Haemoglobin C and S Role in Acquired Immunity against Plasmodium falciparum Malaria

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A recently proposed mechanism of protection for haemoglobin C (HbC; β6Glu→Lys) links an abnormal display of PfEMP1, an antigen involved in malaria pathogenesis, on the surface of HbC infected erythrocytes together with the observation of reduced cytoadhesion of parasitized erythrocytes and impaired rosetting in vitro. We investigated the impact of this hypothesis on the development of acquired immunity against Plasmodium falciparum variant surface antigens (VSA) encoding PfEMP1 in HbC in comparison with HbA and HbS carriers of Burkina Faso. We measured: i) total IgG against a single VSA, A4U, and against a panel of VSA from severe malaria cases in human sera from urban and rural areas of Burkina Faso of different haemoglobin genotypes (CC, AC, AS, SC, SS); ii) total IgG against recombinant proteins of P. falciparum asexual sporozoite, blood stage antigens, and parasite schizont extract; iii) total IgG against tetanus toxoid. Results showed that the reported abnormal cell-surface display of PfEMP1 on HbC infected erythrocytes observed in vitro is not associated to lower anti-PfEMP1 response in vivo. Higher immune response against the VSA panel and malaria antigens were observed in all adaptive genotypes containing at least one allelic variant HbC or HbS in the low transmission urban area whereas no differences were detected in the high transmission rural area. In both contexts the response against tetanus toxoid was not influenced by the β-globin genotype. These findings suggest that both HbC and HbS affect the early development of naturally acquired immunity against malaria. The enhanced immune reactivity in both HbC and HbS carriers supports the hypothesis that the protection against malaria of these adaptive genotypes might be at least partially mediated by acquired immunity against malaria.

INTRODUCTION

Red blood cell disorders including haemoglobin variants and thalassaemias provide with their unusually high prevalence and distribution in malaria endemic areas [1–3] the most compelling evidence of genetic factors controlling the susceptibility to any infectious disease in humans. Conclusive evidence exists on the protective role of Haemoglobin C (HbC; β6Glu→Lys) against clinical Plasmodium falciparum malaria [4–6]. Recently, an abnormal display of PfEMP1, an antigen involved in malaria pathogenesis, was reported [7,8] on HbAC and HbCC infected erythrocytes that showed reduced cytoadhesion and impaired rosetting in vitro. On this basis it has been proposed that HbC protection might be attributed to the reduced PfEMP1-mediated adherence of parasitized erythrocytes in the microvasculature. Given the reported observations, it could be hypothesized that HbC carriers may develop an altered immune response to PfEMP1. Intriguingly, an enhanced immune recognition of variant surface antigens (VSA) was initially observed in HbAS individuals by Marsh et al., [9] and further demonstrated in a study showing that the presence of HbAS genotype was associated with enhanced recognition of two randomly selected clinical isolates amongst Gabonese children [10]. A cohort study in Kenya lends further support to the hypothesis of an accelerated acquisition of immunity against mild clinical malaria in HbAS children <10 years [11].

The coexistence of HbC and HbS in a hyperendemic malaria context such as Burkina Faso provided a unique opportunity to explore this question in a comparative approach. In the present study, we compared the humoral response to PfEMP1 in individuals belonging to the Mossi ethnic group carrying different β-globin genotypes (AA, AC, CG, AS, SC, SS) living in urban and rural areas of Burkina Faso, West Africa by measuring: i) total IgG against a single VSA (variant surface antigen), A4U, and against a panel of VSA from severe malaria cases; ii) total IgG against recombinant proteins of P. falciparum sporozoite (CSP), blood stage (AMA1, EBA-175, MSP-119, MSP-2, MSP-3) antigens, parasite schizont extract, and iii) total IgG against a non malaria antigen, tetanus toxoid.

MATERIALS AND METHODS

Study population

Healthy individuals belonging to the Mossi ethnic group of Burkina Faso were recruited after oral informed consent was obtained. The study population was compared to a control group consisting of Healthy individuals belonging to the Mossi ethnic group carrying different β-globin genotypes (AA, AC, CG, AS, SC, SS) living in urban and rural areas of Burkina Faso.

