Influence of Carriage of Hemoglobin AS and the Fcγ Receptor IIa–R131 Allele on Levels of Immunoglobulin G2 Antibodies to Plasmodium falciparum Merozoite Antigens in Gabonese Children

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Background. To extend our previous finding showing an imbalanced distribution of immunoglobulin G2 (IgG2) antibodies to Plasmodium falciparum merozoite surface protein 2 (MSP2) and a higher frequency of infection with multiple P. falciparum strains in Gabonese children with sickle cell trait (hemoglobin AS), human Fcγ receptor (FcγR) IIa (CD32) polymorphism and the rate of in vitro invasion of red blood cells (RBCs) from subjects with either hemoglobin AA or AS by multiple P. falciparum strains were investigated.

Methods. FcγRIIa mutation at amino acid position 131 (arginine or histidine) was detected by polymerase chain reaction, and in vitro cultures for parasites were used to assess the invasion rate.

Results. FcγRIIa polymorphism is normally distributed in this population, with no preferential carriage by children with hemoglobin AS. Lower levels of IgG2 subclass antibodies to MSP2 peptides were independently associated with the FcγRIIa-R131 allele and with carriage of hemoglobin AS. Our data suggest that IgG3 antibody responses to MSP2 epitopes could be exacerbated by lower IgG2 levels in children with hemoglobin AS.

Conclusions. The higher rate of invasion of RBCs in the presence of multiple strains may indicate that several invasion pathways are solicited simultaneously, and the longer persistence of ring forms in RBCs from the subjects with hemoglobin AS might reflect a slower multiplication phase, leading to a longer circulation and enhanced phagocytosis of these nonpathogenic parasite forms. This may contribute to the protection against P. falciparum malaria observed in children with hemoglobin AS.

In 1949, Haldane [1] hypothesized that malaria has a strong impact on the human genome. Several decades later, polymorphisms in multiple human genes that affect susceptibility to Plasmodium falciparum infection have been described, and, among these, the sickle cell trait (hemoglobin AS) appears to be frequent in sub-Saharan Africa. Early and recent case-control epidemiological studies have demonstrated that persons with the sickle cell trait do become infected with P. falciparum but that a smaller proportion of those with hemoglobin AS than of those with normal hemoglobin AA develop severe malaria symptoms [2, 3]. The mechanisms by which the sickle cell trait confers protection are still unclear. Among the most common hypotheses, intraerythrocytic polymerization of hemoglobin S and accelerated sickling of parasitized red blood cells (RBCs) in vivo might predispose such infected cells to early removal from the blood circulation [4, 5], and
higher levels of cellular and humoral immunity to \( P. falciparum \) in carriers of hemoglobin AS have also been evoked for contributing protective effects [6–8].

Initiated in 1997, molecular epidemiological studies of children carrying the sickle cell trait and harboring asymptomatic \( P. falciparum \) infections [9–11] have been conducted in an area of hyperendemicity in Gabon (Central Africa), where the prevalence of the sickle cell trait is \( \sim 22\% \) [10]. An influence of hemoglobin type on the frequency of \( P. falciparum \) infections [9] and on the prevalence of IgG subclass antibodies to merozoite surface protein 2 (MSP2) [11] were reported. Specifically, cytophilic IgG3 and “noncytophilic” IgG2 subclass antibodies were predominantly detected in plasma samples from children with hemoglobin AS. These observations are of particular interest in light of recent studies showing the importance of IgG2 in protection against clinical malaria in a West African population [12, 13].

Of the 3 classes of human Fcy receptors (Fc\( _{\gamma} \)R) described to date, only Fc\( _{\gamma} \)RIIa (CD32) can bind to IgG2 subclass antibodies. This 40-kDa glycoprotein, expressed on a variety of cells (such as granulocytes, monocytes/macrophages, B cells, and some subsets of T cells), contains a polymorphism in the second immunoglobulin-like domain of the extracellular region that results in an amino acid change from arginine to histidine in the ligand-binding epitope at position 131 [14, 15]. Fc\( _{\gamma} \)RIIa-H\( _{131} \) (mutant type) has a higher affinity for IgG2 than does Fc\( _{\gamma} \)RIIa-R\( _{131} \) (wild type), which preferentially binds to subclasses IgG1 and IgG3 [14]. The variation in ligand specificity of mutant Fc\( _{\gamma} \)RIIa has clinical relevance in bacterial infections [15, 16]. C-reactive protein is also able to bind to Fc receptors with high affinity thus, recognition of both IgG and C-reactive protein by Fc\( _{\gamma} \)RIIa are influence by this polymorphism.

