CONCISE COMMUNICATION

Association of Severe Malaria with a Specific Plasmodium falciparum Genotype in French Guiana

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Why severe Plasmodium falciparum malaria occurs in only a small percentage of patients is unclear. The possibility that specific parasite characteristics contribute to severity has been investigated in French Guiana, a hypoendemic area, where parasite diversity is low and all patients with severe cases are referred to a single intensive care unit. Parasite genotyping in geographically and temporally matched patients with mild and severe disease showed that the association of a specific msp-1 allele (B-K1) with a specific var gene (var-D) was over-represented among patients with severe versus mild disease (47% vs. 3%, respectively; P < .001). Moreover, this genotype combination was consistently observed in the most severe clinical cases. Reverse-transcription polymerase chain reaction demonstrated programmed expression of var-D in vivo, which is consistent with its potential implication in severe disease. These results provide field evidence of an association of severe malaria with specific genetic characteristics of parasites and open the way for intervention strategies targeting key virulence factors of parasites.

Why severe Plasmodium falciparum malaria occurs in only a small percentage of patients remains poorly understood. Massive colonization of some organs by parasites that express specific adhesin(s) and excessive production of proinflammatory cytokines are believed to contribute to pathogenesis. Paradise virulence heterogeneity has long been invoked [1], but field evidence for this is still lacking. The population of French Guiana, a geographically isolated area where P. falciparum is hypoendemic, is not immune to malaria [2]. The P. falciparum population presents such a limited polymorphism that circulation of specific genotypes within the area can be visualized [3]. Such a situation is propitious for identifying the clinical impact of specific parasite characteristics. We hypothesized that parasite factors contributing to severity would similarly present a restricted polymorphism. Thus, we compared the parasite genotypes in patients in French Guiana with mild and severe malaria, to determine whether specific genotype characteristics are associated with severe P. falciparum malaria.

Patients, Materials, and Methods

Study area. French Guiana, an overseas French Department covered with equatorial forest, has a population of ~150,000. Malaria transmission is low and is restricted to localities disseminated along the rivers. There are ~6000 malaria cases annually, occurring primarily in 2 distinct areas. Local private practitioners and dispensaries diagnose malaria cases and treat patients rapidly. Patients usually reach these peripheral health facilities by pirogue. The incidence of severe malaria is low, with 4–17 patients with severe cases hospitalized each year [2]. Patients with severe cases are transferred to the Referral Hospital in Cayenne by helicopter, where the intensive care unit (ICU) facilities are of the highest standards for the management of severe malaria.

Patients. Patients with malaria were recruited from the 2 endemic areas of French Guiana from October 1994 through January 1996 (a period encompassing 2 transmission seasons). A total of 51 patients was recruited, of whom 50 were adults. Subjects were divided into 2 clinical groups—those with severe and those with mild disease. The severe malaria group included 19 patients admitted to an ICU in Cayenne. At admission, full clinical histories were recorded, and complete physical, biochemical, and biological tests were done. Clinical manifestations of malaria were classified according to the

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Informed consent was obtained from the families of patients or the patients themselves.

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definitions and associated criteria of the World Health Organization (WHO) [4]. Respiratory manifestations (edema and acute respiratory distress) were defined according to criteria outlined elsewhere [5], and multiple organ dysfunction syndrome (MODS) criteria were as defined elsewhere [4, 6, 7]. Patients with severe disease were treated with intravenous quinine formate (loading dose, 20 mg/kg and then 10 mg/kg every 8 h) and doxycycline (200 mg/day for adults) or erythromycin (200 mg/day for the child). Symptomatic intensive supportive care (i.e., mechanical ventilation, hemodialysis, hemofiltration, vasoactive drugs, plasma exchange, or blood transfusion) was provided as required. A 5-mL blood sample was obtained at admission for in vitro assessment of the drug-resistance profile (chloroquine, quinine, halofantrine, or mefloquine) and parasite DNA extraction.

The mild malaria group included 32 patients recruited during the same period and in the same areas as the severe malaria patients. The patients in this group were febrile, had asexual P. falciparum parasites, and had no other explanation for their fever, no WHO-defined or associated criterion for severe malaria, and no sign of severe organ disorder. No patient presented with hyperparasitemia. The ethnic groups of French Guiana were equally represented in both patient groups. The male:female ratio was 13:6 for the severe malaria group and 27:5 for the mild malaria group ($P = .16$).

**Extraction of DNA and polymerase chain reaction (PCR).** DNA was extracted, and PCR genotyping for the single-copy msp-1 block 2, msp-2, and glurp loci was done as described elsewhere [3]. PCR of var genes was done using UNIEBP primers [8, 9] and var-D-specific primers (var-D3 5′-AGTTCACCTGTTCCCGGC-3′ and var-D5 5′-CCCTGAAGATTTTAAGCGTC-3′). The amplification products were analyzed for size polymorphism by agarose or polyacrylamide gel electrophoresis [3].

