MOLECULAR EPIDEMIOLOGY OF MALARIA IN CAMEROON. XVIII.

POLYMORPHISMS OF THE PLASMODIUM FALCIPARUM MEROZOITE SURFACE ANTIGEN-2 GENE IN ISOLATES FROM SYMPTOMATIC PATIENTS

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Abstract. Merozoite surface antigen-2 (MSA-2) is a polymorphic genetic marker that is highly discriminatory for characterizing Plasmodium falciparum field isolates. Genetic diversity of isolates obtained from symptomatic patients residing in Yaoundé, Cameroon was analyzed by an allele-specific polymerase chain reaction and direct sequencing of amplification products. Of 137 isolates, 25 (18%) had only FC27-type alleles, 40 (29%) had only 3D7-type alleles, and 72 (53%) had multiple parasite populations with both alleles. Of 295 fragments, 145 (49.2%) and 150 (50.8%) belonged to FC27 and 3D7 alleles, respectively. There were 23 different MSA-2 alleles (10 FC27-type and 13 3D7-type that yielded 44 different combinations in multiple infections). DNA sequencing showed distinct individual sequences. Sequences belonging to the FC27 allelic family were relatively conserved, with most of the polymorphism arising from differences in the number of repeat units. In contrast, the sequences within the GSA-rich region in 3D7 allelic family were less conserved, but many of the sequences in Cameroonian isolates have been identified in other isolates from geographically distant origins. Our results show an extensive diversity of the central region of MSA-2 in size, allelic family, combinations of these two features in multiple infections, and sequence variations underlying the complex population structure of P. falciparum clinical isolates in Yaoundé, Cameroon.

INTRODUCTION

Merozoite surface antigen-2 (MSA-2) is one of the four integral membrane proteins characterized to date that are present on the surface of Plasmodium falciparum merozoites.1,2 Although it has been shown to be antigenic and specific antibodies directed against MSA-2 epitopes inhibit merozoite invasion of erythrocytes in in vitro experiments,3 its biologic function during the erythrocytic phase of malaria life cycle or its possible immunologic function in developing immune evasion mechanisms is not established. Two related aspects of MSA-2 highlight its importance for malaria research. First, it is a candidate target for a subunit vaccine against the asexual erythrocytic stage of P. falciparum. Second, as a highly polymorphic protein, it may serve as a tool to analyze the complexity of parasite population structure in the field. Studies on the extent of antigenic diversity of a target protein are also a necessary step before field evaluation of vaccine candidates.

The primary structure of MSA-2 is characterized by highly conserved N- and C-terminal domains and a variable central domain that gives rise to size polymorphisms. The central region is further characterized by the presence of one of two unrelated dimorphic allelic families, designated FC27- and 3D7-types, which refer to the sequences of the corresponding P. falciparum reference clones.4 These alleles are defined by specific nonrepetitive sequences flanking the central repeat region. The central tandem repeats belonging to the FC27 family of alleles consist of varying number of relatively conserved units of 32 amino acid residues and 12 amino acid residues. In comparison, the repeat units belonging to the 3D7 family are much less conserved and are highly variable in length (tetramer to decamer) and sequence.

Among genetic markers used to assess parasite diversity in past studies, including MSA-1, circumsporozoite protein (CSP), and glutamine-rich protein (GLURP), MSA-2 was found to be the most discriminatory for field isolates.5–7 These polymorphic features of MSA-2 may be exploited for genotyping naturally-occurring parasites. As an initial effort to assess the extent of genetic diversity and complexity of population structure of malaria parasites in Cameroon, MSA-2 of consecutive P. falciparum clinical isolates from a single study site was analyzed by comparison of multiplicity, size variation, allelic diversity, and DNA sequences. Since an ideal vaccine candidate should protect human hosts against parasite strains that are responsible for malarial disease giving rise to signs and symptoms in patients, more than against malarial infection (i.e., against the parasites that are present in asymptomatic carriers in intense transmission zones), this study was limited to clinical isolates obtained from symptomatic Cameroonian patients.