Here, we present 2 sets of data that extend and clarify the finding of our previous studies through analyses of human Fc\( _{\gamma} \)RIIa in the same Gabonese children presenting with an imbalanced distribution of IgG2 antibodies. Additionally, to extend our understanding of our results showing a higher frequency of infection with multiple \( P. falciparum \) strains in the children with hemoglobin AS, we hypothesized that RBCs from carriers of hemoglobin AS are more susceptible to invasion in the presence of multiple \( P. falciparum \) strains. This hypothesis was tested by measuring the in vitro rate of invasion of RBCs from subjects with hemoglobin AS by single versus multiple \( P. falciparum \) strains.

**SUBJECTS, MATERIALS, AND METHODS**

**Study site.** A cross-sectional study was conducted from May to June 1999 in the city of Bakoumba in southeastern Gabon, where malaria is endemic, with marked transmission during 2 rainy seasons from February to May and October to December. The most predominant malaria-causing species is \( P. falciparum \) [17].

**Study population and blood collection.** This study was initiated in 1999, and detailed description of the participants, the inclusion criteria, and hematologic and parasitological methods have been reported elsewhere [11]. Briefly, children with asymptomatic \( P. falciparum \) infections who were between 6 months and 10 years old were enrolled, after informed consent was obtained from their parents, in a cross-sectional study during the transmission season. Venous blood was collected into EDTA and heparinized tubes for determination of hematologic parameters, such as blood group and hemoglobin type, and for subsequent immunologic and molecular assays. Plasma samples obtained from healthy European donors with no history of malaria were used as negative controls for the immunologic assays. This study was approved by the Ethics Committee of the Centre International de Recherches Médicales de Franceville.

**Extraction of genomic DNA.** Pellets obtained from 25% (159/641) of these children were available for analysis of human DNA. Human DNA was extracted using a QIAamp DNA Blood Mini Kit (Qiagen), in accordance with the manufacturer’s protocol.

**Indirect ELISA with MSP2 biotinylated peptides.** IgG antibodies and subclasses were detected by indirect ELISA, using 12 biotinylated peptides reproducing parts of MSP2, as described elsewhere [11]. Optical density was measured at 450–650 nm (end point). The plasma samples from the healthy European donors who had no history of malaria were tested using the same procedure and served to determine the specificity of the immunologic assays. Plasma samples giving a signal 2 times greater than the cutoff value of the wells without peptide were considered to be positive.

**Fc\( _{\gamma} \)RIIa (CD32) 131 point mutation analysis.** Fc\( _{\gamma} \)RIIa-H/ R\( _{131} \) polymorphism was determined by an allele-specific restriction enzyme digestion method [18]. The polymerase chain reaction (PCR) conditions were modified as follows: 1 cycle of 5 min at 96°C; 35 cycles of 92°C for 40 s, 55°C for 30 s, and 72°C for 10 s; and 1 cycle of 10 min at 72°C. The PCR product of the H\( _{131} \) allele contains 1 BstUI site, and the PCR product of the R\( _{131} \) allele contains 2 BstUI sites. Digestion products were analyzed by electrophoresis on a 3% agarose gel stained with ethidium bromide. Each run included DNA samples previously genotyped as either H/H\( _{131} \), H/R\( _{131} \), or R/R\( _{131} \). Expected lengths of fragments were as follows: R/R\( _{131} \), 322 bp; H/R\( _{131} \), 343 bp and 322 bp; and H/H\( _{131} \), 343 bp.

