30 kb downstream of *dhfr*. The size of a selection valley is determined by several different parameters: the strength of the selection pressure on the mutant allele, the frequency of recombination, the transmission intensity, and the number of parasite generations since the emergence of the selected allele (16). In this analysis, these four parameters could be considered equal because all isolates were sampled from the same area. Thus, these results indicate that two ancestors of the *dhfr* double mutant in Papua New Guinea emerged coincidentally: one came from Southeast Asia, and the other arose independently within Melanesia. Although the appearance of the two resistant lineages emerged nearly simultaneously, we consider that the Melanesian-resistant type might have appeared slightly earlier than the influx of the SEA-resistant type. This is because if the SEA-resistant type migrated to Papua New Guinea earlier, it would have swept away microsatellite polymorphisms linked to the wild *dhfr*. Therefore, the possibility that a novel *dhfr*-resistant type having distinct microsatellite haplotypes appeared soon after the sweep in Papua New Guinea seems very unlikely.

The way drugs are used within regions of endemicity affects the generation and selection of resistant alleles. SP was widely used in Thailand and Cambodia during the 1970s and 1980s as the first-line treatment for uncomplicated malaria. In Melanesian countries, SP was introduced as a first-line treatment during the mid-1990s. Up until this time, pyrimethamine monotherapy was infrequently used for the treatment of malaria and other infections. It is difficult, therefore, to explain the nearly simultaneous emergence of pyrimethamine-resistant parasites

in this area. It is, however, possible to attribute the emergence and spread of the triple and quadruple *dhfr* mutants to the widespread use of SP in Southeast Asia during the 1970s and 1980s.

We consider that weak and persistent pyrimethamine pressure by medicated salt projects, a form of mass drug administration, may explain the first selection of pyrimethamine-resistant parasites (15, 25). In the late 1950s and early 1960s, medicated salt projects were carried out in four different endemic regions: Indonesian Papua, the Thailand-Cambodia border, Ghana, and Iran (15, 25). In Southeast Asia and Melanesia, this project was carried out from 1959 to 1962 and from 1960 to 1961, respectively. Pyrimethamine resistance in *P. falciparum* was reported within 3 months of the start of the project (1960) in Indonesian Papua (7). At the Thailand-Cambodia border, resistance also developed quickly and pyrimethamine was replaced by chloroquine beginning in 1961. A large number of people treated with subcurative doses of antimalarial drugs present ideal conditions for the emergence of drug resistance (4, 27, 28). This, combined with the long half-life of pyrimethamine (116 h) (29), would have facilitated the emergence of drug resistance within the areas covered by the medicated salt project: Indonesian Papua and the Thailand-Cambodia border.

The *dhfr* double mutant (CNRNI), which confers moderate resistance to pyrimethamine, is widely distributed in Africa, West Asia, and Melanesia. However, whether this mutant is regularly selected de novo or whether it has spread from a limited number of foci of emergence is not known. In the present study, only two distinct microsatellite haplotypes (SEA and Melanesia haplotypes) were observed in a total of 184 *P. falciparum* isolates harboring the *dhfr* double mutant from Papua New Guinea, Vanuatu, and the Solomon Islands, suggesting that the generation of two mutations at positions 59 and 108 in *dhfr* is not frequent. In laboratory isolates, key point mutations in *dhfr* have occurred at frequencies as high as 2.5×10^{-9} per parasite replication, which predicts the generation of one mutant parasite in every malaria patient, assuming the number of parasites to be 10^{10} to 10^{12} in every infection (14). Consistently, the expected heterozygosities at microsatellite markers around *dhfr* were comparable between the wild-type and single *dhfr* mutant parasites. Thus, the initial mutation at position 108 in *dhfr* may occur relatively frequently (12, 20), but the generation and selection of an additional mutation at position 59 appear to be considerably less frequent. Mutations that render pathogens resistant to drug treatment are often associated with a loss of fitness (8, 11, 26). Resistant mutants may themselves develop compensatory mutations, which could then allow them to grow and survive in competition with wild-type forms (5, 26). The discrepancy between the frequent generation of the mutation at position 108 in *dhfr* and the rare occurrence of the *dhfr* double mutant as observed in this study may thus be reconciled by the requirement of complex compensatory mutations in a locus other than *dhfr* for restoring parasite fitness in natural populations.

In the present study, our samples of Papua New Guinea and Thailand were from individuals with clinical malaria, while samples from other sites were from cross-sectional studies of asymptomatic individuals. Symptomatic patients usually have higher parasite densities than do asymptomatic individuals. Thus, we cannot exclude the possibility that prevalences of microsatellite haplotypes may differ between isolates that cause disease and those that do not produce symptoms. However, we do not consider it very likely because there was no significant difference in the frequency distribution of genotypes of an antigen gene (*msp1*) between clinical patients and asymptomatic individuals in Melanesia (23).

In conclusion, this study provides strong evidence for the unique and independent origin of pyrimethamine resistance in Melanesia. The *dhfr* mutant, perhaps emerging from West Papua, has the same double mutations found in other geographic areas but distinct microsatellite haplotypes flanking the gene. Our results also show that the generation of double mutants with mutations at positions 59 and 108 of *dhfr* is a rare event, and this double mutation may be a first rate-determining step for the stable persistence of pyrimethamine resistance in *P. falciparum*.

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Production of High-Affinity Human Monoclonal Antibody Fab Fragments to the 19-Kilodalton C-Terminal Merozoite Surface Protein 1 of *Plasmodium falciparum*[▽]

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A combinatorial immunoglobulin gene library was constructed from peripheral blood lymphocytes of eight patients infected with *Plasmodium falciparum* and was screened for the production of human monoclonal antibody Fab fragments to the C-terminal 19-kDa fragment of *P. falciparum* merozoite surface protein 1 ($MSP\text{-}1_{19}$). Three Fab clones recognized recombinant $MSP\text{-}1_{19}$ under nonreducing conditions. Indirect immunofluorescence microscopy demonstrated that three Fab clones stained the surfaces of late trophozoites/schizonts and merozoites of the FCR3 and 3D7 strains, suggesting the Fabs' reactivities to a conserved epitope. Sequence analysis of the heavy-chain genes revealed that the closest germ line V segments were VH1-8 and VH7-81, with 91% to 98% homology. The closest germ line D segment was D3-10, and the closest germ line J segment was JH4 or JH5, with 90% to 97% homology. In the light-chain genes, the closest germ line V segment was A27 for the Jκ2, Jκ4, and Jκ5 segments. The dissociation constants of these Fab fragments for recombinant $MSP\text{-}1_{19}$ ranged from 1.09×10^{-9} to 2.66×10^{-9} M. The binding of the three Fab fragments to $MSP\text{-}1_{19}$ was competitively inhibited by the anti-$MSP\text{-}1_{19}$ mouse monoclonal antibody 12.8, which inhibits erythrocyte invasion by merozoites. However, the human Fab fragment with the highest affinity did not inhibit in vitro growth of *P. falciparum*. This is the first report of gene analysis and bacterial expression of human monoclonal antibodies to *P. falciparum* $MSP\text{-}1_{19}$. The combinatorial immunoglobulin gene library derived from malaria patients provides a potential tool for producing high-affinity human antibodies specific for *P. falciparum*.

Malaria caused by *Plasmodium falciparum* is a major public health problem in tropical countries, where it is responsible for 300 to 500 million cases and more than 1 million deaths annually (36). The development of malaria vaccines is urgently needed for improved malaria control. Proteins expressed on the surface of the merozoite, an invasive form of the parasite, seem to be important targets of host immunity and therefore could be potential candidates for the development of malaria vaccines. The *P. falciparum* major merozoite surface protein 1 (MSP-1) is a leading vaccine candidate antigen (21). Antibodies against MSP-1 are protective against human, monkey, and rodent malaria parasites, and immunization with MSP-1 affords antiparasite protection in experimental animals (7, 8, 30, 35). MSP-1 is synthesized as a 195-kDa precursor on the surfaces of late trophozoites/schizonts, and it is proteolytically processed to form four fragments, of 83 kDa, 30 kDa, 38 kDa, and 42 kDa, during merozoite maturation (14). The C-terminal 42-kDa fragment is further cleaved into N-terminal 33-kDa and C-terminal 19-kDa fragments ($MSP\text{-}1_{19}$) (3). All of the fragments, except for $MSP\text{-}1_{19}$, are shed from the merozoite surface upon erythrocyte invasion. $MSP\text{-}1_{19}$, which contains two epidermal growth factor-like modules, is anchored to the surface via a glycosylphosphatidylinositol moiety (13, 14). Although the *P. falciparum* MSP-1 gene (*msp1*) is highly polymorphic, the *msp1* region coding for $MSP\text{-}1_{19}$ is well conserved among parasite isolates. There is accumulating evidence suggesting that sera from individuals living in areas where malaria is highly endemic contain antibodies against the 19-kDa fragment that inhibit merozoite invasion into red blood cells (9, 24, 25, 31).

