Increased Complement Deposition on Red Blood Cells in Children with Sickle Cell Trait

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Authors' contributions

This work was carried out in collaboration between all authors. Author WO helped in study design, conducted all experiments, helped in data analysis and drafted the manuscript. Author JAS designed the study, directed the work, drafted the manuscript, helped in data analysis and data interpretation. Author BBAE helped in study design, data analysis, data interpretation and helped in drafting of the manuscript. Author JRA helped in study design, data analysis, data interpretation and helped in drafting of the manuscript. Author BG helped in study design, data analysis, data interpretation and helped in drafting of the manuscript. Author MMO helped in study design, data analysis, data interpretation and helped in drafting of the manuscript. Author COO helped in study design, data analysis, data interpretation and helped in drafting of the manuscript. Author SMOG helped in study design, data analysis, data interpretation and helped in drafting of the manuscript. All authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Aims: Immune-mediated mechanism, such as deposition of complement (C3b) on erythrocytes leading to enhanced receptor-mediated uptake by macrophages has been proposed to contribute

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partly to the destruction of non-infected cells leading to anaemia. The extent of complement deposition on RBC (red blood cells) may therefore influence an individual's resistance or susceptibility to severe malarial anaemia. Our objective was to see if RBC of sickle cell trait individuals have increased susceptibility to deposition of complement *in vivo*. Under oxygenated and deoxygenated conditions, cytofluorometry was used to determine susceptibility of RBC from individuals with normal haemoglobin and those with heterozygous sickle cell trait to complement deposition.

Methods: Children aged 0-192 months (n=116) were enrolled in the nested case controlled study and were stratified into HbAS (n=47) and HbAA (n=69). The 47 HbAS individuals were matched to the 69 HbAA individuals of similar age (± 2 months or ± 24 months for those below or more than 192 months, respectively) at a ratio of 1:1 or 1:2. We measured the red cell C3b by flow cytometry under normal and reduced oxygen saturation. Individuals who were positive for malaria were treated and blood was collected when they were free of parasitemia. Analysis of variance was used to identify independent variables associated with the complement C3b positive red cells and Hb level.

Results: The mean complement C3b-positive cells for the HbAS was significantly higher than HbAA (P=0.0191). This was also true when this was repeated under deoxygenated conditions (P=0.00065). When the study volunteers were grouped by age cohorts into 0-12, 13-48 and 49-192 months, it was noted that generally; the mean complement C3b positive red cells was higher in the HbAS compared to HbAA but was not statistically significant. Under deoxygenated conditions, the trend was the same. However, between the ages of 49-192 months, the difference was statistically significant.

Conclusion: Increased complement C3b deposition on red cells of HbAS cells may predispose the HbAS individuals to increased RBC destruction and therefore protection from severe manifestations of malaria.

1. BACKGROUND

Malaria infection is a significant cause of anaemia, particularly in children living in areas of high transmission [1]. The pathogenesis of *P. falciparum* malarial anaemia is complex and cannot be explained solely on the basis of haemolysis of parasitized red blood cells as often the anaemia is disproportionate to the level of parasitaemia. In addition to the destruction of parasitized erythrocytes by rupture of cells after completion of the parasite's intra-erythrocytic life cycle as well as opsonization and clearance of intact infected RBC [2], increased clearance of uninfected erythrocytes, has been proposed to contribute significantly to the development of malarial anaemia [2,3]. The mechanisms responsible for clearance of uninfected erythrocytes in malaria have not been clearly defined. Several factors including immune-mediated mechanism, such as deposition of complement (C3b) on erythrocytes leading to enhanced receptor-mediated uptake by macrophages has been proposed to contribute partly to the destruction of non-infected cells leading to anaemia [2]. Therefore, the extent of complement (C3b) deposition on red cells may influence an individuals' resistance or susceptibility to severe malarial anaemia.

