Fc Gamma Receptor 3B (FCGR3B-c.233C>A-rs5030738) Polymorphism Modifies the Protective Effect of Malaria Specific Antibodies in Ghanaian Children

Bright Adu,1,2,a Micha Phill Gramholm Jepsen,1,2,a Thomas A. Gerds,3 Eric Kyei-Baafour,4 Michael Christiansen,1 Daniel Dodoo,4 and Michael Theisen1,2

1Department of Clinical Biochemistry and Immunology, Statens Serum Institut, 2Centre for Medical Parasitology at Department of International Health, Immunology, and Microbiology and Department of Infectious Diseases, Rigshospitalet, and 3Department of Biostatistics, University of Copenhagen, Immunology, and Microbiology and Department of Infectious Diseases, Antigens in Ghanaian Children Protecting Effect of Malaria Specific Antibodies (FCGR3B-c.233C>A-rs5030738) with protection against malaria. The FCGR3B-c.233C>A polymorphism thought to influence the interaction between IgG and FcγRIIB was recently associated with malaria. We studied the statistical interaction between glutamate rich protein antibodies and FCGR3B-c.233C>A genotypes on risk of malaria in a cohort of Ghanaian children. The absolute risk of malaria decreased more rapidly with increasing antibody levels for 233AA/AC individuals compared with 233CC children. This genotype related effect modification may significantly influence malaria sero-epidemiological and vaccine trial studies.

Keywords. malaria; GLURP; FCGR3B-c.233C>A; FcγRIIB; Plasmodium falciparum; effect modification; neutrophils.

Malaria-specific cytophilic immunoglobulin G (IgG) antibodies (IgG1 and IgG3) are considered crucial in immunity against clinical malaria. Presently, most malaria immunity studies have either focused on antibody data [1, 2] or genetics data [3, 4] to ascertain correlates of protection or susceptibility. Studies where both host genetics and antibody data from the same cohort are interrogated simultaneously for markers of protection against and/or susceptibility to malaria are curiously lacking. The antibody-dependent respiratory burst, mediated by neutrophils, was recently found to correlate with protection from febrile malaria [5]. Human neutrophils constitutively express Fc gamma receptor IIA (FcγRIIA) and Fc gamma receptor IIB (FcγRIIB) [6]. FcγRIIA crosslinking by IgG triggers phagocytosis, whereas FcγRIIB crosslinking leads to neutrophil degranulation and generation of reactive oxygen species [7], which has been shown to inhibit intraerythrocytic malaria parasites development [8]. FcγRIIA and FcγRIIB both exist in polymorphic forms having variable IgG binding affinities. We recently reported an association between the FCGR3B-c.233C>A, but not FcγRIIA, genotypes and febrile malaria in a cohort of Ghanaian children [3]. Here, we hypothesized that the interaction between FCGR3B-c.233C>A genotypes and malaria-specific antibodies influences the risk of febrile malaria. Clinical, parasitologic, demographic, and FCGR3B genotyping data were combined with antibody measurements against 2 regions (R0 and R2) of the malaria antigen, glutamate rich protein (GLURP), from a well-characterized longitudinal cohort [3]. The effect modification of FcγRIIB polymorphisms on the associations between malaria-specific antibodies and Plasmodium falciparum infection outcome was investigated.

METHODS

Ethics Statement
Ethical approval for the study was given by the institutional review board of the Noguchi Memorial Institute for Medical Research (NMIMR) of the University of Ghana, Accra. Written informed consent was given by the parents and guardians of children before they were enrolled into the study.

Study Population and Study Design
The data presented here were generated from a longitudinal malaria cohort study conducted in Asutsuare and environs in the Damgbe West District of Ghana, described in detail elsewhere [3]. Briefly, 798 healthy children aged 1–12 years were enrolled before the malaria transmission season and followed-up actively and passively for malaria case detection for 42 weeks. At the end of the study, the cohort was divided into 3 groups: (1) those susceptible, in which parasitemia was associated with febrile malaria (axillary temperature ≥37.5°C
measured or reported and at least 1 other sign of malaria such as vomiting, diarrhea, or malaise; (2) those apparently protected against clinical manifestation despite parasitemia; and (3) those without detectable parasitemia by microscopy and no clinical manifestations of malaria. It should be noted that this study had 49 more febrile malaria cases than what was previously reported in the same cohort [3]. Of these individuals, 32 had baseline plasma available for serology but not DNA and hence were included in only antibody analysis. The remaining 17 individuals were asymptomatic but were only confirmed to carry very low level parasitemia upon subsequent quality control reading of case report slides by 2 independent microscopists.