MATERIALS AND METHODS

Parasites. Fresh clinical isolates of P. falciparum were obtained by venipuncture (5–10 mL of whole blood) from symptomatic patients ≥ 12 years old presenting spontaneously at the Nlongkak Catholic missionary dispensary in 2000–2001. To reduce a potential source of bias, notably in the number of identifiable parasite populations before treatment, only samples from patients with no recent intake of antimalarial drugs, as confirmed by a negative Saker-Solomons urine test result, were included in the study.9 Younger children and infants were excluded from the study due to self-treatment practiced at home in a large majority of patients before consultation and due to the difficulties in obtaining urine samples for confirmation of the absence of antimalarial drugs. In addition, pregnant women and patients with signs and symptoms of severe and complicated malaria were excluded. The enrolled patients were treated with amodiaquine, the first-line drug for uncomplicated P. falciparum malaria in Cameroon. This study was reviewed and approved by the Cameroonian National Ethics Committee and Cameroonian Ministry of Public Health.
Polymerase chain reaction. An aliquot of 1.5–2 mL of red blood cell pellet was stored at −20°C until DNA extraction was performed by phenol-chloroform method, as described previously. The primary polymerase chain reaction was designed to amplify the entire coding region of the MSA-2 gene using primer pairs MSA2-1, 5′-ATGAAGGTAATTAAACATTTGCTATTATA-3′ (forward primer, first 30 bases at 5′-end of the gene, including the start codon) and MSA2-2, 5′-TTATATGAATATGGCAAAAGATAAAAAACAG-3′ (reverse primer, last 30 bases at 3′-end, including the stop codon). The reaction mixture consisted of ∼100–200 ng of genomic DNA, 15 pmol of each primer, buffer (50 mM KCl, 10 mM Tris, pH 8.3), 1.5 mM MgCl₂, 200 μM of deoxynucleoside triphosphates (dNTPs), and one unit of Taq polymerase (Roche Diagnostics, Meylan, France) in a final volume of 50 μL. The PTC-100 thermal cycler (MJ Research, Watertown, MA) was programmed as follows: 94°C for two minutes for the first cycle and 30 seconds in subsequent cycles, 50°C for one minute for the first cycle and 30 seconds in subsequent cycles, and 72°C for one minute for all cycles, for a total of 30 cycles, followed by a 15-minute extension step at 72°C.

For each sample, two sets of nested polymerase chain reaction were performed with allelic family-specific primers. The common primer for these reactions, MSA2-3 (forward primer, 5′-ATAAAACATGCTTATAATAGTATAAGG-3′), was designed to hybridize within the 5′ conserved region, nine bases upstream from the central variable region. FC27 allelic-specific primer (reverse primer, 5′-AGCAGGATTTTCATTCTCGCGTTTGAGG-3′) and 3D7 allelic-specific primer (reverse primer, 5′-AGATTTGTAATTCGGGGATTACGTGTTGTC-3′) were designed to hybridize to the short conserved region at the 3′-end of the central variable region to allow size determination of the variable region.

Amplification products were subjected to electrophoresis on 2.0% agarose gels, stained with ethidium bromide, and visualized by ultraviolet transillumination to count the number of identifiable distinct bands and estimate the size of each amplified fragment. For samples that yielded a single band corresponding to one, but not both, of allelic families, another nested polymerase chain reaction was performed with primer pairs that hybridize within the conserved domains flanking the central variable region. The purpose of this additional polymerase chain reaction was to determine DNA sequences of the entire central region. The primer pairs were MSA2-5, 5′-TAATATAGCAACACATTTCTATAAACAGC-3′ (forward primer) and MSA2-4R, 5′-TCTTTTGTGACTATTAGAAGTATTTTGGG-3′ (reverse primer). All nested polymerase chain reactions were performed by using the same amplification program as that for the primary amplification.

Sequencing of DNA. Only isolates that yielded a single band corresponding to either the FC27 or 3D7 allelic family, but not both, were selected for further characterization of MSA-2 sequence. The amplified product was purified by High Pure PCR Purification kit (Roche Diagnostics), and the quantity of amplified DNA was estimated by comparing with molecular weight markers with known quantities. The purified product was marked with fluorescent nucleotides in the following mixture: 200 ng of amplified product, 4 μL of Terminator Ready Reaction Mixture (Perkin Elmer Corp., Les Ulis, France), 80 mM Tris-HCl (pH 9.0), 2 mM MgCl₂, and 3.2 pmol of primer 5′-TAAATATAGCAACACATTTCTATAAACAGC-3′ in a final volume of 20 μL. The PTC-100 thermal cycler was programmed as follows: 96°C for 30 seconds, 50°C for 15 seconds, and 60°C for four minutes, for 25 cycles. Residual dye terminators were removed by the ethanol precipitation method. The extension product was sequenced by using the ABI Prism automated DNA sequencer (Perkin Elmer Corp.).

