Cytoadherence and Genotype of *Plasmodium falciparum* Strains from Symptomatic Children in Franceville, Southeastern Gabon

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Background: *Plasmodium falciparum* causes severe clinical manifestations by sequestering parasitized red blood cells (PRBC) in the microvasculature of major organs such as the brain. This sequestration results from PRBC adherence to vascular endothelial cells via erythrocyte membrane protein 1, a variant parasite surface antigen.

Objective: To determine whether *P. falciparum* multiple genotype infection (MGI) is associated with stronger PRBC cytoadherence and greater clinical severity.

Methods: Nested polymerase chain reaction was used to genotype *P. falciparum* isolates from symptomatic children and to distinguish between single genotype infection (SGI) and MGI. PRBC cytoadhesion was studied with cultured human lung endothelial cells.

Results: Analysis of two highly polymorphic regions of the merozoite surface antigen (MSP)-I and MSP-2 genes and a dimorphic region of the erythrocyte binding antigen-175 gene showed that 21.4% and 78.6% of the 42 children had SGI and MGI, respectively. It also showed that 37 (89%) of the 42 PRBC samples expressed MSP-I allelic family KI. Cytoadherence values ranged from 58 to 1811 PRBC/mm² of human lung endothelial cells monolayer in SGI and from 5 to 5744 PRBC/mm² in MGI. MGI was not associated with higher cytoadherence values or with more severe malaria.

Conclusions: These results suggested that infection of the same individual by multiple clones of *P. falciparum* does not significantly influence PRBC cytoadherence or disease severity and confirmed the predominance of the MSP-I KI genotype in southeastern Gabon.

Keywords: Malaria, *Plasmodium falciparum*, Cytoadherence, Merozoite surface antigen, erythrocyte membrane protein 1, multiple genotype infection

Plasmodium falciparum causes the most severe form of malaria and kills up to 3 million people every year, mostly children in sub-Saharan Africa.¹ It is unclear why some children and other non-immune hosts die while others remain asymptomatic or develop uncomplicated illness.² *P. falciparum*-parasitized red blood cells (PRBC) can adhere to host endothelial cells, leading to mechanical blockade of microvessels, as well as cytokine secretion and modification of the T cell repertoire.³ PRBC sequestration obstructs capillaries and venules through various mechanisms, including cytoadherence, agglutination, rosetting and reduced red blood cell deformability.^{4,5} *P. falciparum* erythrocyte

membrane protein-1 (PfEMP-1) is the surface antigen variant that is the best characterized. It is expressed on PRBCs and is responsible for their adhesion to host endothelial cell receptors. PRBC cytoadherence in the microvasculature of organs, such as the brain and lungs is modulated by platelets^{6,7} and mediated by endothelial cell-surface receptors such as thrombospondin, CD36, intracellular adhesion molecule-1, vascular cellular adhesion molecule-1, CD31, integrins and hyaluronic acid.⁸ PRBC adhesion to endothelial cell receptors can induce (1) over-production of various inflammatory cytokines, such as interferon- γ , tumor necrosis factor- α , transforming growth factor- β , interleukin-1 (IL-1), IL-6, IL-10 and IL-18 by endothelial cells,^{3,7} (2) phosphorylation and activation of

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endothelial cell cytoskeletal proteins such as cortactin, which increases junctional permeability through cytoskeleton changes,⁹ and (3) caspase activation and apoptosis.¹⁰

Most studies of interactions between PRBC and endothelial cell receptors have been done *in vitro*. Static and flow-based cytoadhesion assays have given useful information on host-parasite interactions, but few relevant data are available on *P. falciparum* multiple genotype infection (MGI) (co-infection by more than one parasite line). It has been proposed that initial binding of one parasite line may facilitate the adhesion of co-infecting parasite lines, and that initial binding of one clone reinforces PRBC sequestration by altering the endothelial cell surface.¹¹

Human lung endothelial cells (HLEC) are a useful co-culture model for studying the pathophysiology of *P. falciparum* malaria *in vitro*¹² and have been used to determine the cytoadherence and rosette phenotypes of *P. falciparum* isolates from Thailand.¹³ In this study, we used this model to investigate the cytoadherence of *P. falciparum* strains freshly isolated from symptomatic children and to identify possible relationships between MGI, cytoadherence and disease severity.