Academic Editor: Neil Hall, University of Liverpool, United Kingdom

Received July 12, 2007; Accepted September 13, 2007; Published October 3, 2007

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Funding: This project was supported by the Wellcome Trust grant no 071626 (Advanced Training Fellowship in Tropical Medicine to FV). This work is also part of the activities of the BioMalPar European Network of Excellence supported by a European grant (LSH-CT-2004-503578) from the Priority 1 Life Sciences, Genomics and Biotechnology for Health in the 6th Framework Programme. FV is currently funded by the malaria Network sponsored by the Compagnia di San Paolo, Turin, Italy. The above mentioned funders had no role in the design and conduct of the study, in the collection, analysis, and interpretation, review, or approval of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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obtained during the dry seasons 2005/2006 at the “Centre Medical Saint Camille” of the capital city Ouagadougou, and in the villages nearby (Donsin, Kuiti, Nagbagré) of the Kadiogo district, as described in Table 1, within a survey of haemoglobinopathies approved by the Ethics Committee of the Saint Camille Medical Center according to the guidelines of the Ministry of Health of Burkina Faso. Haemoglobin genotype was determined by cellulose acetate electrophoresis (Helena). Parasite genomic DNA was extracted using QIAamp DNA Mini Kit (Qiagen). Plasmodium falciparum genotyping of gldap was carried out according to a standard protocol [12] to evaluate parasite positivity assessed by PCR at the time of sera collection.

**Antibody Assays**

Antibody responses against the parasite infected-erythrocyte surface antigens were tested using the method with modifications of Williams et al. [13] on 85 urban and 85 rural individuals including all haemoglobin genotypes which were processed in a single assay against αi a reference isolate A4U, derived by sequential selection for binding to the monoclonal antibody BC6, which is specific to the expressed A4U var gene, resulting in a population of infected erythrocytes expressing predominantly one specific PfEMP1 variant on their surface, A4U PfEMP1 [14] and αi a composite isolate (CI) derived by pooling a panel of wild isolates obtained from children presenting to the wards of Kilifi district hospital in Kenya with malaria at high parasitemia (T.H.

**FACS analysis**

Mature trophozoite stage parasitized RBC (pRBC) at between 3-5% parasitaemia were thawed from frozen culture using saline solutions according to a gradient from 12% to 0.9% in a suspension at 50% haematocrit with buffer (0.5% BSA/PBS). 1 ml of serum was pipetted into separate wells of a 96-well U-bottomed plate (Falcon, Becton Dickinson, USA) to which was added 11.5 ml of the infected pRBC cell suspension in 0.5%BSA/PBS and 10 µg/ml of ethidium bromide. The mixture was incubated at room temperature for 30 min, following which the cells were washed three times with 0.5%BSA/PBS, centrifuging at 1000 rpm for 3 min per wash. The cells were then re-suspended in 50 µl of carbonate buffer (15 mM Na2CO3, 35 mM NAHCO3, pH 9.3), and incubated overnight at 4°C. The mixture was then incubated in 200 µl/well of blocking buffer (1% skimmed milk in PBS/Tween) for 5 hours at room temperature. Antigen-coated wells were washed and incubated overnight at 4°C with test sera (1/500 dilution) at 100 µl/well. Unbound antibody was washed off, and 100 µl of secondary antibody (HRP-conjugated rabbit anti-human IgG, Dako Ltd.) diluted to 1/5000 in blocking buffer was added into each well and incubated for 3 hours at room temperature before detection with O-phenylenediamine (OPD, Sigma). The reaction was stopped with 25 µl/well of 2M H2SO4. The absorbance was read at OD 492nm using the SPECTRAFluor program, (XFLUOR4 Version: V 4.11) and analysed using Excel [Microsoft]. The coefficient of variation

