Summary:
The adhesion of infected red blood cells (IRBCs) to the cell lining of microvasculature is thought to play a central role in the pathogenesis of severe malaria. Individual IRBC can bind to more than one host receptor and parasites with multiple binding phenotypes may cause severe disease more frequently. However, as most clinical isolates are monoclonal, previous studies were hampered by the difficulty to distinguish whether a multidendritic phenotype was due to one or more parasite population(s). We have developed a tool, based on cytoadhesion assay and GenScan genotyping technology, which enabled us to assess on fresh isolates the capacity of adherence of individual P. falciparum genotypes to human receptors expressed on CHO transfected cells. The cytoadhesion to ICAM-1 and CD36 of IRBCs from uncomplicated and severe malaria attacks was evaluated using this methodology. In this preliminary series conducted in non immune travelers, IRBCs from severe malaria appeared to adhere more frequently and/or strongly to ICAM-1 and CD36 in comparison with uncomplicated cases. In addition, a majority genotype able to strongly adhere to CD36 was found more frequently in isolates from severe malaria cases. Further investigations are needed to confirm the clinical relevance of these data.

KEY WORDS: Plasmodium falciparum, severe malaria, uncomplicated malaria, cytoadherence, ICAM-1, CD36, msp-2.

Résumé: Caractéristiques de cytoadhérence aux récepteurs endothéliaux ICAM-1 et CD36 de populations de Plasmodium falciparum provenant d’isolats présentant des cas graves ou simples de paludisme

L’adhésion des globules rouges infectés aux cellules endothéliales des microvaisseaux est connue pour jouer un rôle central dans la pathologie du paludisme grave. Un globule rouge infecté peut adhérer à plusieurs récepteurs de cellules endothéliales de l’hôte. Il est possible que le parasite possédant de multiples phénotypes d’adhésion causera plus fréquemment des cas graves. Comme la plupart des isolats cliniques sont polyclonaux, les études antérieures ont été restreintes par la difficulté de distinguer si un phénotype particulier d’adhésion était dû à une ou plusieurs populations de parasites. Nous avons développé une procédure basée sur un test de cytoadhésion associé au génotypage par GenScan. Cette procédure nous a permis d’évaluer sur des isolats provenant de patients paludéens présentant des cas graves ou simples, la capacité d’adhérence de génotypes de Plasmodium falciparum à des récepteurs humains, exprimés par des cellules CHO transfectées. La cytoadhésion à deux récepteurs ICAM-1 et CD36 a été étudiée suivant cette méthodologie. Dans cette série de résultats préliminaires effectuée chez des voyageurs non immuns, les globules rouges infectés provenant de cas graves apparaissaient adhérer plus fréquemment et/ou fortement à ICAM-1 et CD36 en comparaison avec ceux provenant de cas simples. De plus, un génotype majoritaire capable d’adhérer plus fortement à CD36 a été trouvé plus fréquemment chez les isolats provenant de cas graves. Des études complémentaires seront nécessaires afin de confirmer l’importance clinique de ces données.


Plasmodium falciparum malaria remains one of the major causes of morbidity and mortality in sub-Saharan Africa, leading each year to the death of an estimated number of 1-2.8 million individuals, mostly children (Breman, 2001). In endemic areas, individuals often harbor a mixture of genetically distinct parasites, which results in a major challenge to immune protection, drug efficacy and malaria control (Contamin et al., 1996). It is apparent that whether an individual develops mild or severe malaria must depend on a complex combination of host and parasite factors (Miller et al., 2002) but the development of immunity remains probably the key factor as older children and adults in endemic areas are less frequently victims of severe malaria. Variations in parasite virulence may also contribute to the wide spectrum of disease severity observed in P. falciparum malaria infection. The main factors of virulence are capacity of parasite multiplication and ability to induce binding of infected red blood cells...
(IRBCs) to the vascular endothelium (cytoadherence) and to non-infected erythrocytes (rosetting) or to other infected erythrocytes (autoagglutination), all phenomena which lead to local occlusions of post-capillary microvasculature (Ho & White 1999; Chen et al., 2000). A single parasite protein, *P. falciparum* erythrocyte membrane protein 1 (PFEMP1), which is expressed at the infected erythrocyte surface, mediates parasite binding to various hosts receptors (Miller et al., 2002).