P. falciparum occasionally causes severe malaria in children and individuals who have less immunity to the parasite. The efficacy of antimalarial drugs is becoming limited due to the high prevalence of multidrug resistance of the parasite. Therefore, new therapeutic measures are needed to treat severe malaria cases. In this context, passive immunotherapy using human antibodies specific to $MSP\text{-}1_{19}$ may provide a valuable therapeutic alternative. Indeed, mouse monoclonal antibodies to $MSP\text{-}1_{19}$ inhibit in vitro growth of *P. falciparum* (3, 5). Mouse monoclonal antibodies are unsuitable for use in humans; therefore, an immunotherapy method that can be used in humans must be developed. However, little is known about the molecular basis of acquired humoral immunity to $MSP\text{-}1_{19}$ in malaria-immune individuals.

Several methods have been developed to produce human monoclonal antibodies (1, 2, 11, 44). We have reported that the bacterial expression system is useful for the preparation of human Fab fragments specific to pathogens (6, 17, 39–42). In the present study, we use a combinatorial immunoglobulin gene library derived from lymphocytes of patients with falcip-

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arum malaria to produce human monoclonal antibody Fab fragments that specifically react to *P. falciparum* MSP-1_{19}. Additionally, we analyze immunoglobulin gene usage in these Fab fragments.

MATERIALS AND METHODS

Cultivation of *P. falciparum*. Asexual blood-stage parasites of *P. falciparum* (strains FCR3 and 3D7) were maintained at 37°C in RPMI 1640 medium supplemented with 10% human type O serum (45). Cultures were gassed with 5% CO_2, 5% O_2, and 90% N_2 and maintained by routine passage in fresh human type O erythrocytes. Parasites were synchronized by Percoll and sorbitol treatment (46).

Preparation of recombinant MSP-1_{19}. Genomic DNA of *P. falciparum* (strain FCR3) was isolated from schizonts by using a DNeasy tissue kit (QIAGEN, Hilden, Germany). The DNA was used as a template for amplification of the *msp1* region coding for MSP-1_{19} (nucleotide positions 4819 to 53 downstream of the 3′ noncoding region; positions are given according to the 3D7 *msp1* sequence [GenBank accession no. Z35327]) with the following primers: forward, 5′-CCCATATGAACATTTCACAACACCAATGCGT-3′; and reverse, 5′-CCCTCGAGTTAGTTAGAGGAACTGCAGAAAATA-3′. To obtain high-fidelity amplification, *Pyrobest* DNA polymerase (Takara, Otsu, Japan) was used. Twenty cycles of PCR were performed as follows: denaturation at 94°C for 15 s (135 s in cycle 1), annealing at 55°C for 30 s, and polymerization at 72°C for 60 s (360 s in cycle 20). The PCR product was digested with NdeI and XhoI, purified, and then ligated with the pET19b vector (Novagen, Madison, WI). The plasmid was introduced into competent *Escherichia coli* JM109 cells, and then a clone containing the insert with the right sequence was selected. *E. coli* BL21 Star (DE3)pLysS cells (Invitrogen, Carlsbad, CA) were transformed with the cloned plasmid. The bacterial clone was cultured in 800 ml of Luria broth containing ampicillin and chloramphenicol until an optical density at 600 nm of 0.6 was achieved. The expression of recombinant MSP-1_{19} tagged with histidine residues was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37°C for 3 h. The protein was purified by affinity chromatography, using His-Bind resin (Novagen) according to the manufacturer's recommendations.

Construction of immunoglobulin gene library. Approximately 10 ml of peripheral blood was obtained from each of eight hospitalized patients with falciparum malaria (six Japanese and two Africans) at Tokai University Hospital, Tokyo Metropolitan Komagome Hospital, and Tokyo Metropolitan Bokutoh General Hospital (Japan). Lymphocytes were separated from the blood by Ficoll-Paque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. Construction of an immunoglobulin gene library from the lymphocytes was performed as previously described (39). Briefly, total RNA was purified from lymphocytes and subjected to reverse transcription-PCR. Genes encoding the light (κ and λ) chain and the Fd region of the heavy (γ and μ) chain were amplified by 30 cycles of PCR. The light-chain genes were first ligated with an expression vector, pFab-His2, and introduced into *Escherichia coli* JM109 cells. The vector with inserts was then ligated with the Fd heavy-chain genes and introduced into *E. coli* cells.

Screening of clones producing anti-*P. falciparum* antibodies. The first screening of positive clones producing anti-*P. falciparum* MSP-1_{19} antibodies was performed as described previously (6). Approximately 5×10^3 *E. coli* colonies per 90-mm plate were grown on Luria broth agar containing 50 μg/ml of ampicillin. Bacterial colonies were transferred to nitrocellulose filters. The filters were replaced on the surfaces of fresh plates containing 1 mM IPTG and then incubated at 30°C for 6 h. The filters were treated with chloroform vapor and lysis buffer containing 100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$, 1.5% bovine serum albumin, 1 μg of DNase per ml, and 40 μg of lysozyme per ml overnight. After being washed with phosphate-buffered saline containing 0.05% Tween 20 (PBST), the filter was blocked with PBST containing 5% skim milk. Each filter was incubated with 125 μg of recombinant MSP-1_{19} and then with plasma from a patient. Positive signals on the filters were detected with a horseradish peroxidase (HRP)-conjugated goat antibody to human whole immunoglobulin G (IgG; ICN Pharmaceuticals, Aurora, OH) and a Konica HRP-1000 immunostaining kit. Positive clones were identified in the original plates and then cultured in 10 ml of super broth (30 g tryptone, 20 g yeast extract, and 10 g 4-morpholinepropanesulfonic acid per liter, pH 7.0) containing ampicillin to an optical density at 600 nm of 0.8. IPTG was added to the bacterial culture at a final concentration of 100 μM, and the culture was then incubated overnight at 30°C for 12 h. Bacteria were pelleted by centrifugation, resuspended in 0.5 ml of PBS containing 1 mM phenylmethylsulfonyl fluoride, and then sonicated. The lysates were centrifuged at 10,000 × *g* for 10 min, and supernatants were subjected to a second screening by an enzyme-linked immunosorbent assay (ELISA). CP2.9 (26), which is a chimera of MSP-1_{19} and domain III of apical membrane antigen 1, was also used for screening. This antigen was kindly provided by W.-Q. Pan, Second Military Medical University, Shanghai, China.

ELISA. Each ELISA well was treated with recombinant MSP-1_{19} or CP2.9 (50 ng/well) diluted in 50 mM sodium bicarbonate buffer. The plates were washed with PBST and then treated with PBS containing 1% skim milk for 1 h. One hundred microliters of the supernatant was added to the wells and incubated for 1 h at room temperature. After being washed, the wells were incubated with 100 μl of HRP-conjugated goat antibody to human IgG Fab (ICN Pharmaceuticals) for 1 h at room temperature and then treated with 200 μl of substrate (0.04% *o*-phenylenediamine in citric acid-phosphate buffer [pH 5.0] including 0.001% hydrogen peroxide). The reaction was stopped by the addition of 50 μl 2.5 N H_2SO_4 after 30 min, and the optical density at 490 nm was determined.

Immunofluorescence microscopy. Indirect immunofluorescence staining was performed with paraformaldehyde-fixed parasites (29) by using fluorescein isothiocyanate-conjugated goat IgG to human IgG Fab (ICN Pharmaceuticals) as the secondary antibody. Propidium iodine was used for counterstaining.

Purification of Fab fragments. Positive clones were cultured in 1 liter of super broth medium, and 20 ml of the resultant supernatant was prepared as described above. Fab fragments were purified with Talon metal-affinity resin (BD Biosciences, Palo Alto, CA) according to the manufacturer's instructions.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting. Purified Fab fragments and *P. falciparum* schizonts (strain FCR3) were solubilized and electrophoresed in 10% acrylamide gels containing sodium dodecyl sulfate (SDS) under reducing and nonreducing conditions, respectively. Protein bands were then transferred to polyvinylidene difluoride membranes. The Fab fragments were detected by an HRP-conjugated goat antibody to the human kappa chain and with HRP-conjugated Ni-nitrilotriacetic acid as previously described (6). Proteins from *P. falciparum* were incubated with 10 μg of purified human Fab fragments and a 1:200 dilution of patient plasma for 1 h and then detected by HRP-conjugated goat antibodies to human IgG Fab and human whole IgG for 1 h. Development was performed with a Konica immunostaining kit. Normal human Fab (OEM Concepts, Toms River, NJ) and normal human sera were used as negative controls.