**Rate of in vitro invasion of RBCs from subjects with hemoglobin AA or AS by culture-adapted lines of \( P. falciparum \).** Blood samples from subjects with hemoglobin AS originating from the French West Indies were collected at the Hospital Henri Mondor (Créteil, France) and were shipped to our laboratory under appropriate conditions within 2 days after blood...
collection for use in in vitro studies the next day. These subjects were tested for *P. falciparum* infection, and all were found to be negative for falciparum malaria. Hemoglobin type was determined by PCR [19]. None of the subjects were glucose-6-phosphate dehydrogenase deficient. Six subjects of both sexes were healthy adults >20 years old. Informed consent was obtained from the donors. RBCs were recovered after centrifugation with 80% Percoll and washing with RPMI 1640 (9.1 g/L HEPES, 2 g/L glucose, 200 μmol/L hypoxanthine, and 2 g/L NaHCO3).

Three culture-adapted lines of *P. falciparum*, Binh, S007, and Muz, were cultured in RBCs at a 5% hematocrit level, using standard procedures [20], in an atmosphere of 1% O2, 3% CO2, and 96% N2 in RPMI 1640 supplemented with Albumax II (GIBCO/Invitrogen). In vitro parasite cultures were stopped when parasite density reached 10%–20%, with mature forms predominating. Mature forms were further enriched in the culture, using magnetic-activated cell sorting [21]. For each line, the resulting inoculum contained >80% schizont forms. The 3 lines were also mixed at this stage, and the resulting inoculum contained one-third of each line. These different *P. falciparum* in vitro cultures were mixed with RBCs from subjects with hemoglobin AA and AS to a final parasite density of 1%. Parasite in vitro cultures with RBCs from hemoglobin AA carriers (from the blood bank in Tübingen) and RBCs from AS carriers were incubated at 37°C, and thin blood smears were prepared at time 0, 4 h later, and every 2 h thereafter, to identify young parasite forms (ring forms) corresponding to newly invaded RBCs. The ring forms were classified as the parasite forms without pigment, including early and late ring forms. All in vitro cultures were *Mycoplasma* free [22]. All invasion assays were done in triplicate. Blood from each donor was tested separately. Slides were examined for 500 RBCs.

**Statistical analysis.** A mean of the number of ring forms per 500 RBCs was calculated by triplicate readings. The non-parametric Kruskal-Wallis test and the Mann-Whitney *U* test were used to compare continuous variables between subjects with genotype H/H131, H/R131, and R/R131 and between subjects with hemoglobin AA and AS, respectively. The χ² test was used to compare nominal data. A multiple linear regression model was used to test the influence of hemoglobin and CD32 polymorphisms on the levels of IgG2 subclass antibodies. Statistical analysis of data was done using JMP software (version 5). Differences were considered to be statistically significant at *P* < .05.

**RESULTS**

The mean age of the 159 children studied for the FcγRIIa polymorphism was 5 years (range, 0.5–10 years). The mean parasite density was 2525 parasites/μL (range, 0–85,334 parasites/μL). Of the children, 90 carried hemoglobin AA and 69 carried hemoglobin AS. Parasite density did not vary with hemoglobin type or with blood group O versus non-O (blood group O has been shown to be protective against malaria in Gabon [23]).

**Distribution of FcγRIIa genotypes in the children with asymptomatic infections.** As shown in table 1, the H/H131, H/R131, and R/R131 genotypes were equally distributed in the population (Hardy-Weinberg equilibrium), with no difference between the 2 hemoglobin groups (*χ² = 4.65; 2 df; *P* > .05), and the frequencies of the H and R alleles at position 131 of FcγRIIa receptor were 48% and 52%, respectively. The H/H131, H/R131, and R/R131 genotypes were found at prevalences of 24%, 50%, and 26%, respectively (table 1). Parasite density was not affected by FcγRIIa polymorphism in this population.