Several studies have attempted to correlate the cytoadherence properties of IRBCs with clinical outcome (Ho et al., 1991; Newbold et al., 1997; Treutiger et al., 1997; Rogerson et al., 1999; Afonso Nogueira et al., 2002). In particular, some endothelial receptors have been studied such as CD36 and thrombospondin (TPS) which showed to be used by all parasite isolates, whereas others such as intercellular adhesion molecule-1 (ICAM-1) or platelet/endothelial cell adhesion molecule-1 (PECAM-1/CD31) were associated with severe malaria. Other investigators have suggested that simultaneous binding of IRBCs to multiple receptors may cause more obstruction than adhesion with single-receptor specificity in the microvasculature, leading to more severe cases of malaria (Heddeni et al., 2001), but specific data are lacking. However, the results of these studies were conflicting due to heterogeneous parasite populations within these samples, inadequate number of clinical samples, and variations in the cytoadhesion assay used. These studies were also performed in endemic areas in subjects having various level of antimalarial immunity, which could be a confounding factor. Moreover, it remains unknown whether the adhesion of IRBCs found in the patients with severe malaria is due to the coexpression of multiple binding events in clonal populations of parasites, or if the observation reflects multiclonal infection, where each clone infecting the patient adheres to one (or several) receptor(s). To address this question, we have studied in mostly non immune travelers the in vitro cytoadhesion to ICAM-1 and CD36 well-known receptors of IRBCs originating from multiclonal isolates obtained in uncomplicated or severe malaria cases. For each isolate, the enumeration and quantification of each msp-2 *P. falciparum* genotype was performed before and after IRBCs were allowed to cytoadhere on Chinese hamster ovary (CHO) cell lines, expressing either human endothelial receptors CD36 or ICAM-1.

**MATERIALS AND METHODS**

Patients admitted to the department of infectious and tropical diseases, emergency department or intensive care unit of five metropolitan French hospitals with a diagnosis of malaria between October 2003 and November 2004 were prospectively included in the present study if they met the following criteria:

1. *P. falciparum* infection with no other associated *Plasmodium* species;
2. parasitemia ≥ 1 %;
3. lack of chemoprophylaxis use, assessed on interrogatory data and drugs concentration in plasma the day of diagnosis;
4. availability of data about age, sex, intake of self-treatment, origin of patient, country of infection and clinical statement (uncomplicated or severe malaria attack);
5. successful maturation of the isolate obtained the day of diagnosis. Patients who reported having taken self-treatment before hospital admittance were not included in the study.

*P. falciparum* malaria attack was defined by fever and other signs of malaria, and asexual *P. falciparum* blood stages on thin and/or thick blood smears. Severity of attack was assessed according to the 2000 WHO guidelines (WHO, 2000). No informed consent was required for this study as following procedures are part of the French national recommendations for care and surveillance of imported malaria. This study was only used blood samples withdrawn for malaria diagnosis and collected by the CNRCP. Therefore, no additional blood samples were collected for this study. Giemsa-stained thin blood smears were examined to calculate *P. falciparum* parasite densities by counting the number of asexual parasite infected erythrocytes, expressed for 100 red blood cells.

The assay was performed in the hours following diagnosis on fresh isolates. RBC suspensions were incubated in RPMI 1640 (Invitrogen, Cergy Pontoise, France) containing 10 % AB Human serum (Biowest, Nuaillé, France) at 37°C, 5 % CO₂, 5 % O₂ and 90 % N₂ (Trager & Jensen, 1976) during 20-24 hours allowing maturation of ring stage parasites to trophozoites. IRBCs containing mature parasite forms, controlled by microscopic observation, were separated from leucocytes and uninfected RBCs by magnetic enriching using Macs columns (Miltenyi Biotec, Paris, France), which resulted in isolates having parasitemias > 90 %. The RBCs were then resuspended at a concentration of 5 × 10⁶ IRBCs/ml in cytoadhesion medium at pH 6.8 (Marsh et al., 1988; Pouvelle et al., 2000).

