Imbalanced Distribution of *Plasmodium falciparum* EBA-175 Genotypes Related to Clinical Status in Children from Bakoumba, Gabon

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Objective: The erythrocyte binding antigen 175 kDa (EBA-175) of *Plasmodium falciparum* is one of the major ligands for red blood cell invasion by merozoites. EBA-175 is a dimorphic antigen but the role that dimorphism plays in host parasite interaction is not fully understood. In this study, we sought to determine the distribution of EBA-175 genotypes and its pathogenetic influence.

Methods: The nested polymerase chain reaction was used to determine the genotypes of *P. falciparum* isolates from asymptomatic and symptomatic Gabonese children.

Results: CAMP strains (C-segment) and FCR-3 strains (F-segment) were found in 13/50 (26%) and 19/50 (38%) symptomatic children, respectively and in 16/66 (24%) and 46/66 (70%) asymptomatic children, respectively. The prevalence of mixed C-/F- infection was 18/50 (36%) and 4/66 (6%) in symptomatic and asymptomatic children, respectively.

Conclusions: These results show that mixed C-/F- infection is associated with clinical malaria (χ^2 , $P < 0.01$) and may have important therapeutic implications.

Keywords: CAMP strains; Erythrocyte binding antigen 175 kDa; FCR-3 strains; *Plasmodium falciparum*

The antigenic diversity of *Plasmodium falciparum* is the main obstacle to antimalarial vaccine development. Erythrocyte-binding antigen 175 kDa (EBA-175), expressed on the merozoite surface, is involved in *P. falciparum* binding to erythrocytes.¹⁻⁵ In animal models, immunization with synthetic EBA-175 peptides encompassing amino acids 1062 to 1103 elicits antibodies that inhibit merozoite invasion by 80% *in vitro*. These antibodies appear to act by preventing EBA-175 binding to erythrocytes.⁶ In addition, a significant reduction in parasitemia was recently reported in monkeys immunized with EBA-175.⁷

EBA-175 is composed of 7 regions (region I-VII) and possesses 2 cysteine-rich segments (F1 and F2) located at the N-terminus in region II (figure 1). These cysteine-rich segments, F1 and F2, are responsible for glycophorin A binding to the erythrocyte membrane⁴ and contain a very small number of polymorphic regions. EBA-175 region III is

located in the central part of the gene, and genomic studies of two *P. falciparum* strains revealed two highly dimorphic segments in region III, named FCR-3 and CAMP.^{5,8,9} Dimorphism is referred to as the F-fragment in the FCR-3 strain and the C-fragment in the CAMP strain. The two segments are inserted at different positions of region III and differ in length by 27 amino acids. These two alleles are both conserved, and *Plasmodium* merozoites, which are haploid, can possess one or the other, but not both.

The role of EBA-175 antigen dimorphism in host-parasite interactions is not fully understood. Preliminary evidence suggests that the initial molecular interaction involves binding of the conserved domain (region II of EBA-175) to glycophorin A sialic acid residues, followed by proteolytic cleavage of EBA-175 and binding of the dimorphic C- and F- segments to the glycophorin A backbone.^{8,9} Likewise, the role of this dimorphism in acquired protective immunity to field strains is not known. It was recently shown that EBA-175 is able to use

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Figure 1. Structure of the EBA-175 gene in *P. falciparum* showing the divergent region III composed of the CAMP segment, 342 base pairs (bp) and the FCR-3 segment, 423 bp.

both sialic and non sialic pathways.¹⁰ We have previously refined EBA-175 genotyping by using nested polymerase chain reaction (PCR) and a set of primers specific to both genotypes.¹¹ In this study, we compared the EBA-175 loci of *P. falciparum* strains isolated from asymptomatic and symptomatic children living in Bakoumba, Gabon.

Patients and Methods

Study site

This study took place in Bakoumba (Haut Ogooué Province), a town in southeast Gabon. Preliminary studies showed that *P. falciparum* transmission is moderate and perennial, with only minor seasonal fluctuations (Touré FS, unpublished data). The main vectors in this area are *Anopheles gambiae* and *Anopheles funestus* with *P. falciparum* responsible for approximately 95% of all diagnosed cases of clinical malaria. The entomological inoculation rate has not yet been established.

Ethical clearance

This study was approved by the ethics committee of the International Centre for Medical Research in Gabon and was performed in accordance with guidelines for human experimentation published by the Gabonese Ministry of Public Health and Population. Informed consent was obtained from the parents or guardians of all participating children.

Blood sampling

Three hundred children from three schools were monitored between March and July 2001 for *P. falciparum* malaria infection. All the children were screened for *P. falciparum* infection by fingerprick blood sampling on day 0, every 2 weeks thereafter and whenever fever occurred. Blood was collected by venipuncture from all *P. falciparum*-infected children using EDTA Vacutainers® (Becton Dickinson, Meylan, France).

Diagnosis

Thick and thin blood films were Giemsa-stained and examined by two readers using standard quality-controlled procedures.¹² Parasite load was expressed as the number of asexual forms of *P. falciparum* per microliter of blood. All infected children displaying symptoms of malaria were treated with quinine (24 mg/kg/day/7days) according to local hospital guidelines.