Antigens
The recombinant antigens GLURP-R0 (amino acids 25–514) and GLURP-R2 (amino acids 705–1178) are the conserved N-terminal nonrepeat and the carboxy-terminal repeat regions respectively of the GLURP molecule. Both antigens were expressed in Escherichia coli [9].

Multiplex Assay for Antibody Measurement
Coupling of antigens to beads and measurement of IgG was performed exactly as described [10]. For the IgG subclass measurements, 50 µL of bead mixture comprising 1250 beads/coupled bead region were added to each well of a prewetted 96-well filter microtiter plate. Plasma samples diluted 1:1000 were added and incubated for 2 hours. Mouse anti-human IgG1 (1:2000) clone NL 16, or IgG2 (1:5000) clone HP-6002, or IgG3 (1:5000) clone ZG4, or IgG4 (1:3000) clone RJ4 was added and incubated for 1 hour. Phycoerythrin-conjugated goat antimouse IgG (1:200) at 50 µL/well was then added and incubated for 1 hour. Plates were washed 3 times with assay buffer E (0.1% Bovine Serum Albumin, 0.05% Tween 20, 0.05% sodium azide in phosphate-buffered saline; pH 7.4) between steps and analyzed on a Luminex 200. Each plate contained positive, negative, and blank controls. To account for interassay variation, the median fluorescence intensity (MFI) was adjusted using positive control as calibrator. Adjusted MFI = (average MFI calibrator all plates)/(MFI calibrator) × (MFI sample).

FcγRIIB Genotyping Data
The FcγRIIB genotyping data on the exact same cohort were recently published [3]. These data were extracted and included in the final antibody dataset for the effect modification analyses.

Statistical Analysis
Children who had neither parasitemia (by monthly blood slide parasitemia surveillance) nor any malaria episodes during the entire follow-up were excluded from further analysis. Age was categorized at 2 levels (aged 1–5 years and aged 6–12 years). Antibody values were log-transformed with base 2, and the 2-fold change of the odds of malaria was analyzed using logistic regression. Multiple logistic regression analyses were adjusted for age, sex, sickle cell trait, and ABO blood group. Interaction terms were introduced to study whether the effects of the antibody levels on the malaria risk depended on the FCGR3B-c.233C>A genotype. The significance of the effect modification was assessed by likelihood ratio tests comparing the model that included additive genotype effects with the interaction model. The latter comparison was adjusted for age groups but not the other potential confounders because of lack of power. The absolute risk of malaria was computed based on the logistic regression models as a function of antibody levels and the different FCGR3B-c.233C>A genotypes under the A allele dominant model of inheritance. We provide raw P values and raw confidence limits and also Bonferroni–Holm corrected P values. The level of significance was set at 5%.

RESULTS

Study Demographics and Parasitemia Prevalence
A total of 669 children completed the follow-up, and the demographic and clinical characteristics of a sub-population (n = 585) were previously described [3]. Because malaria incidence was relatively low during follow-up, children with definitive malaria exposure (n = 168 of 669; 25.1%) were identified as those who were either diagnosed with febrile malaria (n = 101 of 669; 15.1%) or carried asymptomatic parasitemia (n = 67 of 669; 10.0%) without clinical symptoms during the entire follow up. Covariable associations with febrile malaria episodes found significantly fewer malaria cases in the 6–12 year age group (odds ratio [OR], 0.36; 95% confidence interval [CI], 0.19–0.69; P = .003) compared with the 1–5 year age group (Supplementary Table 1). Sex, sickle cell trait, and blood group did not show any associations with febrile malaria episodes in this cohort (Supplementary Table 1). The sickle cell trait is often associated with protection against malaria. However, this effect may have been obscured in the cohort by the overall low malaria incidence and low prevalence of the sickle cell trait in the study population (Supplementary Table 1).