Measurement of affinity of Fab fragments. The affinity constants of the Fab fragments were assessed by surface plasmon resonance, using a BIAcore 3000 instrument (Biacore AB, Uppsala, Sweden). Recombinant MSP-1_{19} was immobilized onto a CM5 chip (Biacore). Association and dissociation constants were determined by using BIAevaluation 3.1.

DNA sequencing. Plasmid DNAs were isolated from immunofluorescence assay-positive clones. Light-chain genes in the expression vector were subcloned into the sequencing vector. Sequencing reactions in both directions were performed with a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA), using M13 primers. The sequences were obtained using an ABI PRISM 3100 genetic analyzer (Applied Biosystems).

Competitive inhibition assay. Each ELISA well was coated with recombinant MSP-1_{19} as described above. For competition between human Fab and a mouse monoclonal antibody, various concentrations of purified Fab fragments (0.01, 0.1, 1, and 10 μg per 50 μl PBS) were mixed with 50 μl of anti-MSP-1_{19} mouse monoclonal antibody 12.8 or 2.2 (3, 12, 22) and then added to the wells. The mouse monoclonal antibodies were kindly provided by J. S. McBride, University of Edinburgh. The plates were incubated for 1 h at room temperature and washed with PBST. Reactions were detected as described above. As a control, normal human Fab fragments (OEM Concepts) were used. Competition ELISA between human Fab and sera or plasmas from patients with malaria was also performed. Human sera immune to *P. falciparum* were obtained from individuals living in the Solomon Islands, where malaria is highly endemic (34). Ten nanograms of Fab labeled with sulfosuccinimidobiotin (Pierce, Rockford, IL) per 50 μl PBS and equal volumes of immune sera (n = 10) diluted 1:10 were premixed and added to the wells of ELISA plates, which were coated as described above. Plasmas from lymphocyte donors (n = 8), diluted 1:10, were also tested by competition ELISA. As controls, serum samples from healthy Japanese individuals (n = 10) were used. The plates were incubated for 1 h and then detected by incubation with streptavidin-biotinylated HRP (GE Healthcare, Buckinghamshire, England) for 1 h. Reactions were also developed as described above and expressed as values relative to those of the control.

Growth inhibition assay. The effect of human Fab fragments on the growth of *P. falciparum* (strain FCR3) was examined in vitro (27, 28). Erythrocytes infected with late trophozoites/schizonts were diluted with complete RPMI 1640 and uninfected human erythrocytes to a final hematocrit of 4% and final parasitemia of 0.5%. A total of 160 μl of this suspension was transferred to wells of a 96-well flat-bottomed microplate, and then 40 μl of PBS containing 40 μg of purified

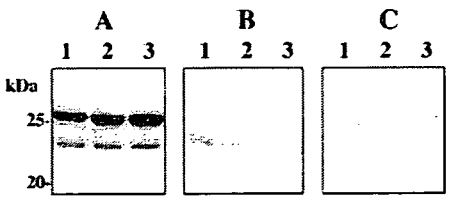

FIG. 1. SDS-PAGE (A) and Western immunoblot analysis (B and C) of purified recombinant Fab fragments from clones Pf25 (lanes 1), Pf143 (lanes 2), and Pf227 (lanes 3). The Fab fragments were subjected to SDS-PAGE in a 10% polyacrylamide gel under reducing conditions and then transferred to polyvinylidene difluoride membranes. The protein bands in panel A were stained with Coomassie brilliant blue. The membranes were treated with an HRP-conjugated goat antibody to the human kappa chain (B) or with HRP-conjugated Ni-nitrilotriacetic acid (C). The numbers on the left indicate the molecular masses of size markers.

recombinant human Fab was mixed with the suspension. As controls, normal human Fab fragments (OEM Concepts) and PBS only were used. Each treatment was tested in two wells. After 24 h of incubation, parasitemia was determined by counting the number of infected erythrocytes in 10,000 total erythrocytes by Giemsa staining. Experiments were repeated three times.

Nucleotide sequence accession numbers. The nucleotide sequence data reported in this paper have been deposited in the DDBJ, EMBL, and GenBank databases under accession numbers AB289325 to AB289330.

RESULTS

Reactivity of human Fab clones. A combinatorial immunoglobulin gene library constructed from peripheral lymphocytes of eight patients with falciparum malaria contained approximately 5×10^7 clones. Colony blotting of 8×10^5 clones yielded 62 positive clones (0.008%). Secondary screening of the positive clones with ELISA, using MSP-1_{19} and CP2.9, followed by screening with immunofluorescence microscopy, identified three positive clones. The positive Fab clones, designated Pf25, Pf143, and Pf227, were reactive to both MSP-1_{19} and CP2.9. Affinity chromatography-purified Fab fragments showed two bands, with molecular masses of 24 and 25 kDa, under reducing conditions (Fig. 1) by SDS-PAGE. These bands were identified as light and heavy chains by Western immunoblot analysis.

Indirect immunofluorescence microscopy revealed the localization of antigens recognized by these purified Fab fragments on the surfaces of late trophozoites/schizonts and merozoites (Fig. 2). This surface staining was demonstrated on strains FCR3 and 3D7, which are representatives with dimorphic allelic variants in MSP-1_{19}, suggesting the Fab fragments' reactivity to a conserved region. Western immunoblot analysis under nonreducing conditions showed that these Fab fragments were reactive to proteins with apparent molecular masses of 16 and 21 kDa (Fig. 3). In addition, 35-, 74-, and 76-kDa bands were also detected. All of these bands were also detected by plasmas from the eight malaria patients used to construct the library in this study.

Primary structure and gene usage of human Fab clones. Deduced amino acid sequences of the heavy- and light-chain immunoglobulin genes of clones Pf25, Pf143, and Pf227 are shown in Fig. 4. The three complementarity-determining regions (CDRs) in the heavy chains of Pf25 and Pf227 were identical. The light-chain CDR1 and CDR2 sequences were identical in the three Fab clones. Only one amino acid was different in CDR3 sequences among these clones. The sequence homology of these clones with germ line sequences was analyzed by IgBLAST at the NCBI website (http://www.ncbi.nlm.nih.gov/igblast/) and by V-QUEST at the international Immunogenetics database (http://imgt.cines.fr:8104/textes/vquest/). For the heavy-chain genes, the closest germ line sequence of the V segments in Pf25 and Pf227 was VH1-8, and that of the

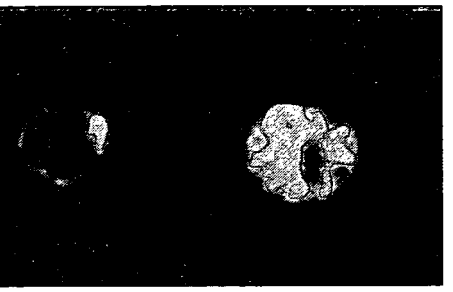

FIG. 2. Indirect immunofluorescence staining of *P. falciparum* (strain FCR3)-infected human erythrocytes with recombinant Fab Pf25. The surfaces of a late trophozoite (left) and of merozoites in the schizont stage (right) were stained. Propidium iodide was used for counterstaining.

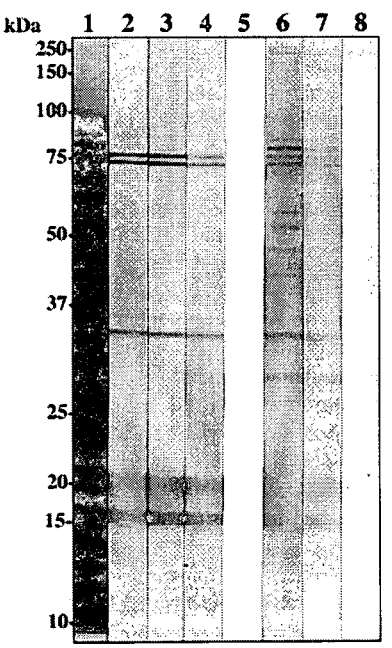

FIG. 3. Western immunoblot analysis of reactivities of human monoclonal antibody Fab fragments to a crude antigen of *P. falciparum* (strain FCR3). Cell lysates were subjected to SDS-PAGE in a 10% polyacrylamide gel under nonreducing conditions and then transferred to polyvinylidene difluoride membranes. The protein bands in lane 1 were stained with Coomassie brilliant blue. Lanes 2 to 8 were treated as follows: lane 2, Pf25; lane 3, Pf143; lane 4, Pf227; lane 5, control human Fab; lanes 6 and 7, plasmas from patients with falciparum malaria; and lane 8, serum from a healthy patient (control). The preparations in lanes 2 to 5 and lanes 6 to 8 were treated with an HRP-conjugated goat antibody to human Fab and an HRP-conjugated goat antibody to human whole IgG, respectively. The numbers on the left indicate the molecular masses of size markers.