1.1 Complement Regulatory Proteins, Immune Complex Binding Capacity and Immune Complex Deposition on Erythrocytes

Severe anaemia is one of the most lethal complications in children infected with *P. falciparum*. The pathogenesis of this anaemia is not completely understood. It has been indicated that the degree of red blood cell (RBC) loss in malaria cannot be explained entirely by the direct destruction of RBC by the parasite [4,5]. However, the destruction of uninfected red cells which also takes place contributes significantly to the anaemia [3]. Indeed, several investigations have reported decrease in life span of uninfected red cells in malaria animal models [4,5] and patients with *P. falciparum* [2]. Moreover, a mathematical model of severe malarial anaemia has revealed that with each lysed infected erythrocyte ; a further 8.5 uninfected erythrocytes are destroyed [3]. Complement is activated during malaria infection [6] and C3d which is one of the molecules implicated in the removal of

senescent red cells via erythrophagocytosis have been detected on red cells of children with severe malaria [7,8].

Erythrocye Complement Regulatory proteins CR1 and CD 55 are important in the pathogenesis of severe malaria and their level of expression vary with age; being low in young children and increases with age [9]. In sickle cell trait individuals, the levels of these complement regulatory proteins were however comparable in the younger age groups but beyond the age of 49 months and this was thought to be a factor in protecting those with HbAS beyond 49 months of age [10]. On the other hand, the immune complex binding capacity for the HbAS was significantly higher than that of HbAA under both normal and deoxygenated conditions. The levels were lowest in the 7-12 months age category; pointing to the fact that the protection afforded by HbAS against severe manifestations of malaria may be partly due to higher immune complex binding capacity (ICBC) in the HbAS cells compared to HbAA cells [11]. Recently, Odhiambo and colleagues [12] demonstrated deposition of the opsonin C3b on red cells of patients with malaria; thus suggesting a role for complement-mediated damage of RBC.

The factors that contribute to reduced susceptibility to anaemia in heterozygous sickle cell traits are unclear. This study aimed to determine if there are differences in susceptibility to C3b deposition between red cells of individuals with normal haemoglobin and those with sickle cell trait.

2. MATERIALS AND METHODS

2.1 Study Site and Design

This study was done in Kombewa division, Kisumu West District, Nyanza Province in Western Kenya. This area borders Lake Victoria and has previously been used as a site for many epidemiological studies in both adults and children [13,14]. Kombewa has a population of about 65,000 people. Malaria transmission in this area occurs all year round with peak seasons following the long rains (March to May) and the short rains (October to December). The annual inoculation rates are estimated to be 31.1 infective bites per person per year [14].

The rest of the site details and study design have been previously reported elsewhere [11,15].

2.2 Study Population

This was a cross-sectional survey with nested case-control study. It was part of a larger study entitled "Changes in Erythrocyte Immune Complex Binding Capacity and Complement Sensitivity with Age in Populations with Different Malaria Risks". It was open to healthy male and female aged 0 to 45 years who were residents of Kombewa Division of Kisumu West District and was conducted between October and December 2004. The potential participants were assessed for any acute or chronic illness which could interfere with the parameters under investigation. In cases of an acute illness, the potential participants were assessed, treated and asked to come again for re-evaluation. At re-evaluation, the potential participants were enrolled when they were deemed well. Haemoglobin electrophoresis was carried on blood from all participants. Individuals who were less than 192 months and HbAS positive were identified and matched by age (±2 months or ±24 months for those below or more than 192 months, respectively) at a ratio of 1:1 or 1:2 with those with HbAA and these formed the nested case control cohort.

Forty seven (47) HbAS individuals aged 0-192 months were matched to 69 individuals with HbAA of similar age (±2 months or ±24 months for those below or more than 96 months, respectively) at a ratio of 1:1 or 1:2. Acute and chronic conditions known to interfere with the parameters under investigation such as complement regulatory proteins formed the exclusion criteria [16-21]. In cases of an acute illness, the potential participants were assessed, treated and asked to come again for re-evaluation. At re-evaluation, the potential participants were enrolled when they were deemed well. For more details see authors previous publications [11,15].

2.3 Ethical Consideration

Recruitment of study participants and procedures were in accordance with all applicable regulations. Informed consent was obtained from all participants or parents/guardians of children. This study was reviewed and approved by the Kenya National Ethical Review Committee of the Kenya Medical Research Institute and by the Human Subjects Research Review Board of the Office of the Surgeon General, U.S. Army.