Materials and Methods

Patients

The study was conducted in the pediatric wards at Hôpital Amissa Bongo and Hôpital de l'Amitié Sinogabonaise in Franceville (Haut Ogooué province, Gabon) between July and November 2004. The study was approved by the Centre International de Recherches Médicales de Franceville Ethics Committee and by the Gabonese Ministry of Health. Children with symptoms of acute malaria were enrolled after informed consent of their parents or guardians was obtained. Clinical status was classified using the World Health Organization criteria.¹⁴ Malaria was diagnosed using the thick-film method. Blood was collected in sterile EDTA tubes for culture when it contained more than 5000 *P. falciparum* asexual forms/ μ L. The children were treated according to local guidelines, and care was provided until hospital discharge.

Parasites

Thick and thin peripheral blood films were stained with Giemsa and examined by microscope. Parasite load is expressed as the number of asexual forms of *P. falciparum*/ μ L of blood, assuming an average leukocyte count of 8000/ μ L. Parasites from children with more than 5000 asexual forms/ μ L of blood were selected for *ex vivo* cultivation. White blood cells were separated using Ficoll gradient and 4 washes with RPMI 1640 medium and PRBC were cultured immediately, as previously described.¹⁵ RPMI 1640 medium was supplemented with 8.3 g/L HEPES, 2.1 g/L sodium bicarbonate, 0.1 μ g/mL gentamicin, 1 μ g/mL Fungizone, 2 g/L of D-glucose and 0.4% Albumax II (Invitrogen Cergy Pontoise, France). All experiments were performed during the first cycle of *in vitro* parasite growth. Parasite growth to the schizont form was checked by examination of Giemsa-stained

thin blood films after 24 or 48 hours. Mature parasites were collected and counted after enrichment by gelatine flotation after 24 to 48 hours.^{10,16} A schizont suspension was prepared using RPMI 1640 medium, pH 6.8.

Culture of HLECs

HLEC were derived from one batch, as described elsewhere.¹² HLEC were cultured in flasks (Corning, NY) at 37°C with 5% CO₂ using medium 199 containing 10% (vol/vol) fetal calf serum, 50 units/mL of penicillin-streptomycin and 0.25 μ g of Fungizone (Invitrogen). The cells were used at passage 8 and were characterized for their expression of von Willebrand factor, ICAM-1, VCAM-1, CD31, CD36, E/P-selectin and chondroitin sulfate A, as previously published.¹⁰ The fact that HLEC express a multitude of potential PRBC host receptors opens the way for a follow-up study where the relative importance of these receptors for PRBC adhesion is studied in more detail (e.g., by using antibodies to block PRBC adhesion to specific receptors). HLEC were seeded at a density of 0.5 to 1 x 10⁴ cells/cm² and grown until confluence was achieved.

Cytoadherence Assay

Endothelial cells were subcultured at a density of 3,000 cells/well in 8-well tissue culture plates and cultivated for 24 to 48 hours under standard conditions to reach confluence. The cells were then either used in a cytoadherence assay directly or kept at 4°C in 300 μ L of phosphate buffered saline (PBS) (for a maximum of 2 months) after fixation for 20 minutes at 37°C with 2% paraformaldehyde. For binding tests, 300 μ L of the schizont suspension at 5% hematocrit were deposited onto cell monolayers in duplicate and incubated for 1 hour at 37°C. After removal of non-adherent schizonts with 3 washes in PBS, the preparation was fixed for 20 minutes with 2% glutaraldehyde, rinsed with PBS and stained with Giemsa. Cytoadherence was expressed as the number of PRBCs adhered in mm² of cultured HLEC.