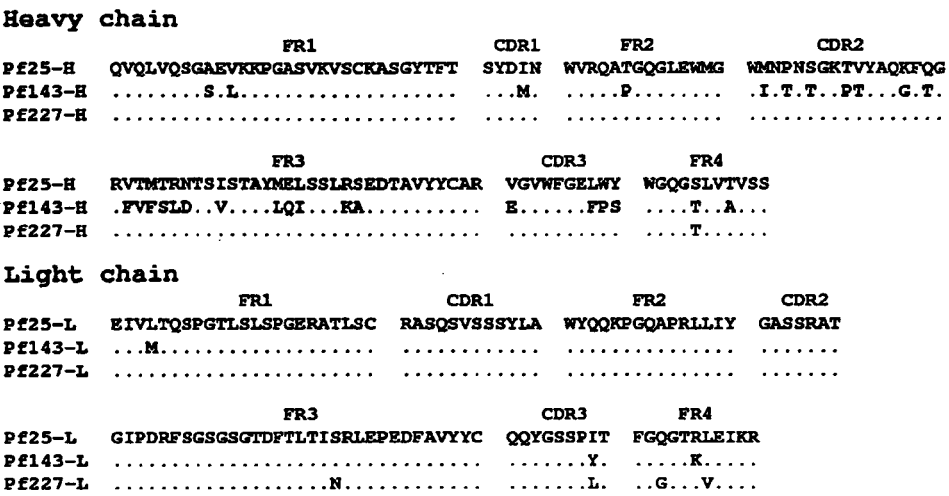

FIG. 4. Deduced amino acid sequences of genes coding for heavy- and light-chain variable regions of human anti-*P. falciparum* MSP-1_{19} Fab fragments. FR, framework regions. The dots indicate identical residues.

V segment in Pf143 was VH7-81 (Table 1). The closest germ line sequence of the D segments was D3-10 for these clones. For the J segment, JH4 was used in Pf25 and Pf227, and JH5 was used in Pf143. All of the light chains belonged to the Vκ1 family. The closest germ line sequence of the V segment was A27 for all three clones, but the closest germ line sequence of the J segment was different for each of the three clones (Table 2).

Affinities of human Fab clones. The affinities of Pf25, Pf143, and Pf227 for recombinant MSP-1_{19} were measured by surface plasmon resonance. The dissociation constants of the three Fab clones ranged from 1.09×10^{-9} to 2.66×10^{-9} M (Table 3). The affinity of Pf25 was approximately two to three times higher than those of Pf143 and Pf227.

Analysis of an epitope recognized by human Fab fragments. To examine whether the three Fab fragments recognized inhibitory epitopes on MSP-1_{19}, a competition assay was performed using an anti-MSP-1_{19} mouse monoclonal antibody, 12.8, which is known to inhibit parasite growth (3, 22). Although competition between the three human Fab fragments and the monoclonal antibody 12.8 was observed, comparable competition was also detected between the Fab fragments and the blocking monoclonal antibody 2.2, which is known to block the binding of the inhibitory monoclonal antibody 12.8 to MSP-1_{19} (12) (Fig. 5).

To examine whether the epitope for these Fabs was recognized by immune sera, competition ELISA was also performed using sera from 10 malaria-immune individuals from the Solomon Islands or plasmas from eight donors of lymphocytes. Only three of the immune sera and three of the donor plasmas showed significant inhibition compared with control sera. No significant difference in mean inhibition levels was demonstrated between the donor and the immune groups (Fig. 6).

Inhibitory activity of Fab fragments on parasite growth. To evaluate whether Pf25, which had the highest affinity among the three Fab clones, inhibits parasite growth, it was added to the culture of *P. falciparum* at a concentration of 200 μg/ml. Although Pf25 suppressed parasite growth compared with the PBS control (Table 4), no difference was seen between Pf25 and a control human Fab fragment.

DISCUSSION

To the best of our knowledge, this is the first report of the successful production of human monoclonal antibodies reactive to *P. falciparum* MSP-1_{19}, a malaria vaccine candidate. Previous attempts to produce human monoclonal antibodies to *P. falciparum* with Epstein-Barr virus-transformed lymphocytes did not induce stable secretion of antibodies (16, 18, 47). Recombinant technology has been used to produce human Fab and scFv fragments to *P. falciparum* proteins (19, 32, 37, 49), and a human scFv fragment to the N-terminal block 2 region has been reported (37). However, the scFv fragment was reactive to only a limited number of parasite isolates, probably because of extensive sequence polymorphism in N-terminal block 2 of MSP-1 (43). In contrast, the three human Fab

TABLE 1. Comparison of gene usage for heavy-chain variable regions of anti-*P. falciparum* MSP-1_{19} human Fab fragments

Clone	V segment		D segment		J segment	
	Closest germ line	% Identity	Closest germ line	% Identity	Closest germ line	% Identity
Pf25-H	VH1-8	97	D3-10	100	JH4	94
Pf143-H	VH7-81	91	D3-10	100	JH5	90
Pf227-H	VH1-8	98	D3-10	100	JH4	97

TABLE 2. Comparison of gene usage for light-chain variable regions of anti-*P. falciparum* MSP-1_{19} human Fab fragments

Clone	V segment		J segment	
	Closest germ line	% Identity	Closest germ line	% Identity
Pf25-L	A27	100	Jκ5	100
Pf143-L	A27	99	Jκ2	100
Pf227-L	A27	99	Jκ4	100

TABLE 3. Association and dissociation constants for binding of recombinant human Fabs to *P. falciparum* MSP-1_{19}, measured by surface plasmon resonance[a]

Fab	K_A (1/M)	K_D (M)
Pf25	9.17×10^8	1.09×10^{-9}
Pf143	5.86×10^8	1.71×10^{-9}
Pf227	3.76×10^8	2.66×10^{-9}

[a] K_A, association constant; K_D, dissociation constant.

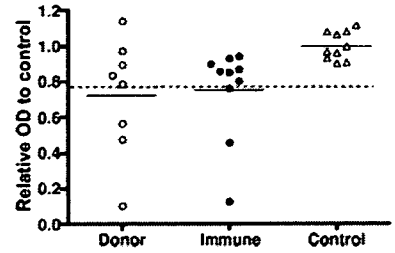

FIG. 6. Competitive binding of MSP-1_{19} in ELISA between recombinant human Fab Pf25 and plasmas from donors of lymphocytes, sera from immune inhabitants of the Solomon Islands, or sera from healthy control individuals. Donor, plasmas from donors of lymphocytes (○); immune, immune sera from individuals with *P. falciparum* infections (●); control, sera from healthy controls (△). Horizontal bars indicate mean values. The dashed line indicates the cutoff value (mean of controls minus 3 standard deviations).

clones, Pf25, Pf143, and Pf227, obtained in the present study are reactive to a conserved region of MSP-1_{19} (14, 15, 33) because the clones were reactive with strains FCR3 and 3D7. These two strains are representatives of dimorphic allelic variants, showing five amino acid substitutions in MSP-1_{19} (K. Tanabe, unpublished data). Therefore, the epitope recognized by these Fabs seems to be conserved in *P. falciparum* isolates. The binding of the three Fab fragments was competitively inhibited by two mouse monoclonal antibodies, 12.8 and 2.2, both of which react to conserved epitopes in MSP-1_{19} (3, 22), also suggesting that the three Fabs recognize a conserved epitope in MSP-1_{19}.

The three Fab fragments share a similar CDR structure in both the light and heavy chains. Therefore, the epitopes recognized by these Fabs are considered identical. Since the three human Fab fragments did not react with MSP-1_{19} under reducing conditions, the epitope is likely formed by the conformation of the two epidermal growth factor-like domains in MSP-1_{19}. The equilibrium dissociation constants (K_Ds) of the three human Fab fragments ranged from 1.1 to 2.7 nM. These values are considerably higher than those reported for other human Fab fragments. For example, the K_D of a human Fab fragment to the recombinant spike protein of severe acute respiratory syndrome-associated coronavirus, which we produced using the same expression system, is 19.8 nM (17), and the K_Ds of neutralizing human Fab fragments to the recombinant LecA domain of *Entamoeba histolytica* lectin are 7.7 to 13.9 nM (41). The K_Ds of anti-*P. falciparum* MSP-3 human Fab fragments reported by Lundquist et al. (19) are 20 to 46 nM.