**FcγRIIa genotypes and IgG subclass responses to MSP2 peptides.** Levels of total IgG to MSP2 synthetic peptides [11] were not found to be different between the H/H131, R/R131, and H/R131 groups (*P* < .001). Regarding the IgG subclasses, levels of IgG2 subclass antibodies were significantly lower in the R/R131 group regardless of the peptide considered, as shown in table 2. No statistical difference was found for the IgG3 and IgG4 antibody subclasses between the 3 groups. Carriage of the R131 allele, (P < .0001) and hemoglobin AS (P < .0001) were independently associated with lower IgG2 levels when a multiple linear regression model was used and differences between peptides were controlled for. Cumulative carriage of hemoglobin AS and the R131 allele had a stronger impact on IgG2 levels than did unique carriage of the R131 allele, as shown in table 3. The number of persons with detectable IgG2 was higher (*P* = .02) among those with hemoglobin AS than among those with hemoglobin AA. Conversely, the levels of IgG2 were higher (*P* < .0001) among those with hemoglobin AA. Neither hemoglobin type nor receptor genotype had an impact on IgG3 levels, the most prevalent subclass of IgG antibody to MSP2 molecules.

**Rate of invasion of red blood cells from subjects with hemoglobin AA and AS by different *P. falciparum* strains.** Ring (early and late) forms were identified and enumerated from 500 RBCs. As presented in figures 1 and 2, using 3 different culture-adapted lines of *P. falciparum* (Binh, S007, and Muz),

<table>
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<tr>
<th>Table 1. Distribution of Fcγ receptor RIa genotypes in Gabonese children from Bakoumba with hemoglobin AA or hemoglobin AS.</th>
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</thead>
<tbody>
<tr>
<td>Genotype</td>
</tr>
<tr>
<td>----------</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>H/H131</td>
</tr>
<tr>
<td>H/R131</td>
</tr>
<tr>
<td>R/R131</td>
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<tr>
<td>Total</td>
</tr>
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</table>

**NOTE.** Data are no. (%) of children. Expected nos. of children in each genotype group were determined using the Hardy-Weinberg equilibrium (*χ² = 4.65; 2 df; *P* > .05). Comparisons of proportional data between children with hemoglobin AA or AS (χ² test) were not significant.
we clearly observed that invasion was facilitated in RBCs from both subjects with hemoglobin AA and hemoglobin AS when the strains were mixed in the inoculum. The time point 12–14 h corresponds approximately to the peak of the presence of ring form stages in RBCs, and no difference was noticed between RBCs from subjects with hemoglobin AA versus those from subjects with hemoglobin AS. Interestingly, ring forms persisted for a longer time in RBCs from subjects with hemoglobin AS, suggesting slower parasite maturation in these cells. Indeed, after 20 h of in vitro culture, ring forms were still present in RBCs from subjects with hemoglobin AS, whereas they almost disappeared in RBCs from subjects with hemoglobin AA (mean, 19/500 RBCs vs. 2/500 RBCs; $P < .05$).

### DISCUSSION

In the present study, we have undertaken an analysis of human FcγRIIa polymorphism in a population of children in whom the prevalence of IgG2 subclass antibody to MSP2 epitopes was imbalanced according to the carriage of hemoglobin type. The data presented here show that FcγRIIa mutations at position 131 were evenly distributed in the Central African population of Gabon, with no preferential carriage by persons with sickle cell trait. The frequencies of the H/H$_{131}$, H/R$_{131}$, and R/R$_{131}$ genotypes in the Gabonese population were quite similar to those observed in the Gambian population [24], where the H/ $H_{131}$ genotype has been associated with susceptibility to severe malaria [24]. In western Kenya, it has been reported that the R/R$_{131}$ genotype offered protection against high levels of *P. falciparum* parasitemia during the first year of life [25]. In our study, we did not find any impact of the FcγRIIa polymorphism on parasite density, whatever the age. This might be due to the fact that only children with symptomless infections were investigated and to the limited size of our study population.

There is accumulating evidence that IgG1 and IgG3 play roles in protection from disease [26], and the contribution of parasite-specific IgG2 in protection against clinical malaria [12] and/or in susceptibility to the disease by reducing parasite clearance [27, 28] needs to be further explored. In our working hypothesis, we proposed a protective role for IgG3 and IgG2 [11], which may activate effector cells through FcγRIIa in children with hemoglobin AS. This hypothesis implies that the frequency of this mutation is higher in persons with hemoglobin AS. This was not the case. In addition, low levels of IgG2 to MSP2 peptides were independently associated with the simultaneous carriage of the R/R$_{131}$ genotype and the sickle cell trait. Our results suggest that IgG3 responses to asexual blood stage epitopes of MSP2 are exacerbated by lower IgG2 levels and also that the levels of IgG2 are more important than the presence of this subclass for efficient immune responses to MSP2 epitopes. This is not in contradiction with our previous