Cell lines used for cytoadhesion assays were CHO transfected cell lines, expressing human endothelial receptors CD36 or ICAM-1 prepared and tested previously (Hasler et al., 1993; Ringwald et al., 1993). Cell lines were cultivated on coverslips and used when semi-confluent monolayer cell growth was obtained in synchronization with maturation of parasites. The order of ICAM-1 or CD36 CHO cells and the time of incubation were tested to maximize the adhesion. The IRBCs suspensions were distributed in quadruplicate on the coverslips of ICAM-1 CHO cells and incubated in wet chambers in a 5 % CO₂ at 37°C under soft continuous agitation for 30 minutes. IRBCs suspension was softly removed and deposited in quadruplicate on CD36 CHO cells and incubated in the same conditions.
for one hour. The samples were then washed several times by immersion in RPMI 1640 and controlled by inversion microscopic observation to release all non-adherent IRBCs. Adherent IRBCs were removed from two other samples by energetic washings in RPMI 1640 for DNA extraction which was performed using QIAamp® DNA micro kit (Qiagen, Courtaboeuff, France), following manufacturer. The number and the proportions of genotypes within isolates were determined for each patient using fluorescence-labeled polymerase chain reaction and GeneScan sizing (Jafari et al., 2004). The description of each isolate included the number of merozoite surface protein 2 (msp-2) genotypes, the size of the corresponding PCR products and the proportion of each genotype (given in percentage) within the isolate. This GeneScan-based genotyping methodology allowed detecting all clones accounting for more than 1% of the whole. This quantitative and qualitative analysis was performed for each isolate three times: after maturation and concentration, after binding of IRBCs to ICAM-1, and after binding of IRBCs to CD36. The data were entered into a database in Excel 2003 and statistical analysis was performed using Epi Info®, version 3.3 (Centers for Disease Control, Atlanta, USA 2004). Comparisons were done using Chi-square or Yates’ corrected Chi-square for categorical data and Student test for continuous data. P-values < 0.05 were considered significant.

RESULTS

Nine patients (3 males; 6 females) with uncomplicated malaria and thirteen patients (7 males; 6 females) with severe malaria (2 cerebral malaria and 11 others) were included in the study. The median age was 37.5 years (range 5-61 years); the mean age was 35.7 (standard deviation, SD = 17.7) years. All patients acquired the infection in various countries of sub-Saharan Africa, excepted one case contracted in India. Ten patients were European travelers (persons born and residing in non-endemic areas) and 12 were African travelers (persons born in Africa residing mostly in France, usually called “visiting friends and relatives”). Parasitemias observed the day of diagnosis ranged from 1% to 40%. Not surprisingly, the mean parasitemia was higher in severe group than in uncomplicated group (mean parasitemia: 16.51% (SD 11.79) versus 2.58% (SD 1.14), \( P = 0.001 \)). Treatment was administered as recommended by the French Ministry of Health: uncomplicated malaria cases were given oral quinine (Quinimax®) or atovaquone-proguanil (Malarone®); severe malaria cases and patients who vomited were given intravenous quinine. All patients showed adequate clinical response.

As shown in Table I, IRBCs from three isolates showed a low adhesion to CD36 and ICAM-1, six isolates showed positive adhesion to only one receptor, either CD36 or ICAM-1. This quantitative and qualitative analysis was performed for each isolate three times: after maturation and concentration, after binding of IRBCs to ICAM-1, and after binding of IRBCs to CD36.
Fig. 1. – Prevalence of different sized alleles of *msp-2* (merozoite surface protein 2) in the study samples from patients with uncomplicated (a) and severe (b) malaria, before maturation (black), after maturation and concentration (grey), after cytoadhesion to ICAM-1 (hatched) and after cytoadhesion to CD36 (white).
(n = 4) or ICAM-1 (n = 2), two isolates showed a positive adhesion to both receptors, ten isolates showed a positive adhesion to ICAM-1 and a strong adhesion to CD36 and one isolate showed a strong adhesion to both receptors. In this preliminary series, IRBCs from severe or uncomplicated malaria were not restricted to a particular cytoadherence profile. However, a positive adhesion to ICAM-1 and a strong adhesion to CD36, or a strong adhesion to both receptors was found more frequently in severe cases (8/13) than in uncomplicated cases (3/9) (but the small number of isolates prevented a statistical significant difference, \( P = 0.39 \)).

In all isolates, all msp-2 genotypes identified before maturation were found after maturation and concentration, sometimes with slight variations in the proportion of genotypes within isolates (Fig. 1). Severity of attack was associated with lower complexity of infection. The mean MOI of isolates was 3.15 (SD 1.34) in severe group and 5.67 (SD 3.67) in uncomplicated group (\( P = 0.08 \)). This trend maintained during adhesion to ICAM-1 (2.77 (SD 0.93) vs 4.67 (SD 2.45); \( P = 0.05 \)) and to CD36 (2.23 (SD 1.01) vs 6 (SD 3.5); \( P = 0.01 \)).