| Table 1. Study samples of Mossi ethnic group of Burkina Faso. |
|-------------------------------------------------------------|
| **Geographic samples (EIR>100)** | Period of blood collection | Haemoglobin genotype |
|---------------------------------|-----------------------------|----------------------|
| Rural samples                   |                             |                      |
| Donsin                          | Dry season (April 2006)     | AA (16.0)            |
|                                 |                             | AC (11.7)            |
|                                 |                             | CC (9.0)             |
|                                 |                             | AS (8.5)             |
|                                 |                             | SC (13.0)            |
|                                 |                             | SS (18.0)            |
|                                 |                             | Total: 70            |
| Kuiti                           | Dry season (April 2006)     | AA (10.7)            |
|                                 |                             | AC (10.2)            |
|                                 |                             | CC (9.2)             |
|                                 |                             | AS (10.2)            |
|                                 |                             | SC (9.0)             |
|                                 |                             | SS (9.5)             |
|                                 |                             | Total: 48            |
| Nagbagré                        | Dry season (April 2006)     | AA (15.0)            |
|                                 |                             | AC (15.0)            |
|                                 |                             | CC (15.0)            |
|                                 |                             | Total: 3             |
| Urban samples (EIR>10-100)      |                             |                      |
| Ouagadougou                     | Dry season (January–May 2005)| 80 (30.0)           |
|                                 |                             | 10 (27.4)***         |
|                                 |                             | 40 (29.5)            |
|                                 |                             | 8 (19.2)³             |
|                                 |                             | Total: 140            |
| Ouagadougou                     | Dry season (January–May 2006)| 86 (29.0)³           |
|                                 |                             | 26 (24.5)³           |
|                                 |                             | 3 (32.5)³³           |
|                                 |                             | 3 (18.3)             |
|                                 |                             | Total: 126           |
| Total                           |                             | 152                  |
|                                 |                             | 133                  |
|                                 |                             | 28                   |
|                                 |                             | 55                   |
|                                 |                             | 15                   |
|                                 |                             | 4                    |
|                                 |                             | 387                  |

Individuals are indicated for each genotype and origin; age is given in brackets. recruited in December 2004; *age available for 50 subjects; **age available only for 5 subjects; *age available only for 29 subjects; ³age available only for 6 subjects; a*age available only for 63 subjects; ³³age available only for 22 subjects; ³³³age available only for 6 subjects.

doi:10.1371/journal.pone.0000978.t001
(CV) between duplicate wells was calculated in Excel (Microsoft) using the formula below to ensure accuracy:

\[
CV = \frac{\text{standard deviation}}{\text{mean OD of duplicate wells}} \times 100
\]

The mean OD of each duplicate was used as the final read-out. The cut-off for positive samples was determined by taking the mean plus 3 standard deviations of twenty (20) non-immune sera for all antigens. All samples were tested for each antigen and its conjugated molecule in a single assay to avoid any daily variation. OD values were log-transformed for normal distribution and differences in means of antibody levels and MFIs according to the different genotypes were tested by Kruskal-Wallis and pairwise comparisons by Two Sample T test. For all tests, P values of less than 0.05 were considered significant. Logistic regression was used to examine the relationship between antibody levels and the chosen variables: Hb genotype, age categories (1/9, 10/max years), gender and parasite positivity assessed by PCR. All analyses were carried out in STATA (StataCorp.1999, Release (9.2)).

RESULTS

Serological reactivities to \textit{P. falciparum} VSA

No differences in the Mean Fluorescence Intensity (MFI) according to the haemoglobin genotype were detected when looking at urban and rural samples all together. Further comparisons were carried out separately with the urban and the rural samples due to the different EIR (entomological inoculation rate) and mean age of the two subsets whose characteristics are described in Table 1. No differences were observed in the MFI of the different haemoglobin genotypes against the A4U isolate in both subsets, whereas we found significant differences in the urban sera (\(P = 0.04\) amongst all adaptive genotypes and HbAA; \(P = 0.02\) between HbAA and HbAC; \(P = 0.03\) between HbAA and HbAS) when testing the panel of composite isolates (CI) of severe malaria VSA from Kilifi (Figure 1).