**Table 2. Comparisons of the levels of total IgG and IgG2 subclass antibodies among subjects with the H/H$_{131}$, H/R$_{131}$, or R/R$_{131}$ Fcγ receptor RIIa genotype.**

<table>
<thead>
<tr>
<th>Peptide 1</th>
<th>H/H$_{131}$</th>
<th>H/R$_{131}$</th>
<th>R/R$_{131}$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total IgG</td>
<td>.043 (.039)</td>
<td>.051 (.041)</td>
<td>.047 (.040)</td>
<td>.308</td>
</tr>
<tr>
<td>IgG2</td>
<td>.047 (.012)</td>
<td>.038 (.021)</td>
<td>.029 (.012)</td>
<td>.016</td>
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</tbody>
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**Table 3. Influence of hemoglobin type and Fcγ receptor (FcγR) RIIa polymorphism on the levels of IgG2 antibodies to merozoite surface protein 2 peptide 1.**

<table>
<thead>
<tr>
<th>Model</th>
<th>$P$</th>
<th>Slope</th>
<th>SE</th>
<th>$t$</th>
</tr>
</thead>
<tbody>
<tr>
<td>FcγRIIa (H vs. R)</td>
<td>.040</td>
<td>-.001</td>
<td>0.005</td>
<td>-2.14</td>
</tr>
<tr>
<td>Hemoglobin (AA vs. AS)</td>
<td>.017</td>
<td>-.001</td>
<td>0.004</td>
<td>-2.52</td>
</tr>
</tbody>
</table>

**NOTE.** The association between hemoglobin type and the H/R$_{131}$ allelic polymorphism was tested using a multiple linear regression model. Similar observations were made for the other peptides. H and R are the 2 FcγRIIa alleles at position 131.
study showing a higher prevalence, but not higher levels, of IgG2 antibodies in children with hemoglobin AS.

In the same population of Gabonese children, the higher frequency of infections with multiple strains in children with hemoglobin AS was interpreted as a higher capacity of RBCs to be invaded by *P. falciparum* parasites. We addressed this issue by evaluating in vitro invasion in RBCs from subjects with hemoglobin AA and hemoglobin AS by single versus multiple *P. falciparum* strains. To our knowledge, this is the first study to investigate this aspect. Clearly, higher infection rates were achieved with a mixture of 3 strains than with each strain alone, and this was observed regardless of the type of RBC and *P. falciparum* strain. *P. falciparum* is able to invade erythrocytes by using multiple receptor-ligand interactions define as invasion pathways [29], and the existence of a significant diversity of invasion pathways in field isolates has been confirmed [30]. It is likely that competition among multiple strains simultaneously facilitates the invasion by soliciting different points of entry into RBCs. This hypothesis needs to be tested further.

Our in vitro data showed that, under appropriate oxygenation conditions, ring forms persist longer in RBCs from carriers of hemoglobin AS. These results suggest that, in vivo, the longer carriage of ring-parasitized RBCs in persons with hemoglobin AS leads to a longer peripheral circulation of these parasite forms. This fact may contribute to a longer exposure of parasite surface antigens of this nonpathogenic stage of the parasite to the immune system. As reported by Ayi et al. [5],
phagocytosis of ring-parasitized RBCs from persons with hemoglobin AS was enhanced compared with that of ring-parasitized RBCs from persons with normal hemoglobin. Their finding fully supports the data presented here. Therefore, we suggest that the presence of persistent ring forms in children with hemoglobin AS contributes to the mechanisms of protection against malaria, even though parasite growth in abnormal RBCs is impaired by the sickling phenomenon [31, 32].

In conclusion, in the present work we have demonstrated the influence of FcγRIIA polymorphism and carriage of hemoglobin AS on the levels of IgG2 subclass antibody and the longer persistence of ring forms in RBCs from persons with hemoglobin AS, suggesting a slower multiplication phase and enhancement of phagocytosis.

Acknowledgments

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