Data interpretation. Based on the visual inspection of amplified fragments separated by agarose gel electrophoresis, MSA-2 alleles were classified according to the number of fragments, size of each fragment, and allelic family to which each fragment belongs. Fragment size was estimated by comparing with molecular weight standards. Each fragment was assigned to one of the following size ranges in basepairs (bp): 1, <440 bp; 2, 440 to <460 bp; 3, 460 to <480 bp; 4, 480 to <500 bp; 5, 500 to <520 bp; 6, 520 to <540 bp; 7, 540 to <560 bp; 8, 560 to <580 bp; 9, 580 to <600 bp; 10, 600 to <620 bp; 11, 620 to <640 bp; 12, 640 to <660 bp; 13, 660 to <680 bp; 14, 680 to <700 bp; 15, 700 to <720 bp; 16, 720 to <740 bp. For compari-

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| 2               | 0 | 0 | 0 | 0 | 0 | 2 | 3 | 0 | 0 | 4 | 2 | 1 | 0 | 1 | 0 | 1 |
| 3               | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |

| **FC27 + 3D7** |   |   |   |   |   |   |   |   |   |    |    |    |    |    |    |    |
| 1               | 2/0 | 9/0 | 11/3 | 0/3 | 9/9 | 3/7 | 7/7 | 0/5 | 0/8 | 2/3 | 1/4 | 1/2 | 0/0 | 0/0 | 0/0 | 0/0 |
| 2               | 0/0 | 5/1 | 5/1 | 4/2 | 4/5 | 2/1 | 11/5 | 1/2 | 1/3 | 1/0 | 0/1 | 0/0 | 0/0 | 0/1 | 0/0 | 0/0 |
| 3               | 2/0 | 2/2 | 3/2 | 1/0 | 5/0 | 1/3 | 4/3 | 1/4 | 1/4 | 0/2 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 |
| 4               | 0/0 | 0/0 | 2/0 | 0/1 | 1/0 | 0/1 | 2/0 | 0/1 | 2/0 | 0/1 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 |
| 5               | 0/0 | 1/0 | 1/0 | 0/0 | 1/0 | 0/0 | 1/0 | 0/0 | 1/0 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 |

* Band sizes were assigned to 20-basepair increments between 440 and 740 basepairs. See Materials and Methods for the corresponding sizes of band sizes 1–16. For isolates presenting both allelic variants, the number of isolates with FC27-alleles is given first, followed by the corresponding number of isolates with 3D7-type alleles.
son of sequences, deduced amino acid sequences were aligned manually to those of 3D7 (GenBank accession number M28891) and K1 (FC-27 type; GenBank accession number M59766) reference clones.4,9

RESULTS

In 2000–2001, 137 consecutive samples in Yaounde were analyzed for MSA-2 polymorphisms. Of 137 isolates, 25 (18%) had single (n = 21) or multiple (n = 4) bands belonging to only FC27-type allelic family, 40 (29%) had single (n = 32) or multiple (n = 8) bands belonging to only 3D7-type allelic family, and 72 (53%) had multiple parasite populations with both alleles. A large majority of patients (61%) had multiple infections consisting of at least two distinct parasite populations. Of 295 fragments, 145 (49.2%) and 150 (50.8%) belonged to FC27 and 3D7 alleles, respectively. The extent of MSA-2 polymorphism in terms of the number of distinct parasite populations, size of the central variable domain, and allelic family is summarized in Table 1. There were 23 different MSA-2 alleles, comprising 10 FC27-type alleles and 13 3D7-type alleles, as determined by the number of different size fragments belonging to each allelic family, which yielded 44 combinations in multiple infections. The complexity of multiple parasite populations constituting individual isolates with both FC27 and 3D7 allelic families is illustrated in Figure 1. Only a few pairs of isolates showed identical patterns.

To evaluate the genetic diversity of individual alleles among isolates with a single band belonging to either the FC27- (n = 21) or 3D7-allelic family (n = 32), but not both, 37 randomly selected isolates (19 FC27-type and 18 3D7-type) were further analyzed by DNA sequencing. Sequence alignment showed distinct individual sequences at the deduced amino acid level due to amino acid substitutions, deletions, insertions, sequences of tandemly repeated units, and number of repeat units (Figure 2). The 32-amino acid repeat unit characteristic of FC27-allelic family, ADTISASGGSTNSASTSTTNNGESQTTTPTA, or its variants with the same characteristic of FC27-allelic family, ADTISASGGSTNSASTSTTNNGESQTTTPTA, or its variants with the same characteristic of FC27-allelic family, ADTIASGSQSST−of repeat units (Figure 2). 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To analyze the sequence of isolates with multiple bands, other labor-intensive strategies, such as vector cloning of amplification bands and in vitro cultivation of parasites followed by cloning of individual parasites by micromanipulation or limiting dilution, would be required. Despite these methodologic limitations, our study shows the highly polymorphic nature of clinical isolates in Yaounde, derived not only from the differences in MSA-2 sequences, but also from multiple parasite populations infecting a patient at a given time.