DNA Amplification for *P. falciparum* Genotyping

P. falciparum DNA templates were extracted using 100 μ L of each peripheral blood sample as previously described.^{17,18} Genotypes of the parasite populations were analyzed by nested polymerase chain reaction (PCR). Block 2 of merozoite surface antigen (MSP)-1 and block 3 of MSP-2 were amplified as previously reported.¹⁹ Erythrocyte binding antigen (EBA)-175 genotype was also determined as described elsewhere.²⁰ After the nested-PCR amplifications, 10 μ L of each amplification product were analyzed by electrophoresis on a 1.5% agarose gel. The gel was stained with ethidium bromide, and the DNA visualized under ultraviolet light.

Statistical Analysis

Data were analyzed using the χ^2 and Student's *t*-tests. The Mann-Whitney *U* test was also employed when variable distribution was not normalized. Associations between quantitative variables were assessed by the Spearman's rank test of correlation. For all tests, *P* values <0.05 were considered significant.

Table 1. Parasitological, biological and clinical characteristics of *Plasmodium falciparum* (Pf) from single genotype infections.

Pf isolates	Number of schizont*	Cytoadherence PRBC/mm ² HLEC	Clinical signs	PCR		
				EBA-175	MSP-1	MSP-2
D3	47025	1748		FCR-3	k1	–
F37	80190	197	h	CAMP	k1	FC27
F42	40095	1561	h	FCR-3	k1	3D7
F5	47025	1741		FCR-3	k1	3D7
F51	37620	1811		FCR-3	k1	3D7
F52	23513	603		FCR-3	k1	FC27
F58	3762	58		FCR-3	k1	3D7
F61	28215	874		FCR-3	k1	FC27
F68	10890	600		FCR-3	k1	3D7

* Number of schizont x 10³.

h, hyperparasitemia; EBA-175, erythrocyte binding antigen 175 kiloDalton; HLEC; human lung endothelial cells; MSP, merozoite surface protein; PRBC, parasitized red blood cells.

Results

Patients

We studied 42 children ranging in age from 1 to 10 years who were symptomatic with *P. falciparum*. The children were divided into two groups based on the *P. falciparum* MSP-1, MSP-2 and EBA-175 genotypes: 9 children (21.4%) had single genotype infection (SGI) and the other 33 children (78.6%) had MGI. Two (22.2%) of the 9 children with SGI had severe malaria diagnosed solely on the basis of high-level parasitemia ($\geq 20\%$). Nine (27.2%) of the 33 children with MGI had severe malaria. Four of these children had neurological signs (3 with altered consciousness; 1 with cerebral malaria), one had isolated severe anemia (4 g Hb/dL) and four had only high-level parasitemia (tables 1 and 2).

Parasites

Parasitemia ranged from 0.4% to 39%. The numbers of schizonts in the specimens used for cytoadherence assays ranged from 3762 x 10³ to 80,190 x 10³ in SGI and from 396 x 10³ to 81,972 x 10³ in MGI (tables 1 and 2); the respective mean numbers of schizonts were 35,370 x 10³ and 30,818 x 10³.

Genetic Characteristics of *P. falciparum* Isolates

MSP-1, MSP-2 and EBA-175 fragments that could be assigned to specific allelic families were used to characterize parasite subpopulations. Of the 42 *P. falciparum* isolates, 7 (16.7%) comprised at least three different genotypes, 26 (61.9%) with two genotypes and the remaining 9 (21.4%) with a single genotype. In MGI, the family distributions were 23/33 (69.7%) of FCR3, 15/33 (45.4%) of CAMP for the EBA-175 locus; 29/33 (87.9%) of Ro33, 28/33 (84.8%) of K1, 15/33 (45.4%) Mad20 for MSP-1; 21/26 (80.7%) of

FC27 and 13/26 (50%) of 3D7 for MSP-2. In SGI, all parasites were genotype K1 for the MSP-1 locus; 87.5% of FCR3, 12.5% of CAMP for EBA-175; 62.5% of 3D7 and 37.5% of FC27 for MSP-2. In total, the allelic family distributions were 37/42 (89%) of K1, 30/42 (71%) of Ro33, 15/42 (36%) of Mad20 for MSP-1; 16/42 (39%) of CAMP, 31/42 (74%) of FCR3 for EBA-175 and 18/42 (43%), 24/42 (57%) for the MSP-2 locus.