Study population

Children, age 6 to 15 years, with uncomplicated malaria were matched for age, gender and place of residence. The children asymptomatically infected (asymptomatics) had fewer than 5000 asexual blood stage parasites per microliter of blood, an axillary temperature below 37.5°C on the day of recruitment, no history of fever in the 24 hours before or the week after recruitment, and were free of sickle cell disease. Their hemoglobin level was more than 10 g/dl and/or their hematocrit was more than 30%. The children with uncomplicated malaria (symptomatic) all had an axillary temperature $\geq 37.5^\circ\text{C}$, between 5000 and 250,000 parasites/ μl of blood, a hemoglobin level ≥ 5 but < 10 g/dl, and were free of sickle cell disease. These children had no concomitant active infections and did not have severe malaria as defined by the World Health Organization (WHO).¹³

EBA-175 genotyping

The EBA-175 genotype was determined by nested PCR as described elsewhere.¹¹

Statistical analysis

Analyses were performed using StatView for Windows 5.01 (SAS Institute Inc., Cary, NC). Differences were considered significant if two-tailed *P* values were < 0.05 .

Results

One hundred sixteen (38.7%) of the 300 children were infected by *P. falciparum*. Fifty children had symptomatic malaria and 66 had asymptomatic infection. Average age of symptomatic and asymptomatic children was 11.3 and 11.2 years, respectively. The geometric mean parasite loads for symptomatic and asymptomatic children was 9613/ μ l and 948/ μ l, respectively.

Thirteen (26%) of the 50 isolates from children with symptomatic infection were CAMP-positive and 19 (38%) were FCR-3-positive (figure 2). CAMP/FCR-3 mixed infection was detected in the 18 remaining samples (36%). Sixteen (24%) of the 66 samples from children with asymptomatic infection were CAMP-positive, 46 (70%) were FCR-3-positive and 4 (6%) were positive for both CAMP and FCR-3. In single infections, the FCR-3 genotype predominated irrespective of the sampling period and clinical status ($P < 0.001$, Mann-Whitney U test). A significant association was found between mixed C-/F- infection and symptomatic malaria (Cochran's chi-square of 0.30 on 1 degree of freedom of linear trend for proportions; $P < 0.01$) (figure 2). No correlation was found between age and either mixed infection or parasite load (not shown).

Discussion

P. falciparum genotyping can help to unravel host-parasite interactions, including strain-specific immune responses. Previous studies of merozoite genes (MSA-1 and MSA-2) demonstrated that some patients were infected by more than one genotype¹⁴⁻¹⁷ and have also identified genotypes associated with clinical malaria.^{18,19} However, the results of MSA-1 and MSA-2 genotyping have shown major geographic variations.²⁰ In this study we used a dimorphic marker, EBA-175, which greatly simplified data analysis (see figure 1). This study is the first to analyze the EBA-175 allelic dimorphism among pediatric *P. falciparum* isolates according to clinical status. The CAMP and FCR-3 genotypes were encountered in both symptomatic and asymptomatic patients, but the FCR-3 genotype predominated regardless of clinical status and the sampling period. In total, 77% (89/116) were carrying the FCR-3 fragment. Our data indicate that the FCR-3 genotype is more prevalent in Bakoumba, which is consistent with a previous study that showed a higher frequency (>70%) of the F-fragment in central and western African populations.^{21,22} However, we found that the frequency of coinfection was far higher in symptomatic subjects than in asymptomatic subjects, whereas Cramer et al²² found fewer cases of coinfection in children with severe malaria than in asymptomatic children. Significant differences in EBA-175 genotype distribution between the north and the south province of the Lao People's Democratic Republic have also been reported.²³ Together, these results suggest that the distribution of EBA-175 genotypes between different clinical groups differs across geographic regions.

The difference between the symptomatic and asymptomatic children in our study was not related to the sickle-cell trait,

Figure 2. Distribution of EBA-175 genotypes in Gabonese children with symptomatic and asymptomatic *P. falciparum* infection.

which is associated with multiple genotype infections.²⁴ The possibility that other genetic characteristics influence the risk of coinfection was not studied here. In addition, although our method permits EBA-175 genotyping of all samples that are positive by direct examination, the possibility that a minor allele was not detected in some cases of coinfection cannot be ruled out.

Symptomatic malaria appears to be due to parasite genotypes that the host has not yet encountered and to a resulting lack of specific immunity.^{25,26} If an individual encounters a CAMP or FCR-3 strain for the first time, he or she may develop specific antibodies (if not major histocompatibility complex [MHC]-restricted) and may thus remain asymptomatic. When an individual encounters genotypes CAMP and FCR-3 simultaneously for the first time, he or she may develop immunity to both strains and remain asymptomatic. In such cases, antibodies directed against the two genotypes inhibit erythrocyte invasion by merozoites.

In our study, children infected by both the CAMP and FCR-3 genotypes were very often symptomatic suggesting that they had no acquired immunity. These children may thus be protected against one genotype but not both, possibly due to MHC restriction.

Coinfection can be due to a single mosquito bite if the mosquito was carrying the two parasite genotypes or due to two different bites where each mosquito transmitted one particular genotype. This latter scheme may occur frequently in areas with a high entomological inoculation rate. It would be interesting to analyze the specific humoral response to the CAMP and FCR-3 strains according to clinical status. On the basis of our results, we would expect antibody titers against

the two alleles to be higher in asymptomatic coinfecting subjects than in their symptomatic counterparts.

Specific immunity to allele EBA-175 cannot alone prevent *P. falciparum* multiplication for several reasons. First, most field isolates use molecules other than EBA-175 to invade red cells.²⁷ Second, it was recently shown that *P. falciparum* can switch its invasion phenotype, thereby avoiding the host response and becoming virulent.²⁸ This points to the existence of virulence factors associated with clinical status and/or severe malaria that can co-segregate with EBA-175.

In conclusion, this comparative analysis of the allelic dimorphism of EBA-175 antigen in *P. falciparum* isolates from Gabonese children shows a significant association between coinfection and clinical status. If confirmed, these findings should be taken into account in antimalarial strategies.

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