Association Between Antibody Levels and Febrile Malaria
Multivariable logistic regression analyses adjusting for age group, sex, sickle cell trait, and ABO blood group showed significant protection from febrile malaria with increasing levels of all anti-GLURP-R2 IgG and IgG subclass antibodies (P < .05). On the other hand, only increasing levels of anti-GLURP-R0 IgG (OR, 0.88; 95% CI, .77–.98; P = .03), and IgG3 (OR, 0.85; 95% CI, .75–.95; P = .007) antibodies were associated with protection against febrile malaria in this cohort (Table 1).

FCGR3B-c.233C>A Effect Modification on Association Between Antibody Levels and Febrile Malaria
Of the 168 individuals with definitive exposure, genotype data were available for 121 individuals (69 febrile malaria cases and
52 asymptomatic parasitemia carriers). The minor allele (233A) frequency in the study population was 0.214, which was in Hardy–Weinberg equilibrium [3]. The likelihood ratio test (LRT) for interaction adjusted for age group showed that the FCGR3B-c.233C>A genotype modifies the protective antibody effects on the risk of malaria significantly for all antibodies except for anti-GLURP-R0 IgG, IgG2, and IgG4 (P_{LRT} > 0.05) (Supplementary Table 2). The absolute risk of malaria as a function of increasing antibody levels showed a sharper decrease for individuals with the c.233AA/AC genotype compared with those who possess the c.233CC genotype (Figure 1). Thus, the A-allele of the FCGR3B-c.233C>A polymorphism appears to enhance the protective effect of malaria-specific antibodies.

DISCUSSION

In summary, we show that the IgG-FcγRIIB ligand–receptor interaction contributes significantly to the outcome of the malaria infection in Ghanaian children. Cross-linking of FcγRIIB by IgG in immune complexes results in neutrophil degranulation and respiratory burst, releasing molecules such as proteases and reactive oxygen species [11], which have been associated with inhibition of intraerythrocytic *P. falciparum* growth [8, 12] and protection against malaria [5]. We previously demonstrated that the A allele of the FCGR3B-c.233C>A polymorphism was associated with protection against febrile malaria in a cohort of Ghanaian children [3]. However, the possible mechanism of action was unknown. Here, we tested the hypothesis that the interaction between malaria-specific IgG and FcγRIIB bearing different FCGR3B-c.233C>A genotypes would significantly modify the risk of febrile malaria. After adjusting for multiple testing by Bonferroni–Holm correction, our data show that when the genotypes are ignored in the analyses, the general conclusion indicates increasing levels of anti-GLURP-R2 IgG and IgG1, and IgG3 against both GLURP antigens are protective against malaria, as previously reported [1, 13].

### Table 1. Antigen-Specific Antibody Association With Febrile Malaria

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antigen</th>
<th>OR (95% CI)</th>
<th>P Value Adjusted P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>GLURP-R0</td>
<td>0.88 (.77–.98)</td>
<td>.027 .13</td>
</tr>
<tr>
<td></td>
<td>GLURP-R2</td>
<td>0.84 (.78–.92)</td>
<td>.0001 0.001</td>
</tr>
<tr>
<td>IgG1</td>
<td>GLURP-R0</td>
<td>0.95 (.85–1.08)</td>
<td>.46 .92</td>
</tr>
<tr>
<td></td>
<td>GLURP-R2</td>
<td>0.87 (.78–.95)</td>
<td>.004 0.03</td>
</tr>
<tr>
<td>IgG2</td>
<td>GLURP-R0</td>
<td>0.95 (.82–1.10)</td>
<td>.49 .92</td>
</tr>
<tr>
<td></td>
<td>GLURP-R2</td>
<td>0.90 (.82–.97)</td>
<td>.009 0.051</td>
</tr>
<tr>
<td>IgG3</td>
<td>GLURP-R0</td>
<td>0.85 (.75–.95)</td>
<td>.007 0.049</td>
</tr>
<tr>
<td></td>
<td>GLURP-R2</td>
<td>0.84 (.77–.93)</td>
<td>.0005 0.005</td>
</tr>
<tr>
<td>IgG4</td>
<td>GLURP-R0</td>
<td>0.82 (.57–1.14)</td>
<td>.24 .73</td>
</tr>
<tr>
<td></td>
<td>GLURP-R2</td>
<td>0.84 (.72–.98)</td>
<td>.03 0.13</td>
</tr>
</tbody>
</table>