The three Fab clones reacted not only to 16- and 21-kDa proteins but also to 74-, 76-, and 35-kDa proteins. We believe that the 16-kDa protein is the C-terminal 19-kDa molecule under nonreducing conditions. The 16-kDa protein may be a fragment processed from the 21-kDa protein after excision of the C-terminal sequence for glycosylphosphatidylinositol anchoring. The 35-kDa band observed in Western immunoblots seems to be the 42-kDa molecule under nonreducing conditions, as reported previously (3). The 74- and 76-kDa proteins may share the same epitope as the N-terminal 83-kDa molecule of MSP-1, which is further processed into slightly smaller fragments of 67 to 75 kDa (20, 38). MSP-1_{19} contains the following three distinct types of epitopes: (i) epitopes recognized by inhibitory antibodies that inhibit processing of the C-terminal 42-kDa molecule and erythrocyte invasion by the merozoite, (ii) epitopes recognized by blocking antibodies that block the binding of the inhibitory monoclonal antibodies to MSP-1_{19}, and (iii) epitopes recognized by antibodies which are

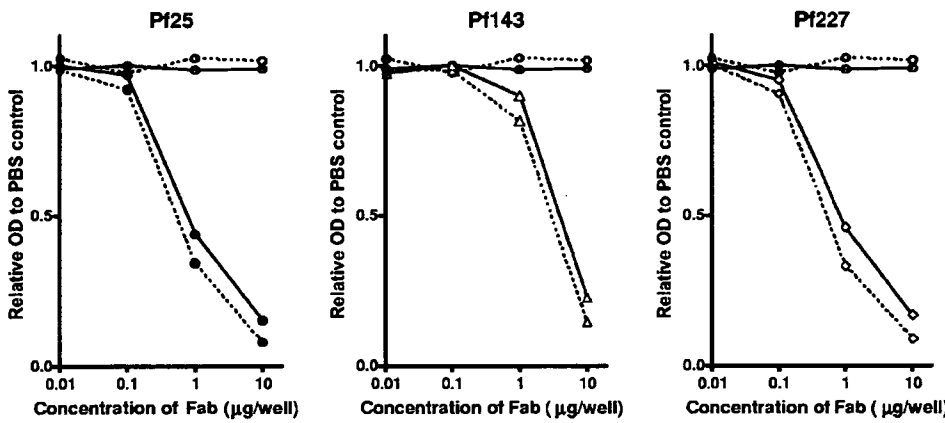

FIG. 5. Competitive binding between recombinant human Fab fragments and mouse monoclonal antibodies to MSP-1_{19} in ELISA. Various concentrations of recombinant Fab fragments (Pf25, ●; Pf143, △; Pf227, ◇; and control Fab, ○) in PBS or PBS only was premixed with 200 ng per well of the inhibitory antibody 12.8 (solid lines) or 20 ng per well of the blocking antibody 2.2 (broken lines) and then added to wells. Reactions were detected with an HRP-conjugated goat antibody to mouse IgG and with a substrate. Optical densities (OD) were measured at 490 nm and expressed as relative values to the PBS control values.

TABLE 4. In vitro effect of Fab fragments on growth of *P. falciparum*

Antibody	Concn (mg/ml)	% Parasitemia[a]	% Inhibition
PBS only	0	2.48 ± 0.34	0
Control Fab	0.2	1.69 ± 0.18	32
Pf25	0.2	1.74 ± 0.34	30

[a] Mean ± standard deviation of data from three experiments.

neither inhibitory nor blocking (4, 48). In the present study, the binding of three human Fabs to MSP-1_{19} was competitively inhibited by both inhibitory and blocking mouse monoclonal antibodies to MSP-1_{19}, suggesting that the epitope recognized by these human Fab fragments overlaps epitopes recognized by these mouse monoclonal antibodies. Importantly, the binding of inhibitory monoclonal antibodies is blocked by naturally acquired human antibodies specific to the 83-kDa fragment of MSP-1 (12). In Western immunoblot analysis using our human Fab fragments as well as reactivity with patient plasmas, approximately 74- and 76-kDa bands were detected under nonreducing conditions. Therefore, we cannot exclude the possibility that the three Fab fragments may cross-react with the 83-kDa molecule.

In the present study, a human Fab clone did not inhibit the parasite's growth at a concentration of 200 μg/ml. It has been reported that the mouse monoclonal antibody 12.8 inhibits processing of MSP-1_{42} by 96% at a concentration of 300 μg/ml (12). This concentration of whole IgG molecule is equivalent to 200 μg/ml of Fab fragments. The reason for the failure in obtaining inhibitory antibodies could be the source of our immunoglobulin gene library. We constructed the library from lymphocytes of eight malaria patients having clinical symptoms. It is generally known that individuals with asymptomatic malaria—but not those with clinical malaria—are immune to *P. falciparum*, and such protective immunity is acquired after repeated infections in areas where malaria is endemic, such as Tanzania (10). In the present study, the epitope for three human Fabs was strongly recognized by only 3 of 10 immune sera. Therefore, construction of a new library from peripheral blood lymphocytes of asymptomatic individuals highly immune to malaria may be required to obtain an inhibitory Fab clone, in addition to further screening of the library prepared for the present study. Indeed, we previously observed that a library constructed with cells from asymptomatic cyst passers of *E. histolytica* demonstrates a higher positive ratio of antibodies recognizing the adherence-inhibiting epitope of a surface lectin than that for a library prepared from a symptomatic patient with amebiasis (41). Recently, the preparation of whole IgG molecules from Fab fragments was reported (19). Whole IgG containing the cloned Fab fragments may induce antibody-mediated killing of merozoites by complement, antibody-dependent cellular cytotoxicity, and growth inhibition by cross-linking of antigen molecules (28, 29). Since the human Fabs reported in the present study possessed high affinities and specificities for a conserved epitope in MSP-1, we believe that there remains the possibility that these human Fabs are still candidates for malaria immunotherapy.

Meanwhile, the human Fab clones are useful for epitope mapping of antigens by analysis of the antigen-Fab fragment complex (23). An important observation in this context was the identification of the primary structure of anti-MSP-1_{19} human antibodies. The usage of germ line sequences in antibodies to MSP-1 would be useful for understanding the molecular basis of acquired humoral immunity to malaria and for analyzing the epitopes of MSP-1_{19}. We believe that the present study will set the stage for further production and development of human anti-MSP-1_{19} monoclonal antibodies for the possible development of passive immunotherapy for falciparum malaria.

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HIGH FREQUENCY OF RECOMBINATION-DRIVEN ALLELIC DIVERSITY AND TEMPORAL VARIATION OF *PLASMODIUM FALCIPARUM MSP1* IN TANZANIA

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Abstract. A major mechanism for the generation allelic diversity in the *Plasmodium falciparum msp1* gene is meiotic recombination in the *Anopheles* mosquito. The frequency of recombination events is dependent on the intensity of transmission. Herein we investigate the frequency of recombination-driven allelic diversity and temporal variation of *msp1* in Rufiji, eastern coastal Tanzania, where malaria transmission is intense. We identified 5′ recombinant types, 3′ sequence types, and *msp1* haplotypes (unique associations of 5′ recombinant types and 3′ sequence types) to measure the extent and temporal variation of *msp1* allelic diversity. The results show that *msp1* haplotype diversity is higher in Tanzania as compared with areas with lower transmission rates. The frequencies of individual polymorphic regions/sites remained stable during the study period. However, the frequency distribution of *msp1* haplotypes varied between 1993 and 1998. These results suggest that frequent recombination events between *msp1* alleles intermittently generate novel alleles in high transmission areas.

INTRODUCTION

The 200-kDa merozoite surface protein-1 (MSP-1) of *Plasmodium falciparum* is a leading vaccine candidate antigen.[1,2] MSP-1 contains at least two regions targeted by host immunity: block 2 near the N terminus and block 17 at the C terminus. Human antibodies against block 2 are associated with protection from clinical malaria in highly endemic areas in Africa.[3] Block 17 encodes a C-terminal 19-kDa polypeptide, a product processed from MSP-1,[4] which confers protection after immunization against challenge with live parasites in animals.[5,6] Sera from individuals living in highly endemic areas contain antibodies against the 19-kDa fragment that inhibit merozoite invasion into red blood cells.[7–9]

MSP-1 exhibits extensive polymorphism,[10,11] which is a potential obstacle to the development of effective vaccines. In animal models, MSP-1 has been shown to be the major antigen involved in inducing "strain-specific immunity," in which the host mounts an immune response that is more effective against the immunizing strain than it is against genetically divergent strains.[12,13] As is the case for other *P. falciparum* antigen genes, *msp1* polymorphism is generated via a number of different mechanisms; point mutations result in single-nucleotide polymorphisms (SNPs), insertion/deletion of repeats cause repeat length polymorphisms, and meiotic recombination involving the exchange of gene fragments between parental alleles produces novel alleles in the progeny. SNPs in *msp1* appear to be stable through time[14] and may be of ancient origin.[15] Repeat-length polymorphisms are common in *msp1*[10,11,16] to the extent that size polymorphism between alleles is widely used as a marker for parasite genotyping.[17] Aside from repeat-length polymorphisms, meiotic recombination is the major mechanism for the generation of *msp1* allelic diversity.[10] Potential recombination sites have previously been mapped to restricted regions within *msp1* (see Figure 1).[10,11] The frequency of recombination in *P. falciparum* is dependent, to a large extent, on the rate of transmission, because meiotic recombination occurs only in the mosquito host. Recombination-driven allelic diversity in *msp1* is expected to be high in areas of intense malaria transmission and lower in areas with less intense transmission dynamics. The validity of this assumption remains to be tested, however, as very few studies have directly measured recombination-driven *msp1* diversity in areas of high transmission.

