Comparative analysis of the Immunoglobulin G antibodies (IgG and IgG subclass) responses in children (≤15 years) with severe and uncomplicated malaria in Buea, South West region, Cameroon

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Abstract

Studies assessing the immunoglobulin G (IgG) antibody responses in severe malaria are not readily available. This study was designed to compare the IgG and IgG1-4 antibody responses in severe malaria and its major clinical presentations (cerebral malaria, severe malarial anemia and respiratory distress) in children (≤15 years) in Buea, Cameroon. In a hospital-based cross-sectional comparative study, children presenting for consultation at the outpatient department/Emergency unit of the Buea Regional Hospital were enrolled and assigned into one of three groups: severe malaria, uncomplicated malaria and negative controls. Baseline characteristics were determined; blood glucose level was measured by glucometer, complete blood count was performed using an automated heamatology analyser and participants were screened for malaria parasites by light microscopy and severe malaria was categorized based on WHO criteria. Total IgG and IgG1-4 antibodies were measured using standard ELISA with Plasmodium falciparum 19-KDa C-terminal region of merozoite surface protein 1 (P.fMSP-19) antigen as capture antigen. A total of 236 participants were enrolled comprising: 66 severe malaria, 70 uncomplicated malaria and 100 negative controls. The participants in the different groups were similar with regards to their ages (p=0.06) and gender (p=0.900). Children with severe malaria had significantly higher levels of anti-P.fMSP-19 IgG4 (p<0.0001) antibodies and significantly lower levels of anti-P.fMSP-19 IgG1 (p<0.0001) and IgG3 (p<0.0001) antibodies. There was no significant variation in the IgG antibody responses between the major clinical forms of severe malaria. The study finding of significantly higher levels of the non-cytophilic antibody IgG4 is suggestive of the role the antibody plays in the pathogenesis of severe malaria. Larger studies investigating how these immune effector cells vary in the major phenotypes of severe malaria are recommended.

Introduction

Malaria, arguably one of the most important infectious disease today, still constitutes a major public health challenge in the tropical and subtropical parts of the world. According to the World Health Organisation (WHO), there were 228 million cases and 405,000 deaths attributed to malaria globally in 2018.1 A disproportionate 93% and 94% of malaria cases and death respectively, occurred in the WHO African region.1 Five species of Plasmodium are known to cause malaria namely: Plasmodium falciparum, P. malariae, P. ovale, P. vivax and P. knowlesi. Plasmodium falciparum is the most virulent species and is the dominant species in the WHO African region, accounting for 99.7% of all malaria deaths globally.1 This may explain why the morbidity and case fatality rate attributed to malaria is highest in the WHO African region. Children age 5 years and below are most vulnerable group; in 2018, they accounted for 67% of all malaria deaths globally.1 Progression of P. falciparum malaria may lead to complications associated with severe malaria. The rate of severe malaria in malaria endemic areas varies considerably from 6.4% to 74.7%.2 The clinical spectrum of malaria in children usually ranges from asymptomatic infection with parasites to a febrile disease that may
evolve into a severe, life-threatening illness. The main clinical phenotypes of severe malaria include cerebral malaria, severe anaemia and respiratory distress (also referred to as acidotic breathing). Other less life-threatening presentations commonly observed at enrollment include: hyperparasitaemia, multiple or prolonged convulsions, hyperlactaeamia, circulatory collapse, hypoglycaemia, prostration, jaundice, persistent vomiting and intravascular haemolysis.

In Cameroon, malaria is still a major cause of morbidity and mortality especially in children. The disease accounts for 48% of all hospital admissions, 30% of all morbidity and 67% of childhood mortality per year. The entire Cameroonian population of over 22 million is at risk of malaria. Plasmodium falciparum is the predominant species causing malaria in Cameroon. Additionally, severe malaria cases (including cerebral malaria, severe anaemia and respiratory distress) are frequent in Cameroon.

Immunoglobulin G (IgG) antibodies are generally known to play vital roles in malaria immunity. The cytophilic antibodies (IgG1 and IgG3) are considered the most important IgG subclasses in conferring malaria immunity as they are capable of mediating the activation of leukocytes via binding to FcγRI and FcγRIII. Studies in malaria endemic areas have shown the predominance of IgG1 and IgG3 is associated with lower risks of malaria-related complications. Contrarily, the non-cytophilic antibodies especially IgG4 are known to be pathogenic and their presence correlates with the severity of malaria. A balance between cytophilic and non-cytophilic antibodies is therefore required for the development of effective immunity against malaria.