**Sequencing of PCR products.** The PCR products were purified by use of a Qiagen column and were processed for DNA sequencing with an ABI PRISM Dye Terminator (Perkin-Elmer) used according to the manufacturer’s instructions. Each sample was sequenced on both strands, using the var-D3 and var-D5 primers.

**RNA extraction and reverse-transcription (RT) PCR.** Peripheral blood was washed with RPMI, was resuspended in 10 vol of extraction solution, and was stored in liquid nitrogen. RNA was extracted using RNA isolation by guanidinium thiocyanate-phenol-chloroform extraction. In parallel, an aliquot was matured for 24 h in vitro under standard culture conditions and then was treated as described above. First-strand cDNA was synthesized using random hexamers and AMV RT (Promega). PCR was subsequently done using the var-D3/var-D5 primers for 35 cycles.

**Statistical analysis.** Quantitative data were expressed as the mean ± SD and were compared by use of Student’s unpaired $t$ test. Qualitative data were compared by use of Yates corrected $\chi^2$ or Fisher’s exact test. Statistical analysis was done by use of Systat 9 (SPSS).

## Results

During the study period, 19 patients with severe and 32 with mild malaria were recruited. All patients but one were adults. The geographic and temporal distributions of the presumed area of infection were similar for both groups. Among patients with severe malaria, a broad range of WHO-defined severe clinical manifestations [4, 2] was observed, and MODS [5, 6] was observed in all but one (a patient with cerebral malaria) of these patients. Four patients, including 2 who died, had primary MODS. None of the patients with mild malaria had any severe manifestation of disease. In addition, parasitemia was much lower in the patients with mild malaria (0.7% ± 0.14%) than in those with severe disease (11.2% ± 13.7%; $P < .0001$). Thus, there were clear-cut differences between the 2 clinical groups.

Parasite genotypes were studied by PCR amplification of 3 single-copy genes located on different chromosomes [3]. Allelic polymorphism was limited, with 4 alleles for msp-1, 3 for glurp, and only 2 for msp-2. The distribution of the 2 msp-2 alleles 3D7-C and FC27-D (77% and 23%, respectively) was similar in both groups. The glurp alleles also showed a similar distribution (58%, 16%, and 26% for glurpE, glurpF, and glurpG, respectively, among the patients with severe cases vs. 61%, 13%, and 26% among the patients with mild cases). In contrast, the msp-1 allele distribution was distorted: the RO33, Mad20, A-K1, and B-K1 alleles accounted for 25%, 4%, 29%, and 41%, respectively, of the msp-1 alleles detected in isolates from patients with severe cases and for 33%, 16%, 35%, and 16%, respectively, of the msp-1 alleles detected in isolates from patients with mild cases. The B-K1 msp-1 allele was overrepresented in the severe malaria group ($\chi^2$ test, $P = .012$; table 1).

We also investigated var repertoires, which code for the clonally variant PfEMP-1 parasite molecules that mediate cytoadherence [8]. Genomic var diversity was investigated by PCR, using UNIEBP primers [9]. As predicted, multiple products were generated. A 520-bp band (band d) was observed in 11 isolates from patients with severe malaria and in 5 from patients with mild disease. Sequencing of band d isolated from a sample from a patient in the severe disease group allowed for the design of internal var-D primers. Var-D-specific PCR amplified a single 225-bp fragment from samples from 9 patients with severe cases and 4 patients with mild cases. The increased prevalence of var-D in patients with severe cases was significant (Fisher’s exact test, $P = .008$; table 1). Sequencing of the products showed that all 13 var-D fragments coded for the same unique

**Table 1.** Distribution of the B-K1 msp-1 and var-D genotypes in patients with mild (n = 32) and severe (n = 19) malaria.

<table>
<thead>
<tr>
<th>Allele or combined genotype</th>
<th>Mild malaria</th>
<th>Severe malaria</th>
<th>$P$</th>
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<td>10</td>
<td>.0002</td>
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</table>