2.4 Deoxygenation of the RBC for Assay

An equal amount of RBC in wash buffer was added to freshly prepared disodium hydrogen phosphate (Na2HPO4, FW 142g) 0.114M and sodium dithionite (Na2S2O4 FW 174.1g) 0.114M at a ratio of 2:3, filter sterilized through a 0.22µm filter [22]. The disodium hydrogen phosphate was prepared from a stock solution while the sodium dithionite was prepared fresh every day. The RBC were incubated at 37ºC for 1 hour and then washed twice with wash buffer before running the assays side by side. This duration of treatment with the dithionite was found to give the maximal sickling for HbAS RBC. This procedure was done to see the effect of reduced oxygen saturation on the parameters under investigation.

2.5 Measurement of C3b Deposition on Red Cells

All centrifugation steps were at x500g for 5 min. Rabbit polyclonal anti-C3a [negative control antibody, [Nordic immunological laboratories, Tilburg, The Netherlands)] and anti-C3b (Accurate) were pre-adsorbed x3 by adding a 1:50 dilution of antibody in phosphate buffered saline (PBS) pH 7.4 to an equal volume of packed pre-washed erythrocytes from the normal standard control. The cells were incubated for 1 hr at 37ºC with constant rocking followed by centrifugation. The pre-adsorbed antibody was frozen at -20ºC in single use aliquots. Pre-washed freshly thawed erythrocytes (100µl) at 1% hematocrit in Alsever's buffer was added to wells of a 96-well plate and resuspended in 50µl of pre-adsorbed rabbit anti-C3b, anti-C3a or in PBS (unstained control), and incubated for 10 minutes at 37ºC. After two washes in PBS, the cells were resuspended in 1:50 anti-rabbit PE (Sigma Aldrich) for 30 minutes at room temperature, washed twice, and resuspended again in PBS then acquisition was carried out. The %C3b –positive cells were calculated by Overton subtraction of the baseline C3a histogram from the baseline C3b histogram [23].

2.6 Statistical Analysis

Statistical analyses were performed using SPSS for windows version 16.0 software (SPSS Inc, Chicago, IL, USA). The mean complement C3b deposition for the HbAS and HbAA under normal and reduced Oxygen Saturation per red cell data and the mean complement C3b deposition for the HbAS and HbAA for each age cohort are presented graphically for each age group as box plots, where the box represents boundaries between the 25th and 75th percentile, the line through the box represents median and whiskers the 10th and 90th percentile limits. Analysis of variance (ANOVA) was used to detect differences across age groups adjusting for factors and covariates. The independent samples t-test was used for comparisons of normal continuous data between two groups, while the Chi-square (χ2) and Mann-Whitney U tests were utilized to examine differences between proportions and for pair wise comparisons of medians, respectively. Bivariate logistic regression analysis was carried out to determine the Odds Ratio (OR) and the 95% confidence interval (CI) for mean complement C3b deposition for the HbAS and HbAA per erythrocyte and mean mean complement C3b deposition for the HbAS and HbAA HbAS and HbAA. The General Linear Method (GLM) was used to test between subject effects. The Chi-square (χ2) test was used to compare proportions across groups. All tests were two-sided with $\alpha \leq 0.05$.

3. RESULTS

3.1 Differences in complement C3b Deposition between HbAS and HbAA Red Cells under Both Normal and Reduced Oxygen Saturation

The complement C3b deposition on RBC before and after deoxygenation with sodium dithionite for HbAS and HbAA cells are presented. The data are presented as box-and-whisker plots. For each group, the horizontal line in the middle of the box marks the median of the sample. The box represents the interquartile range and the central 50% of the data falls within the range of the box. The whiskers are the vertical lines extending up and down from each box and they represent the upper and the lower 25% of the data (Fig. 1).

The mean percentage C3b-positive cells for the HbAS cells (52.51±SD=6.379) was higher than HbAA cells (48.12±SD=11.59). This difference was statistically significant P=0.0191. When the cells were deoxygenated, the mean percentage C3b-positive cells for the HbAS cells (52.66±SD=7.789) was again higher than HbAA cells (48.29±SD=8.131). This difference was again statistically significant P = 0.00065. See Fig. 1 and Table 1.

3.2 Differences in Complement C3b Deposition between HbAS and HbAA Red Cells in Each Age Cohort

Under normal oxygen conditions, the percentage C3b deposition was observed to be higher in HbAS than HbAA in all the age cohorts. The mean complement C3b deposition for the age cohorts 0-12, 13-48 and 49-192 were consistently higher for HbAS compared to HbAA but these were not statistically significant P

values respectively 0.1515, 0.2801 and 0.3153 (Fig. 2).

