MOLECULAR EPIDEMIOLOGY OF MALARIA IN YAOUNDE, CAMEROON. VIII. MULTIPLE PLASMODIUM FALCIPARUM INFECTIONS IN SYMPTOMATIC PATIENTS

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Abstract. The extent of genetically distinct parasite populations coinfected individual human hosts (i.e., multiplicity) was studied by polymerase chain reaction amplification of 3 polymorphic genetic markers, circumsporozoite protein and merozoite surface antigens (MSA) 1 and 2, in symptomatic children and adults and analyzed in relation with age and initial parasitemia. Of the total of 177 DNA samples analyzed (of which 115 were paired pre- and posttreatment samples), 101 (57%) were composed of multiclonal infections, with up to 7 distinguishable parasite populations. Among the 3 polymorphic markers, msa-2 yielded the highest proportion of clinical isolates with multicolonial populations. Patients with multiclonal infections before treatment had, on average, 2.9 genetically distinct parasite populations. The extent of multiplicity decreased significantly (P < 0.05) in recrudescent parasites, but not with reinfections, as compared with the pretreatment samples. Neither age (5–60 years) nor initial parasitemia was significantly correlated with multiplicity. Further studies in different epidemiological settings are required to understand the role of multiclonal Plasmodium falciparum infections in influencing malaria transmission.

INTRODUCTION

Even before molecular biology techniques were applied to malaria research, it was known that an infected human may carry several distinct populations of malaria parasites with different isoenzymes and variant antigenic types.1,2 Continuous in vitro culture of Plasmodium falciparum strains also provided evidence that the original isolate, defined as the set of parasite populations obtained from a patient at a given time, may be composed of several distinct parasite populations.3–6. The amplification of polymorphic genetic markers by the polymerase chain reaction (PCR) yielding 2 or more variants of the same gene from a single isolate was additional proof of the presence of multiple parasite populations within the same host.7 Multiplicity (i.e., the coinfection of an individual host by 2 or more genetically distinct clones) may arise either from a single mosquito bite with the vector carrying 2 or more parasite populations, or from inoculation by different mosquitoes.8 The presence of distinct parasite populations within the same host has several implications for the understanding of parasite biology, immunology, population structure, transmission dynamics, and drug resistance under natural conditions in the field.

To determine the extent of multiplicity of P. falciparum infections in symptomatic Cameroon patients, 3 polymorphic genetic markers were amplified by PCR, and the number of individual parasite types comprising an infection was analyzed in relation to patients’ age, parasite density, and recrudescence or reinfection after drug therapy. The relationship between age and parasite density is generally thought to be a reliable indicator of malaria endemity.9 The influence of drug treatment on the multiplicity of infections was studied in patients treated with different drugs who had further episodes of malarial infections during longitudinal follow-up.

MATERIALS AND METHODS

Patients. The study was part of randomized clinical trials conducted at the Nlongkak Catholic missionary dispensary in Yaoundé between 1994 and 1998.10,11 Inclusion criteria were as follows: age ≥ 5 years, fever at presentation (or history of fever within the past 24 hr), monoinfection with P. falciparum, parasite density > 5,000 asexual parasites/μL of blood, ready access to the dispensary for daily monitoring, and no recent history of self-medication with antimarial drugs, as confirmed by a negative Saker-Solomons urine test result.12 Patients with signs and symptoms of severe or complicated malaria, as defined by the World Health Organization13 and those with severe anemia, with hemoglobin < 5.0 g/dL, or moderate and severe malnutrition were excluded. Infants and young children aged < 5 years were not included in this study because of the difficulty in obtaining sufficient venous blood samples for hematological and biochemical examinations and in vitro drug sensitivity assays. The patients were treated with standard orally administered doses of chloroquine, amodiaquine, pyronaridine, sulfadoxine-pyrimethamine, or arsunate under supervision. Patients who failed to respond to these drugs were treated with quinine or halofantrine. Informed consent was obtained from either the patients or a guardian accompanying the sick children. The study was approved by the Cameroonian National Ethics Committee and the Cameroon Ministry of Public Health.