Cytoadherence

All the PRBC samples adhered to HLEC (figure 1), with values ranging from 58 to 1811 PRBC/mm² in SGI (figure 2) and from 5 to 5744 PRBC/mm² in MGI (figure 3). Thirty-five (83.3%) of the 42 PRBC samples gave values above 200 PRBC/mm² (arbitrary cutoff); six of the remaining seven samples were from subjects with MGI. This cutoff was not predictive of the severity of malaria (tables 1 and 2). Likewise, the schizont count was not related to the cytoadherence value of the corresponding sample. Several PRBC samples from patients with high-level parasitemia had very low cytoadherence values (see sample F63 for example, table 2). The performance of our adhesion assay was not significantly affected by the use of fixed HLEC within 2 months (figure 4).

Relationship between Cytoadherence, MGI and Severe Malaria

There was no significant difference in the mean cytoadherence values between PRBC from patients with SGI (1021 PRBC/mm²) and those with MGI (1028 PRBC/mm²) ($P=0.55$). Likewise, there was no relationship between severe malaria and either MGI ($P=1.00$) or the cytoadherence value ($P=0.92$).

Table 2. Parasitological, biological and clinical characteristics of *Plasmodium falciparum* (Pf) from multiple genotype infections.

Pf isolates	Number of schizont*	Cytoadherence PRBC/mm ² HLEC	Clinical signs	PCR		
				EBA-175	MSP-1	MSP-2
D1	-	580		FCR3	K1/Ro33	FC27
F1	29122	5744		FCR3	K1/Ro33/Mad20	3D7/FC27
F17	6584	36		FCR3	K1/Ro33	FC27
F26	14108	230	a	FCR3	K1/Ro33/Mad20	FC27
F28	3168	341		FCR3	K1/Ro33	3D7
F29	55688	1169		CAMP	Ro33/Mad20	-
F3	14642	216	p	FCR3/CAMP	K1/Ro33	3D7/FC27
F30	29700	3122		CAMP	K1/Ro33	3D7/FC27
F31	71280	937	h,p	CAMP	Ro33/Mad20	FC27
F32	75240	5619	h	CAMP	K1/Ro33/Mad20	3D7/FC27
F33	18810	513		CAMP	K1/Ro33	3D7/FC27
F34	53460	3012		CAMP	K1/Ro33/Mad20	FC27
F36	1980	6		FCR3	K1/Ro33	FC27
F38	2475	250		FCR3	K1/Ro33	FC27
F39	396	63		CAMP	K1/Ro33/Mad20	3D7
F41	23513	215		FCR3/CAMP	K1/Ro33	3D7/FC27
F43	9405	212		FCR3/CAMP	K1/Ro33	-
F44	9405	212,0		FCR3/CAMP	Ro33/Mad20	3D7
F45	80190	1793	h	FCR3/CAMP	K1/Ro33/Mad20	3D7
F47	23513	235		FCR3	Ro33/Mad20	FC27
F48	24008	998		FCR3	K1/Ro33/Mad20	FC27
F49	6683	687		FCR3	K1/Ro33	-
F50	51728	613		FCR3	K1/Mad20	FC27
F53	80190	592	h	FCR3	K1/Ro33	FC27
F54	23513	610		FCR3	K1/Ro33	-
F55	18810	1873		CAMP	K1	3D7/FC27
F59	9603	7		FCR3	K1/Ro33	FC27
F60	11286	13		CAMP	K1/Mad20	-
F62	11880	437	p	FCR3	K1/Ro33	3D7
F63	56430	5	h	FCR3	Ro33/Mad20	FC27
F64	23513	1561		FCR3	K1/Ro33/Mad20	-
F67	1782	332		CAMP	K1/Ro33	3D7/FC27
F69	81972	1686	h,c,a	FCR3	K1/Ro33	-