Serological reactivities to \textit{P. falciparum} antigens and parasite schizont extract

Prevalence and levels of antibodies tested against all antigens was consistently higher in the villages due to higher exposure to malaria of the rural subset despite the relatively younger age (Table 2, Table 3). Within the urban samples higher levels of total IgG amongst genotypes containing at least one adaptive haemoglobin allele compared to HbAA were observed for most of the \textit{P. falciparum} antigens tested by ELISA, with some degree of statistical significance as measured by Kruskal-Wallis test and pairwise T-test (Table 2, Figure 2). Remarkably, the same observation of higher levels of IgG in all genotypes including HbC and HbS alleles was confirmed by CSP and schizont extract, both considered markers of exposure (Table 2, Figure 2). However, significantly higher levels of total IgG against \textit{P. falciparum} antigens were observed in the rural samples only against EBA-175 in the overall comparisons, and against AMA1 in the comparisons between HbAA versus HbAC, and HbAA versus HbCC (Table 2). Differences observed in the primary analysis were tested by logistic regression including Hb genotype, age categories (1/9, 10/max years), gender and parasite status confirming the role of adaptive genotypes for most of the tested antigens (Table 3). Finally, a non malaria antigen, tetanus toxoid, was tested revealing no differences in any of the subsets (data not shown).

Figure 1. Mean Fluorescence Intensity (MFI) of total IgG against \textit{Plasmodium falciparum} A4 Ultra parasite isolate (A4U) and a panel of Composite Isolates (CI) expressing VSA in urban samples of Mossi from Ouagadougou, Burkina Faso according to the different haemoglobin genotypes.

doi:10.1371/journal.pone.0000978.g001
DISCUSSION

Despite substantial evidence of protection against clinical malaria given by the haemoglobin variants HbC and HbS, the precise mechanism(s) are still under debate. A recently proposed mechanism suggested that the protection conferred by HbC may be attributed to the reduction of cytoadherence and impaired rosetting observed in relation to the altered display of PfEMP1 on HbCC erythrocytes [7,8] which raises the question of whether this would affect VSA recognition, and ultimately the immune reactivity against VSA. Taking on from this hypothesis we anticipated a difference in antibody levels of HbCC homozygous, possibly a reduction due to their impaired VSA expression. Also, an “immunological hypothesis” initially proposed for HbS has been supported by a number of studies in various epidemiological contexts although the functional mechanisms involved in the enhanced acquisition of natural immunity observed in HbAS.

Table 2. Total IgG means of log transformed OD values against several blood stage antigens (AMA1, EBA-175, MSP-119, MSP-2, MSP-3), circumsporozoite protein (CSP), and parasite schizont extract (PSE) of Plasmodium falciparum in urban and rural sera of Burkina Faso.

| Antigen | Prevalence | Pairwise comparisons |
|---------|------------|----------------------|
|         | Urban samples (N = 266) | AA vs AC | AC vs CC | AA vs CC | AA vs AS | Overall |
| AMA1    | 78%        | P = 0.001 NS         |         |         |         | P = 0.001 P = 0.0001 |
| EBA-175 | 73%        | P = 0.04 NS          |         |         |         | P = 0.03 P = 0.02 |
| MSP-119 | 67%        | NS P = 0.03 NS       |         |         | NS (P = 0.06)         |
| MSP-2   | 64%        | P = 0.001 NS         |         |         |         | P = 0.004 P = 0.0001 |
| MSP-3   | 70%        | NS NS NS             |         |         |         | PS = 0.02 NS         |
| CSP     | 65%        | P = 0.05 NS          |         |         |         | P = 0.001 P = 0.03 |
| PSE     | 74%        | NS P = 0.01 NS       |         |         |         | P = 0.01 P = 0.05 |
| Rural samples (N = 121) |         |         |         |         |         |         |
| AMA1    | 90%        | P = 0.03 NS          |         |         | NS       | NS NS |
| EBA-175 | 76%        | NS (P = 0.07) NS     |         |         | NS (P = 0.06) P = 0.05 |
| MSP-119 | 70%        | NS NS NS             |         |         | NS NS NS |
| MSP-2   | 82%        | NS NS NS             |         |         | NS NS NS |
| MSP-3   | 77%        | NS NS NS             |         |         | NS NS NS |
| CSP     | 68%        | NS NS NS             |         |         | NS NS NS |
| PSE     | 78%        | NS NS NS             |         |         | NS NS NS |