**NOTE.** Data are no. of patients. Distributions by clinical group were compared by $\chi^2$ test or Fisher’s exact test.
**Table 1. Clinical manifestations and parasitologic characteristics in patients with severe malaria admitted to the intensive care unit in Cayenne, French Guiana.** Patients are classified according to the presence or absence of the varD/B-K1 msp1 genotype association. The following criteria were used to define clinical manifestations: cerebral malaria, Glasgow coma score $\leq 9$; severe anemia, hemoglobin level $\leq 5$ g/dL or hematocrit $\leq 15\%$; renal failure, serum creatinine level $\geq 265$ $\mu$mol/L or urine output $\leq 400$ mL; pulmonary failure, respiratory rate $\geq 60$ or $\leq 149$ breaths per minute, mechanical ventilation for $\geq 2$ days, and FiO$_2$ $\leq 0.4$ and/or PEEP $\geq 5$ cmH$_2$O; hepatic failure, clinical jaundice or total bilirubin level $\geq 50$ $\mu$mol/L in the absence of hemolysis and serum glutamic-pyruvic transaminase level $\leq 25$ normal; hematologic failure, hematocrit $< 21\%$, leukocyte count $\leq 300$ cells/mm$^3$, platelet count $\leq 50,000$ cells/mm$^3$, and disseminated intravascular coagulation syndrome; neurologic impairment, Glasgow coma score $< 7$. "N" and "Y" stand for the absence or presence of the parameter considered, respectively. Genotypes were determined as described elsewhere [3]. "A-K1" and "B-K1" refer to msp-1 block 2 alleles of the K1 family of 540 and 620 bp, respectively. "RO" and "Mad" indicate RO33- and Mad20-type alleles (540 bp each). "3D" and "FC" indicate the 870-bp, 3D7-type and the 760-bp, FC27-type msp-2 alleles, respectively. "E," "F," and "G" refer to 3 distinct alleles of the glurp locus (1160, 1200, and 1360 bp, respectively). The presence or absence of var-D was determined using the var-D–specific polymerase chain reaction. UNIEBP bands were assigned arbitrary symbols on the basis of gel migration profiles. The band migrating at 520 bp was called “d.” Patient 6 was receiving chemoprophylaxis. The time when patient 1 was ill before referral to the hospital could not be ascertained. *Patients 16 and 17, who lived in the same house, were admitted to the hospital on the same day. Typing generated identical genotypes for all loci investigated, which suggests infection by the same parasite clone. “N.I.,” noninformative; WHO, World Health Organization.

<table>
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<th>Sex</th>
<th>var-D</th>
<th>msp-1</th>
<th>vDlep</th>
<th>UVIEBP</th>
<th>Time to treatment (nos. of days)</th>
<th>Parasites (%)</th>
<th>Cerebral malaria</th>
<th>Severe anemia</th>
<th>Renal failure</th>
<th>Pulmonary failure</th>
<th>Hypertension</th>
<th>Laboratory evidence</th>
<th>WHO, World Health Organization criteria</th>
<th>Additional criteria</th>
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<th>Multiple organ dysfunction criteria</th>
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*Figure 1. Clinical manifestations and parasitologic characteristics in patients with severe malaria admitted to the intensive care unit in Cayenne, French Guiana. Patients are classified according to the presence or absence of the varD/B-K1 msp1 genotype association. The following criteria were used to define clinical manifestations: cerebral malaria, Glasgow coma score < 9; severe anemia, hemoglobin level < 5 g/dL or hematocrit < 15%; renal failure, serum creatinine level > 265 $\mu$mol/L or urine output < 400 mL; pulmonary edema or acute respiratory distress syndrome (ARDS), onset of bilateral pulmonary infiltrates with a PaO$_2$/FiO$_2$ < 300 mm Hg, regardless of positive end-expiratory pressure (PEEP) and a pulmonary artery occlusion pressure < 18 mm Hg or no clinical evidence of left atrial hypertension; shock, systolic blood pressure < 70 mm Hg and persistent hypotension < 90 mm Hg, despite adequate volume repletion; acidemia, plasma bicarbonate concentration < 15 $\mu$mol/L or arterial pH < 7.25; hemoglobinuria; spontaneous bleeding; impaired consciousness but capable of being aroused; prostration and extreme weakness; hyperparasitemia, > 5% parasitemia; jaundice, total bilirubin level > 50 $\mu$mol/L or > 3 mg/dL; hyperpyrexia, rectal temperature > 40°C. Multiple organ dysfunction syndrome (MODS) criteria were as defined elsewhere [5, 6]: cardiovascular failure, mean arterial pressure < 50 mm Hg, need for volume loading, and/or vasoactive drugs to maintain systolic arterial pressure > 100 mm Hg; pulmonary failure, respiratory rate < 6 or > 49 breaths per minute, mechanical ventilation for > 2 days, and FiO$_2$ > 0.4 and/or PEEP > 5 cm H$_2$O; renal failure, serum creatinine level > 279 $\mu$mol/L and need for dialysis or ultrafiltration; hepatic failure, clinical jaundice or total bilirubin level > 50 $\mu$mol/L in the absence of hemolysis and serum glutamic-pyruvic transaminase level > 2 x normal; hematologic failure, hematocrit < 21%; leukocyte count < 300 cells/mm$^3$, platelet count < 50,000 cells/mm$^3$, and disseminated intravascular coagulation syndrome; neurologic impairment, Glasgow coma score < 7. “N” and “Y” stand for the absence or presence of the parameter considered, respectively. Genotypes were determined as described elsewhere [3]. “A-K1” and “B-K1” refer to msp-1 block 2 alleles of the K1 family of 540 and 620 bp, respectively. “RO” and “Mad” indicate RO33- and Mad20-type alleles (540 bp each). “3D” and “FC” indicate the 870-bp, 3D7-type and the 760-bp, FC27-type msp-2 alleles, respectively. “E,” “F,” and “G” refer to 3 distinct alleles of the glurp locus (1160, 1200, and 1360 bp, respectively). The presence or absence of var-D was determined using the var-D–specific polymerase chain reaction. UNIEBP bands were assigned arbitrary symbols on the basis of gel migration profiles. The band migrating at 520 bp was called “d.” Patient 6 was receiving chemoprophylaxis. The time when patient 1 was ill before referral to the hospital could not be ascertained. *Patients 16 and 17, who lived in the same house, were admitted to the hospital on the same day. Typing generated identical genotypes for all loci investigated, which suggests infection by the same parasite clone. “N.I.,” noninformative; WHO, World Health Organization.*
polypeptide sequence, with residues characteristic of a DBL-delta domain (GenBank accession no. AJ277137). However, the nucleotide sequence showed 2 allelic forms, with 2 silent point mutations. Var-D1 was present in 9 isolates (6 severe and 3 mild cases), and var-D2 was present in 4 isolates (3 severe and 1 mild case). Of interest, the var-D2 allele was detected exclusively in patients who came from a precise area of French Guinea where malaria was present. Data not shown). We concluded that the parasites circulating in this patient had abolished its production, as predicted. From their observations of induced mild malaria in vitro. Peripheral blood parasites were cultivated in vitro to demonstrate that specific genotypic characteristics are associated with severe malaria cases and the demonstration of its programmed expression in vivo. The observation of 2 geographically isolated alleles that code for the same amino acid sequence reinforces this interpretation. Cytoadherence is believed to be one of the major contributors to *P. falciparum* pathogenesis, since the *Plasmodium* species that cause mild disease in humans do not cytoadhere. Our finding of a specific var gene associated with the most severe forms is consistent with the prevailing concept that specific ligand or receptor interactions are involved in severe malaria [13]. The var-D gene is absent from all laboratory strains tested (FCR3, Palo Alto, FCC1, Tak9/100, ItG2G1, and Tak9/96). Further characterization of the var-D gene should open the way to identification of the corresponding cytoadherence receptor. This is the critical foundation on which to base subsequent identification of additional var products that share similar binding specificity and from which to derive a rational approach to prevent these interactions.