Although the complement C3b deposition after treatment with sodium dithionate was generally higher for the HbAS red cells compared to HbAA red cells, this was not significant in the age cohorts 0-12 and 13-48; P =0.0843 and 0>2643 respectively. For the age group 49-192 months, the complement C3b deposition was significantly higher for the HbAS compared to the HbAA red cells, P =0.0407 (Fig. 3).
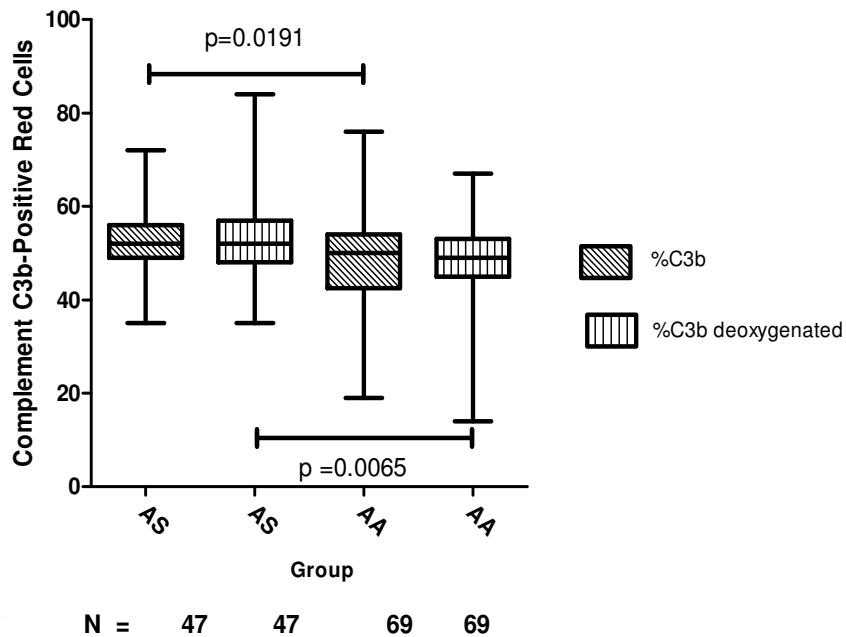

Fig. 1. Percent C3b Deposition on Erythrocytes of HbAS and HbAA under normal and reduced Oxygen Saturation

Table 1. Demographic, clinical and laboratory characteristics of the study population

Characteristic	HbAA (n = 69)	HbAS (n = 47)	P
Gender			
Male n (%)	28 (54.9)	23 (45.1)	0.447[a]
Female n (%)	41 (63.1)	24 (36.9)	
Mean Age in months (95% CI)	56.6 (37.3-75.9)	98.1 (53.5-142.8)	0.059[a]
Haemoglobin levels in g/dL (95% CI)	10.7 (10.3-11.2)	10.9 (10.5-11.4)	0.548[a]
Mean complement C3b deposition /Erythrocyte (95% CI)	48.12 (45.33-50.90)	52.51 (50.64-54.38)	0.00191[b]
Mean complement C3b deposition deoxygenated (95% CI)	48.29 (46.34-50.24)	52.66 (50.37-54.95)	0.0065[b]
Numeric *P. falciparum* read			
Negative n (%)	38 (61.3)	24 (38.7)	0.672
Positive n (%)	31 (57.4)	23 (42.6)	