The current study uses the Plasmodium falciparum 19-KDa C-terminal region of merozoite surface protein 1 (P.fMSP-19) antigen as capture antigen in ELISA to measure the total IgG and IgG subclass responses in the target population. The merozoite surface proteins are involved in the initial attachment of the merozoite to the erythrocyte surface. P.fMSP-19 was selected over the other MSP-1 molecules because of the fine specificity of anti-MSP-19 specific antibodies, as well as its role in the protection against clinical malaria. Furthermore, a study demonstrated that MSP-19 specific antibodies are potent inducers of neutrophil antibody-dependent respiratory burst (ADR), which correlates with acquired clinical protection. In addition, studies have highlighted the role of the cytophilic antibodies (IgG1 and IgG3) in ADR. Understanding the role of the different IgG subclass antibodies in the pathogenesis of severe malaria is necessary to depict a clearer picture of the overall immunologic responses to malaria and in vaccine development. However, studies on the variation of IgG antibody responses in the different clinical phenotypes of severe malaria are not forthcoming. With this in mind, we designed this study to compare the IgG subclass antibodies responses in the different clinical presentations of severe malaria.

**Materials and Methods**

**Study area**

This study was performed at the Buea Regional hospital, located in Buea, Southwest region of Cameroon. The study area has been described in great details elsewhere. The prevalence of malaria and severe malaria in Buea has been reported as 33.0% and 13.8% respectively. In this area, over 95% of malaria has been reported to be caused by Plasmodium falciparum.

**Study design an duration**

This was a hospital-based cross-sectional comparative study conducted between March and June 2018.

**Sample size estimation**

The sample size was estimated using an effect size of 0.33 calculated from data obtained by Kwenti et al. The power of study 0.9 considering alpha 0.05, three groups and the ANOVA function in G*Power, obtained a total sample size of 120 (i.e. 40 per group).

**Participants and sampling**

Children (≤15 years) of both sexes, presenting for consultation at the Outpatient Department/Emergency Unit were consecutively enrolled upon provision of signed consent by their parents/guardians. Their vital signs were collected and they were examined by the consulting physician. Based on the laboratory confirmation of their malaria status, consented participants were enrolled and assigned into one of the following study groups: Severe malaria, uncomplicated malaria and negative controls. Excluded from the study were children who were already on treatment with an antimalarial drug and those for which there was evidence of other infectious causes of fever other than malaria.

**Laboratory analysis**

**Specimen collection**

Following aseptic techniques, about 4 ml of blood was collected from consented participants into EDTA and dry (non-anticoagulated) test tubes. Blood specimen in the EDTA tubes were used for the performance of the complete blood count and preparation of thick and thin blood films for malaria screening. Aliquots of the serum from the dry test tubes were transferred into Eppendorf tubes and stored at − 40 °C for the performance of ELISA.

**Performance of complete blood count**

Complete blood count (CBC) was performed using the Mindray® Auto haematology analyzer (BC-2800, Shenzhen Mindray Bio-Medical Electronics Co. Ltd, China). The white blood cell counts (WBC) were obtained from the CBC results and used in the estimation of the malaria parasite density.

**Measurement of blood glucose level**

Blood glucose level was measured with a glucometer (On Call® Plus Blood Glucose Meter, ACON Laboratories, Inc., USA) by a finger prick.

**Detection of malaria parasites**

The prepared blood films were air-dried and stained with 10% Giemsa (1 in 20 dilution) for 25–30 minutes and examined by light microscopy. Detection and quantification of malaria parasite was performed as previously described.

**Measurement of IgG responses in the study population**

The anti-Pf-MSP-19 IgG and IgG1-4 antibody responses were measured using a standard ELISA technique as earlier described. Briefly, ninety-six (96) well microtiter plates (Nunc MaxisorbTM, Denmark) were coated overnight at 4°C with 100µl MSP-19 antigen (expressed in Escherichia coli and purified to 95%) solution (1xPBS) at final concentration of 1µg/mL. The unbound antigens were removed by washing 3 times with 0.1% Tween/PBS washing buffer. After washing, 150µL/well of 3% skimmed milk powder in Tween/PBS was used to block the antigens for 8h at 4°C followed by plate washing as described above. After the second wash, sera samples (diluted 1 in 100) were added in duplicate, along with positive control serum (a pool of sera from...
eight adults in Muyuka with lifelong exposure to malaria) and 10 negative control sera from non-exposed German adults (kindly donated by Andreas Latz). The plates were then incubated at 37°C for 1 hour before washing as described above. Peroxidase conjugated goat anti-human IgG (Caltag Laboratories, US) for IgG (1/40) and mouse antihuman IgG1, IgG2, IgG3 and IgG4 (Arigo Biolaboratories Corp., Taiwan) for IgG1 (1/6000), IgG2 (1/4000), IgG3 (1/6000) and IgG4 (1/5000) were added (100µL/well) and incubated for 30 minutes at room temperature (RT). Afterward, 100µL/well TMB substrate was added and incubated for 15 minutes in the dark. The reaction was then stopped by adding 100µl/well of 0.2M sulphuric acid and absorbance was read at 450nm with an ELISA plate reader (BioTek® ELx800TM, BioTek Instruments, Inc., USA). Antibody responses were converted to Arbitrary Units (AU) with the aid of a standard curve derived from serial dilution (1:200, 1:400, 1:800, 1:1600, and 1:3200) of positive control sera for all test plates, with the absorbance of the lowest dilution corresponding to 100 AU.