* doi:10.1371/journal.pone.0000978.t002

Table 3. Geometric means and 95% Confidence intervals in brackets of log transformed OD values against several blood stage antigens (AMA1, EBA-175, MSP-119, MSP-2, MSP-3), circumsporozoite protein (CSP), and parasite schizont extract (PSE) of Plasmodium falciparum in urban and rural sera of Burkina Faso.

| Antigen | AA | AC | AS | CC | SC | SS |
|---------|----|----|----|----|----|----|
| Urban Samples (N = 266) |    |    |    |    |    |    |
| AMA1    | 2.90 (2.78–3.03) | 3.14 (3.06–3.22) | 3.20 (3.10–3.31) | 3.27 (3.16–3.38) | 3.23 (3.05–3.41) | 3.40 (3.35–3.46) |
| EBA-175 | 2.75 (2.70–2.81) | 2.82 (2.78–2.87) | 2.84 (2.77–2.92) | 2.91 (2.79–3.04) | 2.82 (2.65–2.99) | 3.15 (3.05–3.25) |
| MSP-119 | 2.45 (2.34–2.58) | 2.56 (2.46–2.66) | 2.67 (2.54–2.81) | 2.83 (2.60–3.08) | 2.60 (2.38–2.85) | 2.81 (2.58–3.05) |
| MSP-2   | 2.97 (2.84–3.10) | 3.19 (3.13–3.26) | 3.22 (3.12–3.32) | 3.26 (3.15–3.38) | 3.38 (3.36–3.40) | 3.40 (3.35–3.46) |
| MSP-3   | 2.44 (2.35–2.53) | 2.50 (2.43–2.58) | 2.60 (2.50–2.71) | 2.55 (2.37–2.74) | 2.66 (2.38–2.97) | 2.52 (2.45–2.61) |
| CSP     | 2.74 (2.69–2.80) | 2.82 (2.77–2.87) | 2.88 (2.82–2.95) | 2.88 (2.75–3.01) | 2.85 (2.70–3.01) |__|
| PSE     | 2.50 (2.44–2.57) | 2.60 (2.56–2.65) | 2.64 (2.56–2.72) | 2.66 (2.55–2.77) | 2.63 (2.46–2.82) | 2.74 (1.26–3.95) |

Rural samples (N = 121) |    |    |    |    |    |    |
| AMA1    | 3.12 (3.04–3.20) | 3.27 (3.18–3.36) | 3.09 (2.54–3.76) | 3.31 (3.23–3.40) | 3.06 (2.59–3.62) | 3.39* |
| EBA-175 | 2.82 (2.74–2.90) | 2.94 (2.85–3.03) | 3.03 (2.76–3.34) | 2.95 (2.81–3.08) | 2.70 (2.28–3.20) | 3.16* |
| MSP-119 | 2.82 (2.60–3.06) | 2.54 (1.31–4.92) | 1.92* |         |         |         |
| MSP-2   | 2.82 (2.70–2.94) | 2.87 (2.75–2.99) | 2.82 (2.37–3.35) | 2.89 (2.57–3.25) | 2.88 (2.41–3.45) | 3.24* |
| MSP-3   | 2.57 (2.48–2.67) | 2.71 (2.58–2.84) | 2.52 (2.11–3.01) | 2.69 (2.41–3.01) | 2.86 (1.03–5.97) | 2.55* |
| CSP     | 2.80 (2.75–2.85) | 2.82 (2.77–2.86) | 2.88 (2.82–2.94) | 2.76 (2.73–2.79) | 2.77 (2.68–2.85) |__|
| PSE     | 2.55 (2.46–2.65) | 2.61 (2.49–2.74) | 2.72 (2.55–2.90) | 2.59 (2.48–2.70) | 2.56 (0.83–7.89) | 2.62* |