Before treatment, venous blood samples (5–10 mL of whole blood) were collected in a Vacutainer tube coated with an anticoagulant (ethylenediaminetetraacetic acid [EDTA]) (Terumo Europe N.V., Leuven, Belgium). Giemsa-stained thin blood films were examined for the identification of Plasmodium species and to count the number of infected erythrocytes against 20,000 erythrocytes. Parasitemia was determined by multiplying the percentage of infected erythrocytes by the erythrocyte count and expressed as the number of asexual parasites per microliter of blood. All blood samples of the enrolled patients had parasitemia > 5,000 asexual parasites/μL, except for 1 sample from a patient (3,690 asexual parasites/μL) who was excluded from the clinical study because of low parasitemia.

Parasite DNA. Venous blood samples were washed 3 times in RPMI 1640 medium by centrifugation (2,000 × g for 10 min) within 3 hr after blood collection. An aliquot of
1.5–2 mL of red blood cell pellet was used to extract parasite DNA (contaminated with human leukocyte DNA). Infected erythrocytes were suspended in 15 mL of ice-cold NET buffer (150 mM NaCl, 10 mM EDTA, 50 mM Tris, pH 7.5) and lysed with 0.015% saponin. The lysate was centrifuged at 2,000 × g for 10 min, and the pellet was transferred to a 1.5-mL microfuge tube and suspended in 500 μL of NET buffer. The mixture was treated with 1% N-lauroylsarcosine (Sigma Chemical Co., St. Louis, MO) and RNase A (100 μg/mL) at 37°C for 1 hr and proteinase K (200 μg/mL) at 50°C for 1 hr. Parasite DNA was extracted 3 times in equilibrated phenol (pH 8), phenol-chloroform-isooamyl alcohol (v/v/v 25:24:1) and precipitated by the addition of 0.3 M (final concentration) sodium acetate and cold absolute ethanol. The extracted DNA was air dried and resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8). Parasite DNA was stored at −20°C until use.

Genotyping of *P. falciparum*. DNA fragments of 3 polymorphic markers, circumsporozoite protein (*csp*), merozoite surface antigen 2 (*msa*-2), and *msa*-2 genes, were amplified by the PCR. The following pairs of species-specific oligonucleotide primers were designed from published DNA sequences: CSP-1, 5′-AAATTACATCTGATGAGAAAATTAGC-3′ (forward primer), CSP-2, 5′-GATGTTCTTATCTTAATATT-AAGGACAAAG-3′ (reverse primer), MSA-2-1, 5′-ATGAAGATCTAATGAGAAATATATGTTGA-3′ (forward primer), and MSA-2-2, 5′-TTATATATATGAGAAATATATGTTGA-3′ (reverse primer).14-16 These primers were designed to amplify the entire *csp* and *msa*-2 genes and block 2 of the *msa*-1 gene.

The reaction mixture consisted of ~200 ng of genomic DNA, 15 picomole of forward and reverse primers, buffer (50 mM KCl, 10 mM Tris, pH 8.3), 1.5 mM MgCl2, 200 μM deoxynucleotide triphosphate (dNTP), and 1 U of Taq DNA 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 2 min for the first cycle and 30 sec in subsequent cycles, 50°C for 1 min for the first cycle and 30 sec in subsequent cycles, and 72°C for 1 min for all cycles (2 min for amplifying the entire *csp* gene), for a total of 30 cycles, followed by a 15-min extension step at 72°C. The amplified DNA fragments were resolved by electrophoresis in a 1.8% agarose gel, stained with ethidium bromide, and visualized under ultraviolet transillumination.

Data interpretation. A given isolate was considered to be monoclonal if a single band was obtained in all genetic markers. The presence of a single band, which was based on the visual evaluation of the number of amplification products, may or may not be associated with a clonal structure of the entire parasite populations infecting the corresponding patients because some parasite populations may be sequenced at the time of blood sampling, 2 distinct parasite clones may be identical for the 3 genetic markers but differ at other loci, and a single band may be composed of variants at the nucleotide sequence level. However, on the basis of the limitations of the methodology that was applied for the present study, the definition of a monoclonal infection cannot be more precise. The proportion of multi-clonality may thus be underestimated in this study. The degree of multiplicity was expressed as the mean number of distinct populations for each genetic marker (excluding the monoclonal isolates).