Categorization of malaria into uncomplicated and severe forms

Malaria was categorized into uncomplicated malaria and the different clinical presentations of severe malaria according to the WHO scheme as described elsewhere.²,⁶

Statistical analysis

Data collected were entered into an Excel spreadsheet and analysed using the Stata® version 12.1 software (StataCorp LP, Texas, USA) and IBM® SPSS® Statistics version 20. Data were log-transformed prior to statistical analysis. The statistical tests performed included the Pearson’s Chi-square test for comparison of group proportions, the student’s T-test for the comparison of group means and the analysis of covariance (ANCOVA) for the comparison of mean antibodies titers adjusting for age. However, Kruskal-Wallis test was used to compare mean antibody titres between the major clinical phenotypes of severe malaria as they were not normally distributed. Statistical significance was set at p<0.05.

Results

Demographic and clinical characteristics of the study participants

In all, 236 participants were enrolled and comprised of 66, 70 and 100 severe malaria, uncomplicated malaria and negative controls respectively. The three groups did not differ in terms of participants ages (p=0.06) and gender (p=0.900) (Table 1).

The mean body temperature was highest in the severe malaria group (p<0.0001) meanwhile the mean haemoglobin concentration and blood glucose levels was lowest in the severe malaria group (Table 1).

Distribution of severe malaria phenotypes in the study population

The common features of severe malaria observed in decreasing order were: hyperpyrexia 44 (66.6%), severe malarial anaemia 42 (63.6%), impaired consciousness 37 (56.0%), respiratory distress 25 (37.9%), hypoglycaemia 19 (28.7%), hyperparasitaemia 17 (25.8%), multiple convulsion 17 (25.8%), circulatory collapse 15 (22.7%), cerebral malaria 13 (19.7%), frequent vomiting 13 (19.7%), coma 13 (19.7%), jaundice 12 (18.1%) and prostration 11 (16.7%). Among the three main clinical features of malaria (cerebral malaria, respiratory distress and severe malaria anaemia), overlap was most frequent between severe malaria anaemia and respiratory distress 18 (27.3%) (Figure 1).

Table 1. Summary of the demographic and clinical characteristics of the participants.

| Parameters                        | Severe malaria (n=66) | Uncomplicated malaria (n=70) | Negative controls (n=100) | p     |
|-----------------------------------|-----------------------|-----------------------------|---------------------------|-------|
| Age category (in years)           |                       |                             |                           |       |
| < 5                               | 37                    | 26                          | 56                        | 0.060 |
| 5 - 9                             | 13                    | 27                          | 24                        |       |
| ≥ 10                              | 16                    | 17                          | 20                        |       |
| Gender                            |                       |                             |                           |       |
| Male                              | 34                    | 34                          | 52                        | 0.900 |
| Female                            | 32                    | 36                          | 48                        |       |
| Mean temperature, °C (±SD)        | 38.4 (0.91)           | 37.7 (0.85)                 | 36.8 (0.84)               | <0.0001|
| Mean haemoglobin concentration, g/dl (±SD) | 7.4 (0.6) | 9.6 (1.3)                  | 11.4 (1.2)                | <0.0001|
| Mean blood glucose, mmol/L (±SD)  | 5.9 (0.6)             | 6.3 (0.5)                   | 6.5 (0.5)                 | <0.0001|

Figure 1. Venn diagram showing the overlap (proportions) of the major clinical subgroups of malaria in the study population. Proportions were obtained by dividing the cases by the total number of severe malaria (66). SMA: severe malarial anaemia; CM: cerebral malaria; RD: respiratory distress; UM: uncomplicated malaria.
Comparison of the IgG levels between uncomplicated malaria, severe malaria and controls groups

Comparison of the levels of anti-Pf-MSP-1_19 IgG and IgG1-4 antibody responses between children with uncomplicated, severe malaria and negative controls adjusting for age and gender, revealed that children with severe malaria had significantly lower levels of anti-Pf-MSP-1_19 IgG, IgG1 and IgG3 meanwhile IgG4 was significantly higher in children with severe malaria (Table 2).