To investigate the nature and frequency of *msp1* allelic diversity in a highly endemic area, we conducted a study of the prevalence of *msp1* haplotypes in isolates collected 1993, 1998, and 2003 in Rufiji, eastern coastal Tanzania, where malaria transmission is intense and perennial.[18] Our results show that the extent of recombination-driven allelic diversity in *msp1* is higher in Tanzania as compared with areas with lower transmission rates. The frequency distribution of *msp1* haplotypes varied through time, but the frequencies of individual polymorphic regions and sites remained stable throughout the 10-year period of study. These results suggest that frequent recombination events in *msp1* intermittently generate novel *msp1* alleles in a high transmission area.

MATERIALS AND METHODS

Study area and sample collection. *P. falciparum* isolates were collected during malaria surveys from individuals living in Nyamisati village in the Rufiji River Delta, 150 km south of Dar es Salaam, in eastern coastal Tanzania in February and March 1993 (N = 120), 1998 (N = 132), and January 2003 (N = 104). Almost all samples were taken from asymptomatic donors of all ages with a mean age of 14.2 years (range, 1–78) and 16.8 years (range, 1–63) in 1993 and 1998, respectively, and from those aged 10–19 years (mean, 13.8 years) in 2003. Malaria in the study area was holoendemic with perennial transmission with some increase during the two rainy seasons, April to June and December.[18] An annual entomological inoculation rate is not available for the study area, but it is known to be in the range of 94 to 667 infective mosquito bites/person/year in eastern Tanzania.[19] Insecticide-impregnated bed nets were distributed to all houses in the village in 1999. Slide-positive parasite rates were recorded for the 1993 sample (46%) but were unavailable for the other sampling dates because of technical reasons. However, para-

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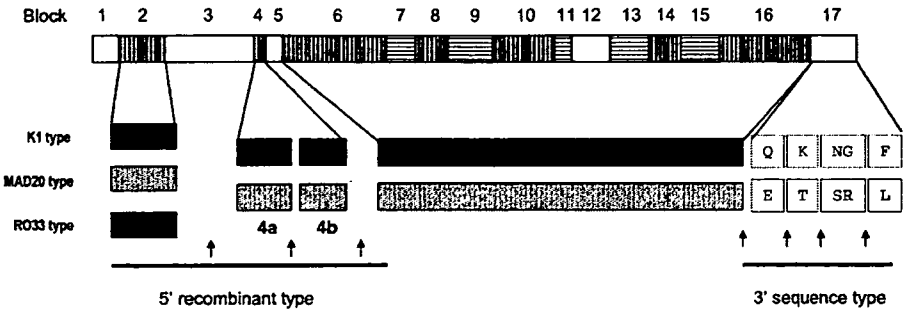

FIGURE 1. Determination of *P. falciparum mspl* haplotype, a unique association of 5′ recombinant types and 3′ sequence types. *mspl* is divided into 17 blocks, in which inter-allele conserved, semi-conserved, and variable blocks are indicated by open, horizontally hatched, and vertically hatched columns, respectively. For K1-type, MAD20-type, and RO33-type variable blocks, sequences are represented by densely toned, half-toned, and black bars, respectively. The 5′ recombinant types were determined by PCR amplification of blocks 2 to 6 using allelic type–specific primers of blocks 2 and 6, followed by nested PCR for blocks 4a and 4b using allelic type-specific primers of blocks 4a and 4b. Five amino acid substitutions in block 17 are indicated by the one-letter codes. The 3′ sequence type is the combination of those residues. Potential recombination sites are shown by arrows.

site positive rates in sampled people as checked by high-sensitivity PCR-based parasite detection (*mspl* typing method used in this study) were 78%, 77%, and 44% in 1993, 1998, and 2003, respectively.

All samples were collected after informed consent had been obtained from the donors or their guardians. Venous blood was collected into EDTA-containing tubes and stored at −20°C. Individuals with signs of clinical disease, i.e., fever and parasites, were treated with Fansidar. Parasite genomic DNA was extracted using the QIAamp DNA Blood Kit (Qiagen, Hilden, Germany). The volume of extracted DNA template was adjusted to be equivalent to the original blood volume. Ethical approval was obtained from the Ethical Committee of the National Institute for Medical Research, Tanzania, and the Ethical Committee of the Karolinska Institute, Sweden. Data previously obtained from clinical samples in Mae Sot in northwestern Thailand in 1995 and from survey samples in Guadalcanal Island in the Solomon Islands in 1994–1996 were used for geographical comparison.[20] We used clinical isolates (N = 111) from patients who attended a malaria clinic in Mae Sot in northwestern Thailand in 1995.[21] The mean age of the donors in Thailand was 24.6 years. A total of 90 isolates were collected in north Guadalcanal, the Solomon Islands: 40 clinical isolates from outpatients with a mean age of 18.3 years of a hospital in Honiara City and 50 isolates from four villages (Kaotave, Tadhimboko, Nugalitav, and Ruavatu).[20] In these rural villages, samples were collected in most cases from parasite-positive asymptomatic individuals during malariometric surveys, and most of the donors were primary-school children aged 8 to 15 years.

Determination of *mspl* polymorphisms. *P. falciparum mspl* (a 5-kb single-copy gene) consists of 17 distinct sequence blocks, according to the degree of sequence similarity among alleles (Figure 1).[10] Sequence variation in *mspl* is principally dimorphic (either one or the other of two major allelic types: K1 type and MAD20 type) in all variable blocks except block 2, which is trimorphic (represented by K1, MAD20, and RO33 types). To monitor the recombination-driven allelic diversity of *mspl*, we divided the gene into three regions: a 5′ 1.1-kb region (blocks 2 to 6), a central 3.5-kb region (blocks 6 to 16), and a 3′ 0.4-kb region (block 17), in which potential recombination sites have been mapped to the 5′ and 3′ regions (Figure 1). No recombination events occur in blocks 6 to 16.[16,21,22] The *mspl* haplotypes are thus defined as unique associations of 5′ recombinant types and 3′ sequence types in this study.

The 5′ recombinant types are defined as unique associations of allelic types of variable blocks 2, 4a, 4b, and 6. In total, 24 distinctive 5′ recombinant types are distinguishable: i.e., 24 = 3 × 2 × 2 × 2 (three allelic types designated as K, M, and R in block 2 and two allelic types designated as K and M in blocks 4a, 4b, and 6). The 5′ recombinant types were determined by our methods described previously.[20] In brief, they were determined by the following two steps: (i) first-round PCR to determine allelic types of blocks 2 and 6 using allelic-type-specific primers, and (ii) nested PCR to determine allelic types of blocks 4a and 4b (≈ 100 bp) using the first-round PCR products and allelic-type–specific primers. The PCR method allows us to determine the rate of multiple 5′ recombinant-type infections, here referred to as "polyinfection rate," and the mean number of 5′ recombinant-type infections per isolate (MORT). One microliter of template DNA was used for first-round PCR. 5′ Recombinant types were fully determined in 94 of 120 Tanzanian isolates collected in 1993, in 102 of 132 isolates in 1998 samples, and in 46 of 104 isolates in 2003. Thus, 68% (242/356) were PCR-positive in all samples obtained through malariometric surveys, indicating that our data represent a *P. falciparum* population in the study area.