Odds ratios and 95% confidence intervals were determined using multivariate logistic regression adjusted for age, sex, sickle cell trait, and ABO blood group (n = 168: 101 febrile malaria cases and 67 asymptomatic parasitemia carriers). The confidence intervals were not adjusted for multiple testing. All antibody values were log-transformed (base 2) so that odds ratios and confidence intervals represent effect associated with 2-fold increase in antibody level. *P* values are shown without adjustment and after Bonferroni–Holm adjustment for multiple testing. Abbreviations: CI, confidence interval; GLURP, glutamate rich protein; IgG, immunoglobulin G; OR, odds ratio.

Figure 1. The genotype FCGR3B-c.233C>A modifies the protective effect of increasing antibody levels. Results from logistic regression for 69 febrile malaria cases and 52 asymptomatic parasitemia carriers (for whom complete serology and genotype data were available) are shown on the absolute risk of malaria scale: 233AA/AC (solid lines) and 233CC (dashed lines).
However, this study also suggests that individuals with different FCG3B-c.233C>A genotypes do not benefit equally from increasing antibody levels in relation to protection against malaria (Figure 1 and Supplementary Table 2). Thus, FCG3B-c.233C>A genotypes significantly modify the protective effect of both GLURP-R0 and -R2 antibodies. Importantly, the risk of malaria was markedly reduced for a given antibody level in homozygous (233AA) and heterozygous (233AC) individuals, whereas the 233CC genotype seemed to benefit much less from high antibody levels in terms of protection against malaria. The c.233A-allele encodes a substitution of the hydrophobic amino acid alanine (A) with the negatively charged aspartic acid (D) at position 78 of the FcγRIIB-NA2 protein resulting in the FcγRIIB-SH allo-type, previously associated with protection against malaria in this cohort [3]. The p.A78D substitution has been proposed to influence a ligand epitope in the membrane distal Ig-like domain of the receptor [14]. Neutrophils of individuals with the 233CC genotype may be much less capable of engaging IgG antibodies to trigger downstream cellular antiparasite multiplication mechanisms, thus allowing parasites to establish symptomatic infection. Although sample size may be a limiting factor as to how far the conclusions of this study reach, it is conceivable that given the central role of neutrophil-mediated mechanisms in malaria immunity [5] a genetic predisposition to a suboptimal neutrophil function would adversely influence risk of malaria. The statistical power for analyzing interactions was not very high in our study. This is reflected in a seeming loss of statistical significance when the Bonferroni–Holm multiple testing correction was applied (Supplementary Table 2). However, similar results and trends were observed for both antigens, which have been shown to be targets of cytophilic antibodies. Also the Bonferroni–Holm procedure applied to dependent tests yields systematically too large P values. Nonetheless, it would be interesting to investigate in a larger cohort whether this genotype effect modification was only GLURP antibody specific or common to other antigens known to be primary targets of cytophilic antibodies such as the merozoite surface protein 3. So far, attempts to explain discrepancies in antibody association from different sero-epidemiological studies have often implicated factors such as parasite antigenic polymorphisms, the lack of clearly defined endpoint of clinical malaria, and the short half-life of malaria-specific antibodies [15]. We speculate that conclusions from different malaria sero-epidemiological studies and vaccine trials may become more consistent and reproducible if the genetic background, particularly with respect to polymorphisms in genes known to influence antibody functionality, are adequately accounted for in the study population.

In conclusion, our data show for the first time that FCG3B-c.233C>A genotypes significantly modify the protective effect of antibody levels on the risk of malaria. This effect modification may influence the outcome of malaria sero-epidemiological and vaccine trial studies because the extent to which individuals benefit from the protective effect of high antibody titers seems dependent on the FCG3B-c.233C>A genotypes, which are often unaccounted for in such studies.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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