Discussion

To our knowledge, our results provide the first field demonstration that specific genotypic characteristics are associated with severe *P. falciparum* malaria. The notion that *P. falciparum* parasites differ in the clinical manifestations they provoke is not new. From their observations of induced mild malaria infections, James et al. [11] concluded in 1932 that “A striking difference can be observed between the clinical virulence of different geographical races or strains of the same morphological species of the parasite; the difference is particularly apparent between various races of *P. falciparum*” (page 1176). Autonomous infection patterns were observed in experimental infection of *Saimiri* monkeys [12]. Field studies have compared phenotypic [13] and genotypic [14] parasite characteristics in mild and severe cases. However, the large parasite polymorphism and the confounding effect of the rapidly acquired immunity to life-threatening forms did not allow for definitive conclusions to be drawn. The unique situation in French Guinea has shown that 2 markers, var-D and the B-K1 msp-I allele, were individually overrepresented in severe cases. More important, their combination was strongly associated with severe malaria and with the most severe clinical presentations. This indicates that parasite virulence factors are intrinsic components of the cascade that leads to severe disease in this setting.

An association of the msp-I locus with disease severity was observed in Senegal [14]. It is unclear whether the msp-I locus itself or a marker in linkage disequilibrium with msp-I is implicated. Since merozoite surface protein–1 has a key role in invasion of red blood cells, it is conceivable that specific msp-I allelic forms favor more efficient invasion than do others. A rapid multiplication rate allows parasites to reach a high density before an effective protective response is established and, as such, contributes to severity [1, 11, 15].

The increased prevalence of var-D in severe falciparum malaria cases and the demonstration of its programmed expression by RT-PCR analysis suggest its involvement in severe pathogenesis. The observation of 2 geographically isolated alleles that code for the same amino acid sequence reinforces this interpretation. Cytoadherence is believed to be one of the major contributors to *P. falciparum* pathogenesis, since the *Plasmodium* species that cause mild disease in humans do not cytoadhere. Our finding of a specific var gene associated with the most severe forms is consistent with the prevailing concept that specific ligand or receptor interactions are involved in severe malaria [13].

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