Globally, 27 msp-2 genotypes were detected. The msp-2 genotype sizes ranged from 508 bp to 975 bp. In six isolates out of 22, some genotypes adhered to one receptor (ICAM-1 or CD36) but not to the other, or with a lesser affinity. In 13 isolates out of 22, genotypes adhered to both receptors with a similar affinity. A majority genotype associated with a strong adhesion to CD36 was found in eight out of 13 isolates from severe malaria and in only one out of nine uncomplicated cases. But globally, no majority genotype was exclusively associated with the adhesion to CD36 or ICAM-1 (Fig. 1).

**DISCUSSION**

The adhesion of IRBCs to the cell lining of microvasculature is thought to play a central role in the pathogenesis of severe malaria (Chen et al., 2000). It has been previously shown that individual IRBC can bind to more than one receptor and parasites with multiple binding phenotypes have already been reported as causing severe disease in field trial (Craig & Scherf, 2001). However, as most clinical isolates are polyclonal, previous studies were hampered by the difficulty to distinguish whether a multiadherent phenotype was due to one or more parasite population(s). We have developed a tool which enabled us to assess on fresh isolates the capacity of adherence of individual genotypes to human receptors expressed on CHO transfected cells. The *P. falciparum* msp-2 gene is highly polymorphic and, combined with fluorescent PCR and GeneScan technology, has revealed high potential for *P. falciparum* infection dynamics studies (Jafari et al., 2004; Falk et al., 2006). ICAM-1 has been implicated previously as being involved in progression to cerebral malaria (Chakravorty & Craig, 2005). Conversely, CD36 is weakly expressed or absent on cerebral endothelium but is present in other organs implicated in severe malaria as liver and lung. In vitro adhesion to CD36 was reported as significantly greater for isolates from patients having non-cerebral severe malaria (Ho et al., 1991). Others reported an association between the degree of binding to ICAM-1 and severity of malaria attack in non anemic patients but did not see such association for binding to CD36 (Newbold et al., 1997). The study by Newbold et al. did not take into account the polyclonality of isolates and used cryopreservate and thawed samples, which could be not fully representative of fresh isolates (Reeder et al., 1994). Rogerson et al. did not find a positive association between adherence to ICAM-1 and CD36 and disease severity (Rogerson et al., 1999). These authors used fresh isolates but did not standardize parasitemias before cytoadhesion assay, which in addition was performed when > 50 % of the parasites were at the trophozoite stage instead of > 95 % as done in Afonso Nogueira’s study and ours. Rogerson et al. recognized that most of their isolates were probably polyclonal, which may limit the interpretation of their data. Interestingly, another recent study by the Rogerson’s group suggested that the dominant clones sequestered in deep organ during severe malaria are usually the same as those in peripheral circulation (Dembo et al., 2006), which supports continued experimental work using circulating parasites. Several studies have used merozoites surface proteins (msp-1 and/or msp-2) polymorphism to describe circulating parasite populations and some authors reported the association of particular genotypes with severity of malaria attack (Ariey et al., 2001; Mockenhaupt et al., 2003; Ranjit et al., 2005; Farooq et al., 2006). However, these associations were described as possibly restricted to geographical areas. In the present work, we did not notice a particular genotype over-represented in severe cases. Moreover, the presence of more than one msp-2 genotype has not excluded the possibility that individual clones were multiple adhesive.

In the present work, severe malaria showed a trend to be associated with a lower complexity of infection. Moreover, fewer genotypes, have adhered to CD36 (\( P = 0.01 \)) in severe malaria in comparison with uncomplicated cases. In endemic areas the association of complexity of infections with the severity of attack was conflicting, as some authors found a lower complexity in severe malaria (Robert et al., 1996) while others claimed opposite results (Mockenhaupt et al., 2003; Ranjit et al., 2005).

This present work allowed validating our methodology. We have studied the adhesion of *P. falciparum* geno-
types to two well-known endothelial receptors; however other receptors as blood group A antigen, CD31, E-selectin could have given better results than ICAM-1 and CD36. The limitation of this work was that our methodology did not allow studying the synergy in adherence of IRBCs to ICAM-1 and CD36, though this phenomenon could be important in the cytoadhesion process (Yipp et al., 2000). The stationary cytoadhesion assay that we used may probably not reflect accurately the intensity of ligand-receptor which occurs in natural conditions. But this methodology is necessary to get back the DNA samples. A supplementary methodology as a continuous flow assay would address this limitation (1).

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