The nucleotide sequence of block 17, which encodes the C-terminal 19-kDa polypeptide, was determined by direct sequencing after amplification of the full-length *mspl*. To see associations of 3′ sequence types (block 17 sequences) and 5′ recombinant types (blocks 2 to 6), only those isolates having a single 5′ recombinant type (i.e., mono-infection) were selected for further analysis. (We did not use cloning of the full-length *mspl* gene because artificial recombination readily occurs during amplification and cloning when samples with mixed genotypes are used.[22]) Because the number of isolates with mono-infections was limited in our Tanzanian samples, we increased the number of mono-infection samples by diluting genomic DNA templates by 20-fold. 5′ Recombinant types were again determined for the diluted samples, and those with a single 5′ recombinant type were selected. The

numbers of isolates sequenced were 38, 23, and 13 in samples collected in 1993, 1998, and 2003, respectively. No significant difference in the frequency distribution of 5′ recombinant types was found between undiluted original samples and diluted samples, indicating no bias of sampling after dilution (not shown). Amplification of the full-length *msp1* was first done with primers UPF1 (5′-GGCTAATGTAAAATGCAAAAATAAATGT) and DWR1 (5′-ACATGACTAAAATATCACTATTCCTGT) in a 20-μL reaction mixture containing 1 μL of template genomic DNA for 37 cycles using LA-*Taq* (TaKaRa, Tokyo, Japan). Two microliters of 10-fold diluted PCR products were amplified by nested PCR using primers UPF3 (5′-AATAAATGTATACATATTTTTGCTAAGTCA) and DWR3 (5′-TTAAGGTAACATATTTTAACTCCTACA) for 20 cycles. The PCR product was purified using the QIA Quick PCR purification kit (Qiagen) and directly sequenced from both directions using primers C17aFs (5′-CAAG(G/A)TATGTTAAACATTTCACAACA) and DWR3 with the BigDye Terminator Cycle sequencing kit (version 3.1) on an automated multicapillary ABI 3100 sequencer (Applied Biosystems, Foster City, CA). Sequences were verified by re-sequencing the PCR products independently amplified from the same DNA. To date, five major amino acid changes have been identified in block 17 from various geographic areas (E or Q at amino acid residue 1644; T or K at 1691; SR or NG at 1700–1701; and L or F at 1716; the positions are numbered according to Ref. 15) (Figure 1).[21,23] Hereafter, we refer to combinations of these residues as 3′ sequence type.

Unique associations of 5′ recombinant types and 3′ sequence types are referred to as *msp1* haplotypes. Partial sequencing of blocks 2 to 6 of the PCR amplicons (full-length *msp1*) confirmed 5′ recombinant types determined by PCR-based typing (Tanabe, unpublished). This indicates that our analysis of linkage between polymorphisms in the 5′ region and 3′ region is not affected by artificial recombination.

Statistical analyses. Frequency distributions of *msp1* 5′ recombinant types, 3′ sequence types, and *msp1* haplotypes were compared using the χ^2 test with Yates correction and Fisher's exact test for data sets fewer than 5. Differences in mean number of 5′ recombinant types per isolate (MORT) were tested for significance using a two-tailed Mann-Whitney *U* test. The diversity level of *msp1* haplotypes was expressed in two ways: (i) relative frequency of the number of unique *msp1* haplotypes per total number of *msp1* haplotypes, and (ii) expected heterozygosity (*h*). *h* and its variance were calculated as previously described.[20] Differences in the relative frequency were tested by *t* test. The frequency of recombination events in *msp1* was inferred from analysis of linkage disequilibrium within and between polymorphic blocks 2 to 6 and polymorphic sites in block 17. To assess linkage disequilibrium within *msp1*, pairs of polymorphic blocks 2, 4a, 4b, and 6 and polymorphic sites in block 17 were subjected to an R^2 test as described elsewhere.[24] Non-informative pairs (frequency < 10% in a polymorphic block or nucleotide site) were excluded from the R^2 test. Significance of linkage disequilibrium was assessed using the χ^2 test with Yates correction or two-tailed Fisher's exact probability test. A $P < 0.05$ was considered statistically significant.

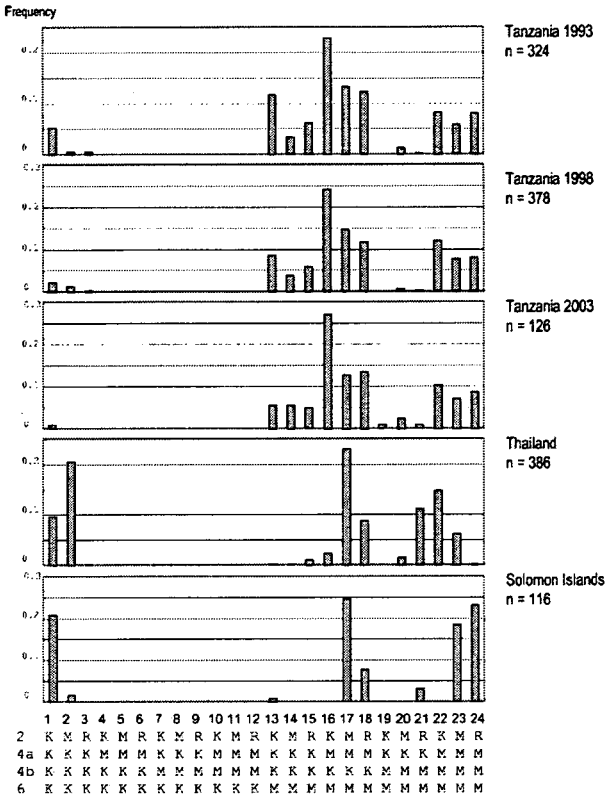

FIGURE 2. Frequency distribution of *P. falciparum msp1* 5′ recombinant types in Tanzania. Twenty-four distinct types—unique associations of allelic types in variable blocks 2, 4a, 4b, and 6—are shown at the bottom of the figure. Data from Thailand and Solomon Islands are from Sakihama et al.[20]

RESULTS

***msp1* 5′ recombinant types (blocks 2 to 6).** The frequency distributions of *msp1* 5′ recombinant types are shown in Figure 2. Types #1 to #12 are those with K1 allelic type in block 6, and types #13 to #24 are those with MAD20 allelic types. Most of the Tanzanian isolates were MAD20 allelic type in block 6 in 1993, 1998, and 2003. The overall pattern of fre-

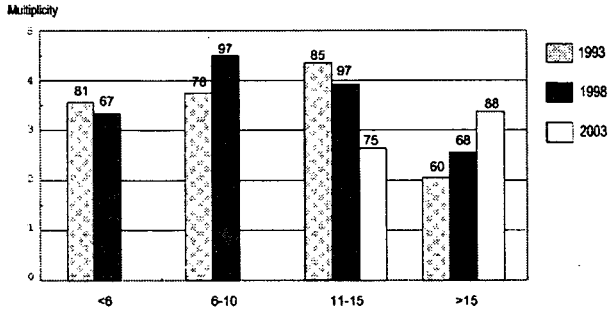

FIGURE 3. Age distribution of mean number of 5′ recombinant-type infections per isolates (MORT) in Tanzania. Ages are categorized into four classes: < 6, 6–10, 11–15, and > 15 yrs. Percentage of multiple 5′ recombinant infections is shown above each bar. Total numbers of isolates are 87, 95 and 44 in 1993, 1998 and 2003, respectively.

quency distribution of 5′ recombinant types was very similar from 1993 to 2003. The frequency distribution of 5′ recombinant types in Rufiji did not differ significantly from that reported previously in Tanga, northeastern Tanzania.[25] Tanzania was, however, significantly different from other geographic areas: Thailand and Solomon Islands ($P < 10^{-10}$), where frequencies of those types having K1 type in block 6 were substantially higher (19% in Solomon Islands and 30% in Thailand) as compared with Rufiji (< 7%).

Rates of multiple infections of the 5′ recombinant types (polyinfection rate) were 76.6%, 87.4%, and 78.3% in 1993, 1998, and 2003, respectively, and the mean number of 5′ recombinant types per isolate (MORT) was 3.48, 3.76, and 2.74, respectively. The reduction of MORT from 1998 to 2003 was significant ($P = 0.008$, Mann-Whitney U test). Both polyinfection rate and MORT are considerably higher in Tanzania than in the Solomon Islands (35.4–60.7% for polyinfection rate and 1.41–1.73 for MORT in Solomon Islands[20]). In Thailand, the polyinfection rate was 96.3% and MORT was 3.61, a level comparable to that observed in Tanzania. Thai isolates, however, were obtained from symptomatic patients, whereas Tanzanian isolates were from asymptomatic carriers, thus making direct comparison somewhat difficult. (There was no significant difference in polyinfection rate and MORT between individuals with clinical malaria and those with asymptomatic malaria in the Solomon Islands.[20])

There was a noticeable difference in age distribution of MORT from Tanzania (Figure 3). In 1993, MORT increased from age group < 6 years to age group 11–15 years and thereafter declined. MORT was significantly lower in age group > 15 years than other age groups ($P = 0.035$ against < 6 years, $P = 0.001$ against 6–10 years, and $P = 0.003$ against 11–15 years). In 1998, a peak MORT was observed in age group 6–10 years, followed by a significant reduction in age group > 15 years ($P = 0.003$). In contrast to the reduction in age group > 15 years in 1993 and 1998, MORT increased from age group