* single individual

* doi:10.1371/journal.pone.0000978.t003
remains elusive [9,10]. Therefore, we investigated haemoglobin phenotype-specific immune reactivity to a composite isolate expressing a wide range of *P. falciparum* VSAs in subjects resident in two different malaria endemic contexts of Burkina Faso. Results showed that the reported abnormal cell-surface display of PfEMP1 on HbC infected erythrocytes observed *in vitro* is not associated to lower anti-PfEMP1 humoral response *in vivo*. In fact, higher immune response against the *P. falciparum* VSA panel and several antigens were observed in all adaptive genotypes containing at least one allelic variant HbC or HbS in the low transmission urban area (Figure 1). Interestingly, cross-reactive antibody responses, i.e., those directed against erythrocyte surface expressed parasite antigens from heterologous parasite isolates are an important component of acquired immunity against malaria [17–19]. However, no differences were detected in the high transmission rural areas. Significantly higher levels of antibodies at several *P. falciparum* antigens were also observed in the same urban samples (Table 2, Table 3, Figure 2). These differences were not explained by age or parasite status at that time point as confounders for most of the antigens examined (Table 4), rather reflected the cumulative effect of a life-long exposure to *P. falciparum* malaria in the presence of a protective factor such as HbC and/or HbS. The enhanced immune reactivity in both HbC and HbS carriers supports the hypothesis that the protection against malaria of these adaptive genotypes might be at least partially mediated by acquired immunity against malaria.

![Figure 2](image_url)

*Figure 2. Means levels of total IgG against several blood stage antigens of* Plasmodium falciparum (*AMA1, EBA-175, MSP-119, MSP-2, MSP-3*) and parasite schizont extract in urban samples of Mossi from Ouagadougou, Burkina Faso according to the different haemoglobin genotypes. The horizontal lines in each box correspond to the median values, the lower edge of each box is the 25% ile and the upper edge is the 75% ile. The whiskers represent the range of the data beyond these percentiles, excluding outliers represented by dots.*

doi:10.1371/journal.pone.0000978.g002

| Antigen | Variable |
|---------|----------|
| Hb genotype | Age | Gender | Parasite + |
| AMA1 | P = 0.001 | NS | NS | NS |
| EBA-175 | NS | NS | NS | NS |
| MSP-119 | NS | P = 0.06 | NS | NS | NS |
| MSP-2 | P = 0.003 | NS | NS | NS |
| MSP-3 | NS | P = 0.08 | NS | NS | NS |
| CSP | P = 0.004 | NS | NS | NS |
| PSE | P = 0.01 | P = 0.01 | NS | NS |
| A4 MFI | NS | NS | NS | NS |
| CI MFI | P = 0.05 | NS | NS | NS |

doi:10.1371/journal.pone.0000978.t004

We believe that the discrepancy between results from urban and rural settings could be the consequence of saturated immunity in high transmission contexts. These observations emphasize the need, when studying candidates of genetic resistance/susceptibility to malaria and their underlying hypothesized mechanisms, to take into full account the epidemiological context as a potential confounder.

Although this study can not conclusively validate any of the different functional mechanisms proposed to explain the protection of haemoglobin variants, a consistently enhanced immune reactivity in both HbC and HbS adaptive genotypes suggests the idea of a convergence in terms of their impact on the acquisition of immunity against malaria. It has been already shown that the model initially proposed for explaining G6PD protection, by enhanced phagocytosis of ring-parasitized altered erythrocytes, fits well also in the case of HbS and β-thalassemia [20]. Thus, it may be possible that this mechanism may also be extended to the...
case of HbC. Further investigation in the rural areas so far examined, focusing on young children, especially below five years, is undergoing to unravel the modulation exerted by HbC (and HbS) on the onset of naturally acquired immunity to malaria.

ACKNOWLEDGMENTS

This work is dedicated to the memory of our colleague, Gaia Luoni (1970–2004). We are grateful to the study participants in Burkina Faso for their cooperation, to the laboratory staff at the Centre Medical Saint Camille of Ouagadougou, Burkina Faso and at KEMRI, Kenya for excellent technical support. We thank all the colleagues who kindly provided us with recombinant proteins of *Plasmodium falciparum*. Also, we are grateful to Lars Hviid and colleagues at CMP for stimulating discussions, and to prof. Coluzzi for his advice and encouragement. This article is published with the permission of the Director of KEMRI.

Author Contributions

Conceived and designed the experiments: TW KM DM PB DC FV. Performed the experiments: KT GW FO. Analyzed the data: KT FV GW FO. Contributed reagents/materials/analysis tools: GF JS TH GB PA IB. Wrote the paper: DM FV.

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