In areas with low transmission of malaria, MSA-2 size polymorphism has been shown to be limited. For example, in Honduras, only three variants (two size variants of the FC27 allele and a single band of identical size of 3D7 allele) were observed.17 Even DNA sequencing of Indian isolates showed very few variations.18 In the African continent, there were considerably fewer MSA-2 variants in Sudan, where malaria transmission is seasonal, as compared with holoendemic areas in Tanzania.19 The same phenomenon may also be observed between neighboring villages with different transmission patterns, as illustrated in Senegalese studies.20 This contrasting difference between areas of low and high transmission also extends to multiplicity, which is much lower in areas of low transmission. These observations were largely confirmed in our study in Yaounde, where malaria infections are characterized by a high level of multiple infections and extensive MSA-2 polymorphisms. Moreover, the frequencies of dimorphic MSA-2 allelic families were similar, which implies random distribution of the genetic marker in our study site. The
The interaction between parasite populations was also shown by the presence of the hybrid FC27/3D7 allele, although the extent of this type of recombination needs to be further characterized in Yaounde.

As reported in previous studies on parasites from different geographic origins, the sequences belonging to the FC27 family found in Cameroonian isolates were generally conserved but varied in the number of repeats. Likewise, most of the sequences of repeat units belonging to the 3D7-type allele found in Cameroonian isolates have been reported in other reference clones and isolates originating from other geographic regions. These repeat units include tetramer GGSA (3D7/unknown origin, Indochina-1, and FCR3/The Gambia clones and Cameroonian isolates D3 and D16), hexamer GGSGSA (7G8/Brazil clone, Wos8/Papua New Guinea [PNG], and Cameroonian isolate D15), and octamer variants.

**Figure 2.** Continued
GAV(G/S)AGSGA (IMR143 and MAD71/PNG, OKS-11U/Indonesia, and Cameroonian isolates D9, D13, D17, and D18), and octamer SGRAGAGA (Thai Tn clone and Cameroonian isolates D1 and D2). The decamers GAGSNPPPA (Wos 17/PNG and Cameroonian isolate D8) and GASGSAGSGD/A (Wos16/PNG and 2 repeat units in D6/Cameroon, a single unit in D2, D7, D10, and D15), as well as their corresponding shortened octamer form GASNPPP (D14/Cameroon) and hexamer form GASGSA (Wos15/PNG, OKS-6/Indonesia, D5 and D12/Cameroon), have also been reported from Papua New Guinea and Indonesia. Other repeat units, such as GAGN and GAVASAGN found in the Nigerian strain NIG32, were either absent or present as a single unit, without repetition, in our series. Some investigators have claimed that novel sequences have been found in their studies. However, as more MSA-2 sequences from *P. falciparum* isolates worldwide are determined, our sequence data seem to suggest that despite the poorly conserved 3D7-type sequences, a unique MSA-2 sequence in relation to the geographic origin may not exist.

The present study shows the extensive MSA-2 polymorphism in terms of size differences in the central repeat domain, allelic families, association of different combinations of alleles in multiple infections, and numerous amino acid sequence variations. Our results are in agreement with those of previous studies involving asymptomatic carriers and symptomatic patients in holoendemic areas, both in the complexity of population structure and multiplicity of parasites in human hosts.\(^\text{4,5,9,10,13,14,19,20}\) However, beyond the apparent extensive diversity of MSA-2, there are features that seem to place a limit to this diversity, possibly due to three-dimensional structural constraint on the surface antigen, human host-parasite-mosquito immunologic interplay, or biologic role played by the protein. First, 5'- and 3'-ends of MSA-2 upstream and downstream of the variable domain are highly conserved. Second, even within the variable domain, the sequences of the FC27-type allelic family seem to be relatively conserved at the amino acid level although the number of repeat units may vary up to a certain extent. Furthermore, for the less conserved 3D7 alleles, similar sequences of repeat units have been identified in parasites originating from geographically distant areas. These restrictions on MSA-2 diversity may be of importance for the successful design of a subunit vaccine.

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