* Number of schizont x 10³.

a, anemia; c, coma; h, hyperparasitemia; p, prostration; EBA-175, erythrocyte binding antigen 175 kiloDalton; HLEC; human lung endothelial cells; MSP, merozoite surface protein; PRBC, parasitized red blood cells; PCR, polymerase chain reaction.

Figure 1. Cytoadherence of *Plasmodium falciparum* parasitized red blood cells (PRBC) to human lung endothelial cells (HLEC): 5619 PRBC/mm² HLEC.

Figure 2. Cytoadherence profiles of *Plasmodium falciparum* single genotype infection isolates.

Relationship between Genotype K1 and Severe Malaria

The K1 genotype was observed in 9 (82%) of 11 individuals with severe malaria and in 28 (90%) of 31 individuals with uncomplicated malaria ($P=0.8$). Therefore, the K1 genotype is not associated with severe malaria.

Discussion

The disappearance of mature PRBC from peripheral blood concomitantly with their sequestration in the microvasculature of major organs, such as the brain, plays a key role in the pathogenesis of *P. falciparum* malaria.²¹ Here we investigated the cytoadherence status of *P. falciparum* strains from symptomatic children and sought possible relationships between MGI, cytoadherence and disease severity.

More than three-quarters of the patients had MGI, confirming the extensive polymorphism of *P. falciparum* in southeastern Gabon.²²⁻²⁴ However, it has been demonstrated that some clones of *P. falciparum* are able to disappear and reappear in the peripheral blood from infected individuals within as little as 2 hours.²⁵ Due to the fluctuation of parasite density and complexity, a single peripheral blood sampling does not reflect the full complexity of the parasite populations harbored by a given individual.²⁶ Thus, it is possible that the numbers of MGI specimens are underestimated in our study for two reasons: first, the blood samples were collected only one time and second, our PCR method may have failed in

detecting minority parasites in MGI or previously sequestered parasites that were not present in the peripheral blood at the time of sampling. Jafari et al²⁵ found that 95% of patients had MGI and showed that minority parasites representing as little as 0.4% of total parasitemia can be detected via quantitative PCR assay. We found no association between MGI and severe disease. This is in contrast to another study of Gabonese children, which demonstrated that clinical malaria attacks were associated with increased complexity of infection.²³ However, the reverse has also been observed in Senegalese, Tanzanian and Papua New Guinean children.²⁷⁻²⁹

In our study, three genetic loci were amplified by nested PCR to discriminate *P. falciparum* SGI and MGI. The Ro33, K1, FC27 and FCR3 genotypes of the MSP-1, MSP-2 and EBA-175 genes, respectively, were predominant in MGI. By contrast, in SGI all the parasites were the K1 genotype. This may be due to the fact that K1 was the predominant MSP-1 genotype. The most prevalent EBA-175 genotype in SGI was FCR3 genotype. This result is therefore consistent with our previously reported data.³⁰ Overall, our study demonstrated the predominance of the allelic family K1 of MSP-1 locus clearly, confirming data reported elsewhere.^{31,32} However, no significant association was found between the occurrence of the K1 genotype and severe malaria; K1 was observed in 82% and 90% PRBC from individuals with severe and uncomplicated malaria, respectively. Nevertheless, possible differences within K1 or other allelic families between individuals with mild versus severe malaria remain to be investigated. For example, in French Guiana, it has been demonstrated that a specific MSP-1 allele (B-K1) bearing a specific variant gene (var-D) has been linked to severe malaria.³³ In Dienga, a village situated in southeastern Gabon approximately 200 km from Franceville where the present study was carried out, the K1 and Mad20 families of MSP-1 were predominantly found in symptomatic individuals compared to asymptomatics.²³ By contrast, in Nigeria it has been shown that the presence of the K1 and MAD20 alleles was significantly associated with asymptomatic malaria, and consequently, a reduced risk of developing the symptomatic disease.³⁴ Ranjit et al³⁵ have also shown that Mad20/MSP-1 and 3D7/MSP-2 alleles were over-represented in severe malaria cases in Orissa (India). Finally, it has also been reported that clinical malaria was associated with 3D7/MSP-2 in some African endemic areas.^{36,37} Together these results demonstrate that the distribution of *P. falciparum* genotypes according to clinical severity differs across geographic regions.