For posttreatment samples, recrudescence was defined as the presence of identical amplification products in pretreatment and posttreatment samples. Subsets of recrudescent cases were defined in our previous study.17 Reinfection was defined as the presence of different alleles at all 3 loci.

Quantitative variables were compared by the *t*-test for unpaired groups and Wilcoxon signed rank sum test for paired groups. The relation between parasitemia and multiplicity was analyzed by Spearman rank correlation test.

## RESULTS

A total of 177 samples were analyzed in this study. Of these samples, 115 (52 Day 0 samples and 63 posttreatment samples) were serial samples from 52 patients presenting with repeated infections or recrudescence (results of these samples were presented in relation to drug regimens and recrudescence-reinfection in our previous study17). Sixty-two additional samples were randomly selected from pretreatment samples. Of 177 samples, 76 (43%) were monoclonal and 101 (57%) were multclonal. All 3 loci (*csp*, *msa*-1, and *msa*-2) were amplified from 115 serial samples to evaluate the extent of size polymorphism among Cameroonian clinical isolates (Table 1). Amplification of the entire *msa*-2 gene provided the most informative data on the extent of the multiplicity of *P. falciparum* infections; next most informative was block 2 of *msa*-1 gene. Among the 115 serial samples, *csp* revealed the highest number of individual populations in only 5 isolates. Four of these isolates originated from either recrudescence or reinfection, whereas 1 isolate was a pre-treatment sample. In all other isolates, *msa*-2, *msa*-1, or both had equivalent, or higher, number of distinguishable parasite populations.

To analyze the effect of drug treatment on multiplicity, samples were classified as Day 0 samples (*n* = 114, includ-
FIGURE 1. Multiplicity of Plasmodium falciparum infection in symptomatic Cameroonian patients before treatment \((n = 68)\) in relation to age. Data are expressed as the mean number (± standard deviation) of parasite populations comprising multiclonal infections in different age groups with merozoite surface antigen \((msa)-2\) (white) and \(msa-1\) (black) as genetic markers.

The trend of multiplicity between the ages of 5 and 60 years old was invariable (Figure 1). The comparison of multiplicity between older children (5–14 years old; \(n = 29\)) and adults (≥ 15 years old; \(n = 39\)) did not show any significant difference for \(msa-2\) (mean ± standard deviation, 2.92 ± 1.20 versus 2.82 ± 1.28) or \(msa-1\) (2.41 ± 1.30 versus 2.62 ± 1.10) \((P > 0.05)\). The initial parasitemia of patients presenting with monoclonal infections on Day 0 \((n = 68)\) and multiplicity were not correlated \((r = 0.003\) for \(msa-2\), \(r = 0.082\) for \(msa-1\); \(P > 0.05)\) (Figure 2). The comparison of Day 0 parasitemia in patients presenting with monoclonal infections \((\text{geometric mean} 47,100 \text{ asexual parasites/µL}; n = 45)\) or multiclonal infections \((\text{geometric mean} 53,600 \text{ asexual parasites/µL}; n = 68)\) showed no significant difference \((P > 0.05)\).

DISCUSSION

Previous studies have demonstrated the extensive size polymorphism of the genes encoding \(msa-1\) and \(msa-2\) in \(P. falciparum\) field isolates collected in sub-Saharan Africa and other geographic areas.\(^{18-22}\) Our preliminary study on Cameroonian clinical isolates obtained in Yaoundé has also suggested substantial polymorphism of these genetic markers.\(^{17}\) These 2 markers allowed the distinction of multiple \(P. falciparum\) infections in 57% of symptomatic patients in our study. We found \(msa-2\) to be more polymorphic than block 2 of \(msa-1\). Thus, among Day 0 multiclonal samples \((n = 68)\), 16 isolates had multiple distinguishable \(msa-2\) alleles, whereas only a single \(msa-1\) allele was detected in these isolates. Conversely, only 4 isolates (5.9%) had multiple \(msa-1\) alleles and a single \(msa-2\) allele. The PCR of \(csp\) was less discriminative. Among multiclonal isolates obtained on Day 0 and tested for 3 loci, 13 of 34 (38%) exhibited a single \(csp\) allele. By comparison, 3% and 17% of the same isolates had a single visible allele in the \(msa-2\) and \(msa-1\) genes, respectively. In the present study, which was based on a single 30-cycle PCR, we found a slightly higher resolution of multiplicity when genotyping of \(msa-2\) and \(msa-1\) was performed. However, a lower or higher resolution may be obtained with these genetic markers, depending on the molecular techniques used.\(^{18-22}\)