Comparison of the anti-Pf-MSP-1_19 IgG and IgG1-4 antibody responses between the major phenotypes of severe malaria

Comparison of the anti-Pf-MSP-1_19 IgG and IgG1-4 antibody responses levels between the three major phenotypes of severe malaria (i.e. cerebral malaria, severe malarial anaemia and respiratory distress) revealed no significant differences adjusting for age and gender (Figure 2).

Table 2. Comparison of the anti-PfMSP-119 IgG and IgG1-4 antibodies between uncomplicated malaria, severe malaria and control groups.

| Antibodies | Type of malaria | n  | Mean ± SD          | p         |
|------------|-----------------|----|-------------------|-----------|
| IgG        | Controls        | 100| 2.27±0.368        | <0.0001   |
|            | Uncomplicated   | 70 | 1.95±0.477        |           |
|            | Severe          | 66 | 1.64±0.485        |           |
|            | Controls        | 100| 2.27±0.375        | <0.0001   |
|            | Uncomplicated   | 70 | 1.90±0.526        |           |
|            | Severe          | 66 | 1.36±0.485        |           |
|            | Controls        | 100| 1.27±0.366        |           |
|            | Uncomplicated   | 70 | 1.47±0.501        | 0.4914    |
|            | Severe          | 66 | 1.42±0.498        |           |
|            | Controls        | 100| 2.26±0.392        | <0.0001   |
|            | Uncomplicated   | 70 | 1.86±0.517        |           |
|            | Severe          | 66 | 1.32±0.469        |           |
|            | Controls        | 100| 1.23±0.433        | <0.0001   |
|            | Uncomplicated   | 70 | 1.50±0.513        |           |
|            | Severe          | 66 | 1.94±0.485        |           |

Figure 2. Variation in the levels of anti-Pf-MSP-119 IgG and IgG1-4 antibody responses between the three major phenotypes of severe malaria (Cerebral malaria [CM], severe malarial anaemia [SMA] and respiratory distress [RD]). There were no significant differences in the levels of the different IgG subclasses between the major phenotypes of severe malaria.
Discussion

Several studies have demonstrated the role of IgG antibodies in protection against malaria and the severe disease.\textsuperscript{18-21} However, an extensive literature search revealed only one study by Lorrorati \textit{et al.}\textsuperscript{24} that had assessed the role of the different IgG subclass antibodies in the protection against the three major phenotypes of severe malaria, \textit{i.e.} cerebral malaria, respiratory distress and severe anaemia malaria, in the same study. This places a void in our understanding of the immunological responses in severe malaria. This study was therefore designed to bridge this knowledge gap.

The current study revealed that severe malaria patients had significantly higher levels of anti-P.f.MSP-1\(_{19}\) IgG4 and lower levels of the anti-P.f.MSP-1\(_{19}\) IgG, IgG1 and IgG3 antibodies compared to uncomplicated malaria and negative controls. The non-cytophilic antibodies especially IgG4 are known to be pathogenic and their presence correlates largely with the severity of malaria.\textsuperscript{18,22,23} In addition, the finding of higher levels of anti-P.f.MSP-1\(_{19}\) IgG1 and IgG3 in participants with uncomplicated malaria and negative controls compared to those with severe malaria further attest to their protective role against severe malaria. These findings are in line with that of a similar study performed in Brazil.\textsuperscript{24}

To the best of our knowledge, this is one of the first study assessing the IgG antibody responses in severe malaria in children residing in a high transmission setting. Malaria transmission in the study area could be described as hyperendemic to holo-endemic, and children and pregnant women are most vulnerable.\textsuperscript{38,40} Comparative analysis of the IgG antibodies titers in the three major clinical presentations of severe malaria (\textit{i.e.} cerebral malaria, respiratory distress and severe anaemia malaria) revealed no significant differences. This could be due to the fact that in reality, there is hardly a situation where you have only one of the phenotypes presenting at any one time. It is therefore not uncommon to have malaria patients presenting with cerebral malaria and severe anaemia and/or respiratory distress or combinations of any of the other clinical phenotypes as seen in the current study. The fewer number of severe malaria cases in the current study may also have influenced the outcome of our analysis. This constitutes a major limitation to our study. Larger studies are therefore required to provide a clearer picture.

Conclusions

This study revealed significantly higher levels of anti-P.f.MSP-1\(_{19}\), IgG4 antibody and lower levels of the total IgG and cytotoxic IgG1 and IgG3 antibodies in severe malaria patients, suggestive of the role of the IgG4 in the pathogenesis of severe malaria. However, the study did not demonstrate any significant differences in the total IgG and IgG1-4 responses in the major phenotypes of severe malaria (cerebral malaria, respiratory distress and severe malaria anaemia). This observation may have been influenced by the small sample of severe malaria in the current study. Larger studies investigating how these immune effector cells vary in the major clinical presentations of severe malaria are recommended.

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