Most PRBC samples tested in this study showed a high level of cytoadherence to HLEC. However, there were significant differences in levels of cytoadhesion between individual PRBC strains from SGI or MGI, suggesting differences in parasite encoding ligands such as PfEMP1, the most important variant surface antigen.³⁸ PRBCs continuously switch expression of PfEMP1 proteins and thereby evade immune destruction. Hence, a single PRBC only expresses a single variant surface antigen type at a given time and can switch to another variant surface antigen at any time,

regardless of the variant surface antigen expressed by other PRBC, whether they are infected with genotypically identical parasites or not. Furthermore, some single variant surface antigen types may have multiple adhesion domains. Consequently, both MGI and SGI are composed of parasite populations expressing a mixture of distinct PfEMP1 proteins that bind to different host receptors. This might explain why MGI was not associated with higher cytoadherence values in our study.

Cytoadhesion involves as many as 11 types of endothelial cell receptors¹¹ and the HLEC used in the study co-expressed CD36 and ICAM-1, the two major receptors for PfEMP-1 ligand, among others. CD36 and ICAM-1 act synergistically in mediating PRBC binding to endothelial cells co-expressing both receptors.^{39,40} Although the binding phenotypes of PRBC were not assessed, our results are consistent with data reported by Newbold et al⁴¹ showing that almost all PRBC isolates assessed in their experiments bind *in vitro* to purified CD36 and most to ICAM-1. Nevertheless, much of the binding could have been due simply to CD36, which is an extremely common parasite adhesion trait. In this respect, it is of interest that the CIDR α domains of PfEMP1 proteins bind to CD36 despite the sequence divergence of these domains.⁴²

In our study, no correlation was found between the levels of cytoadhesion of PRBC isolates and malaria severity. This result confirmed previous data reported elsewhere² and reinforces the conclusion that coma is the only manifestation caused by cytoadhesion of PRBC to the brain vascular endothelium via variant surface antigen.⁴³ Indeed, it has been documented that severe malaria is associated with expression of the variant surface antigen group A,⁴⁴⁻⁴⁶ group B,⁴⁷ groups A or B/A,⁴⁸ groups A and B,⁴⁹ and group D.³³ Moreover, the postmortem analysis of the brain revealed that all coma cases did not have sequestered parasites in cerebral capillaries.⁵⁰ There is also evidence suggesting that severe malaria is associated with expression of PfEMP1 variants that do not bind to CD36.^{51,52} Therefore, the lack of association between cytoadherence and disease severity observed in our study could be explained by the small number of patients with cerebral malaria. In future studies with a larger number of parasite isolates from individuals with cerebral malaria, it would be interesting to explore variant surface antigen expression patterns in relation to the parasite genotypes present and host endothelial cell binding phenotype.

In conclusion, our results suggested that PRBC avidity for HLECs is not influenced by the number of clones infecting a given individual. We also confirm the predominance of the K1 genotype at the MSP-1 locus in southeastern Gabon.

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Figure 3. Cytoadherence profiles of *Plasmodium falciparum* multiple genotype infection isolates.

Figure 4. Comparative analysis of cytoadherence of *Plasmodium falciparum* 3D7 and field isolate (D1) using fresh or fixed human lung endothelial cells (HLEC).

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