By use of similar molecular techniques, other recent studies have found ~83% of asymptomatic carriers with multiclonal infections in holoendemic, rural areas in Senegal and Tanzania.\(^{23,24}\) The lower multiplicity observed in Yaoundé may be due to the difference in the epidemiology of urban and rural malaria. In the central districts of Yaoundé, where many of our patients resided, the entomological inoculation rate ranged 3–13 infective bites per person per year.\(^{25}\) Even in the periurban areas around Yaoundé, the entomological inoculation rate did not exceed an average of 33 infective bites per person per year.\(^{26}\) By contrast, in rural Senegalese
and Tanzanian villages where most of the previous longitudinal studies on multiplicity have been conducted in Africa, an entomological inoculation rate of 100–550 infective bites per person per year has been observed.\(^{27,28}\)

In studies in The Gambia, where entomological inoculation rates ranged 4–24 infective bites per person per year, \(\sim 60\%\) of the infected people had multiclonal infections.\(^{7,29}\) The situations in The Gambia and Yaoundé seem to be similar in terms of both the entomological inoculation rate and the proportion of patients with multiclonal infections. At the other extreme, in a hypoendemic area in Sudan where the entomological inoculation rate has been estimated to be \(< 3\) infective bites per person per year, only 20\% of symptomatic children and adults were infected with multiclonal infections.\(^{24}\) These epidemiologic data from various study sites in Africa suggest that the multiplicity of \(P. falciparum\) infections is directly related to the intensity of malaria transmission and determines the genetic structure of malaria populations in different endemic areas.\(^{30}\)

In asymptomatic parasite carriers residing in holoendemic rural areas in Senegal and Tanzania, multiplicity of infection was positively correlated with parasitemia and negatively correlated with age.\(^{9,23}\) Ntoumi and others\(^{23}\) have observed that mean multiplicity decreased from 4 in children < 14 years old to 2 in adults ≥ 15 years old. In our study subjects, comprising symptomatic older children (> 5 years old) and adults, there were no significant differences in multiplicity of infection with regard to age and parasitemia, as observed by other investigators in symptomatic patients among young Tanzanian children (1–5 years old) and Ugandan children and adults (6 months to 80 years old).\(^{31,32}\) However, studies based on symptomatic patients and asymptomatic parasite carriers may not be comparable. In previous studies, opposing trends between multiplicity and asymptomatic-symptomatic infections have been observed.\(^{33–35}\) Clearly, further studies on the malaria population structure in young children and comparison between symptomatic and asymptomatic people in the same study site are needed for the better understanding of the dynamics of parasite populations within different human hosts.

Multiplicity has important implications for the epidemiology of drug-resistant \(P. falciparum\) and the outcome of drug treatment. The initial presence of several parasite populations with different levels of drug sensitivity or resistance may result in the elimination of sensitive populations and selection of resistant populations. In the present study, this was illustrated by the decrease of multiplicity between Day 0 (average of 2.9 populations with \(msa-2\) as the genetic marker) and recrudescent samples (2.2 populations). Similarly, in Ugandan patients treated with chloroquine, sulfadoxine-pyrimethamine, or trimethoprim-sulfamethoxazole, the mean number of populations declined from 4.2 before treatment to 3.4 on Day 7 after treatment.\(^{32}\)

The degree of multiplicity varies in different epidemiologic context and is directly related to the intensity of malaria transmission. Furthermore, there seems to be a complex interplay between multiplicity and numerous host factors, including the degree of acquired immunity, age, initial parasitemia, and antimalarial drug actions. Experimental studies have also suggested that multiplicity may have a profound influence on the sexual development of malaria parasites and transmission.\(^{36}\) These findings on the importance of multiplicity in the determination of the population structure of malaria parasites, both in the human hosts and mosquito vectors, require further investigation in different epidemiologic settings.

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