TABLE 1
Frequency distribution of *P. falciparum msp1* haplotypes in Tanzania

5′ Recombinant type	3′ Sequence type					Total	No. of *msp1* haplotypes
	QKNGL	QKNGF	EKNGL	EKNGF	ETSRL		
Tanzania 1993							
KKKK	1	0	0	0	0	1	
KKKM	2	0	1	0	0	3	
KMKM	2*	3	7	0	1	13	
KMMM	0	1 (QKSGF)	1	0	0	2	
MMKM	0	0	5	0	0	5	
(M/R)MMM	1	0	0	0	0	1	
RKKM	0	0	1	0	0	1	
RMKM	1	3	2*	2	0	8	
RMMM	3	0	0	0	1	4	
Total	10	7	17	2	2	38	20
Tanzania 1998							
MKKK	1	0	0	0	0	1	
KMKM	2	1	8	0	0	11	
KMMM	1 (QTSRL)	1	1	0	0	3	
MKKM	0	0	1	0	0	1	
MMKM	1	1	1	0	1+1 (EKSRL)	5	
RMKM	0	1	0	0	1	2	
Total	5	4	11	0	3	23	15
Tanzania 2003							
KKKK	1	0	0	0	0	1	
KKKM	1	0	0	0	0	1	
KMKM	3	2	2	0	0	7	
MMKM	0	0	0	0	1	1	
RMKM	2*	1	0	0	0	3	
Total	7	3	2	0	1	13	9
Thailand							
KKKK	2	0	0	0	0	2	
MKKK	9	0	4	0	0	13	
KMKM	0	0	0	0	1	1	
MMKM	0	2	12	0	2	16	
RMKM	0	0	3	0	0	3	
RKMM	0	0	3	0	1 (ETSGL)	4	
KMMM	1	1	2	0	3	7	
MMMM	0	0	1	0	1	2	
Total	12	3	25	0	8	48	16
Solomon Islands							
KKKK	9	0	0	0	0	9	
MMKM	1	0	0	0	13	14	
RMKM	0	0	0	0	1	1	
MMMM	0	1	5	0	3	9	
RMMM	0	0	0	0	14	14	
Total	10	1	5	0	31	47	8

Identical *msp1* haplotypes shared between 1993 and 1998 are boxed.
* A variant having a substitution from S to N at 1699 is included.

TABLE 2
Diversity of *P. falciparum msp1* haplotype in Tanzania

	No. of samples	No. of *msp1* haplotypes	Relative frequency	*P* value vs. Thailand vs. *Solomon*	$h \pm$ SE*	*P* value vs. Thailand vs. *Solomon*
Tanzania						
1993	38	20	0.53	0.040 *0.002*	0.94 ± 0.02	0.212 *0.0002*
1998	23	15	0.65	0.007 *0.006*	0.89 ± 0.06	0.84 *0.21*
2003	13	9	0.69	0.015 *0.002*	0.94 ± 0.05	0.515 *0.022*
Thailand†	52	16	0.31		0.89 ± 0.03	
Solomon Islands	47	8	0.17		0.80 ± 0.03	

* *h*, expected heterozygosity as an index of haplotype diversity.[20]
† Data from Sakihama et al.[20]

11–15 years to age group > 15 years in 2003, but this trend was not statistically significant. (In 2003, sampling was limited to those of age > 10 years for technical reasons in the survey, and therefore MORT in age groups < 6 years and 6–10 years was not shown.) Polyinfection rates also showed similar patterns of age dependency. A sharp fall was noted from 11–15 years to > 15 years: 85% to 60% in 1993 and 97% to 68% in 1998.

3′ Sequence polymorphism (block 17). Five major nucleotide polymorphisms in block 17, all resulting in amino acid replacements, were observed in Tanzanian isolates ($N = 74$). We obtained 10 unique 3′ sequence types: Q-K-NG-L, Q-T-SR-L, Q-K-NG-F, Q-K-SG-F, E-K-NG-L, E-K-NG-F, E-T-SR-L, and E-K-SR-L (Table 1). In addition, minor variants showing Q-K-NNG-L ($N = 2$) and E-K-NNG-L ($N = 1$) were also observed, where the underlined "N" are substitutions for S at 1699, as detected earlier.[21,23] The number of 3′ sequence types was 5 and 4 in Thailand ($N = 48$) and Solomon Islands ($N = 47$), respectively.

Distribution and diversity of *msp1* haplotypes. The numbers of distinct *msp1* haplotypes were 20 in 38 isolates in 1993, 15 in 23 isolates in 1998, and 9 in 13 isolates in 2003 (Table 2). The *msp1* haplotype diversity, as expressed by relative frequency of the number of unique *msp1* haplotypes per total number of samples, was high in Tanzania in 1993 to 2003 (0.53–0.69) (Table 2). These levels were significantly higher than the level observed in Thailand (0.31; $P < 0.04$) and Solomon Islands (0.17; $P < 0.006$). Rare *msp1* haplotypes with a frequency of < 5% were abundant in Tanzania as compared with Thailand and Solomon Islands: 21/26 haplotypes (81%) in Tanzania in 1993 and 1998, 10/16 haplotypes (63%) in Thailand, and 3/8 haplotypes (38%) in Solomon Islands. Expected heterozygosity (h) was also high from 1993 to 2003 (Table 2). The difference in h reached statistical significance in 1993 and 2003 between Tanzania and Solomon Islands but not between Tanzania and Thailand.

Temporal variation in *msp1* polymorphisms. The frequencies of polymorphisms in polymorphic blocks 2, 4a, 4b, and 6 and five major polymorphic nucleotide sites in block 17 were compared from 1993 to 2003 (Table 3). A frequency variation was only observed in block 4a. Pairwise comparisons were also made between 1993 and 1998 ($P = 0.71$) and between 1998 and 2003 ($P = 0.06$). In contrast to the stable frequencies of individual polymorphisms, the frequency distribution of *msp1* haplotypes was clearly different between 1993 and 1998 (Figure 4) (χ^2 test, $P = 0.001$), indicating temporal variation of *msp1* haplotypes during this 5-year interval. (Rare *msp1* haplotypes were excluded from analysis: $N = 4$ in 1993 and $N = 2$ in 1998; see Table 1.) Among 26 distinct haplotypes found in 1993 and 1998 in a total of 61 isolates, only six haplotypes ($N = 35$) were shared between 1993 and 1998. Because of limited numbers of samples, a comparison with samples collected in 2003 was not made. The frequency distribution of *msp1* haplotypes in Tanzania was considerably different from that of Thailand and Solomon Islands (χ^2 test, $P < 10^{-10}$).

Linkage disequilibrium in *msp1*. To determine the frequency of recombination events in *msp1*, we performed linkage disequilibrium (LD) analysis, in which pairs of four polymorphic blocks (blocks 2, 4a, 4b, and 6) and four polymorphic sites were analyzed. Two sites at 1700 and 1701 in block 17 were always linked, and so they were combined for LD analysis. LD was undetectable in most pairs in Tanzania (Figure 5). Only one pair of 10 informative pairs in 1993 ($N = 37$) and one pair of 15 informative pairs in 1998 ($N = 23$) were significant. These pairs were within block 17. LD analysis was not carried out on samples from 2003, due to limited numbers ($N = 13$). These results indicate that the frequency of recombination events in *msp1* is high in the Tanzanian populations. In contrast, in Thailand and Solomon Islands 12 of 21 pairs

TABLE 3
Stable frequency of polymorphism in *Plasmodium falciparum msp1* in Tanzania

Block	Polymorphic type	*n* (frequency) 1993	1998	2003	*P* value
2	K1	156 (0.481)	176 (0.466)	56 (0.444)	0.869
	MAD20	79 (0.244)	104 (0.275)	35 (0.278)	
	RO33	89 (0.275)	98 (0.259)	35 (0.278)	
4a	K1	95 (0.293)	84 (0.222)	26 (0.206)	0.048
	MAD20	229 (0.707)	294 (0.778)	100 (0.794)	
4b	K1	247 (0.762)	271 (0.717)	88 (0.698)	0.261
	MAD20	77 (0.238)	107 (0.283)	38 (0.302)	
6	K1	21 (0.065)	13 (0.034)	1 (0.008)	0.06*
	MAD20	303 (0.935)	365 (0.966)	125 (0.992)	
17	1644:Q†	16 (0.432)	9 (0.391)	9 (0.692)	0.339
	1644:E	21 (0.568)	14 (0.609)	4 (0.308)	
	1691:T	2 (0.054)	3 (130)	1 (0.077)	0.775
	1691:K	35 (0.946)	20 (0.870)	12 (0.923)	
	1700-01:SR	3 (0.081)	4 (0.174)	1 (0.077)	0.772
	1700-01:NG	34 (0.919)	19 (0.826)	12 (0.923)	
	1716:L	28 (0.757)	19 (0.826)	10 (0.769)	0.926
	1716:F	9 (0.243)	4 (0.174)	3 (0.231)	

* Comparison between 1993 and 1998. Frequency in 2003 was not informative.
† Positions are after Miller et al.[11]