Data are presented as means (95% CI). Children (n=116) were categorized according to the haemoglobin types into either HbAA (n=69) or HbAS (n=47). a: Statistical significance determined by the Chi-square analysis. b: Statistical significance determined by Mann-Whitney U test
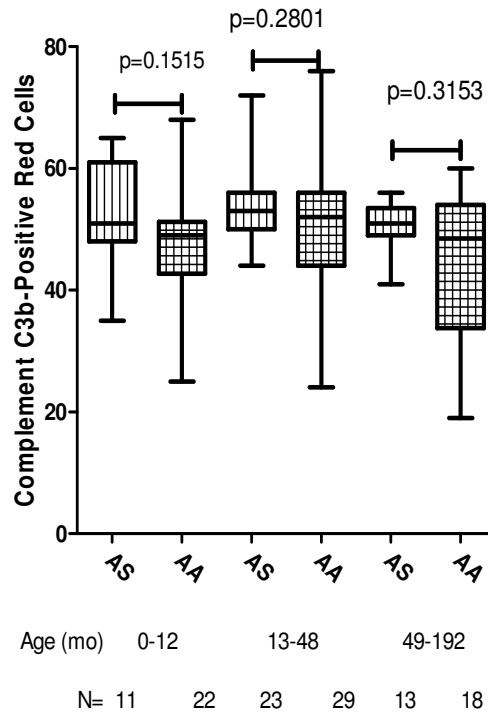
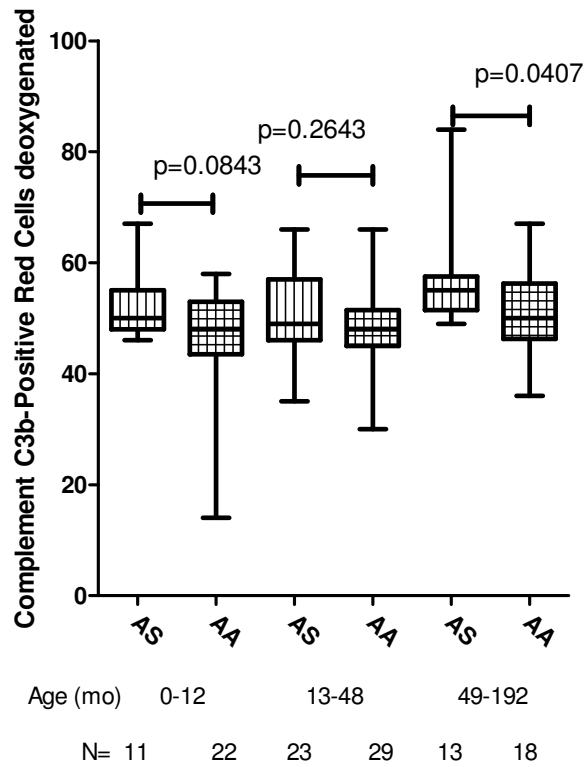**Fig. 2. Complement C3b Deposition for the various Age Cohorts by Haemoglobin Type**

Fig. 3. Complement C3b deposition on erythrocyte of individuals with HbAS and HbAA in each age cohort under reduced oxygen saturation

4. DISCUSSION

Malaria infection leads to complement activation and C3b deposition on red cells [4,24]. Red cells of individuals with low complement regulatory proteins were found to be more susceptible to C3b deposition compared to controls [12]. Moreover, the pattern of C3b deposition on red blood cells has been shown to be opposite that of CR1 levels.

In the present study, C3b deposition was lowest in the 0-6 month age cohort probably due to the corresponding high levels of complement regulatory proteins in this age cohort. This high level of complement regulatory proteins in the neonates may make these children more equipped to handle IC formation. This can explain their reduced susceptibility to complement deposition and therefore protection against severe manifestations of malaria for example severe malarial anaemia. This is in agreement with earlier studies which found similar results [12] and this can explain their reduced susceptibility to complement deposition. This is in agreement with earlier studies which found similar results [12].

This study showed that the mean %C3b deposition for the HbAS was significantly higher than those for HbAA RBC under both normal and reduced oxygenation. This could translate to increased susceptibility of these cells to immune complex destruction which would lead to mopping of these cells especially if infected with malaria. This would in turn lead to destructions of the parasitized cells and can be a factor in the partial protection of HbAS cells from severe manifestations of malaria for example severe malarial anaemia. When the volunteers were grouped into 0-12, 13-48 and 49-192 months age cohorts, the same general trend was maintained but only achieved significance in the 49-192 months age group. It is possible that the general increase in complement deposition increased with age leading to greater protection as the children grew older. These data suggests that differences in susceptibility to C3b deposition between HbAS and HbAA erythrocytes may not be an important factor that determines protection from severe malarial anaemia in individuals with sickle cell traits.

In this study, deoxygenation did not result in any significant difference in complement C3b deposition between the HbAS and HbAA cells.

For the diagnosis of HbAS, we used Hb Electrophoresis as opposed to high performance liquid chromatography (HPLC). The HbAS individuals have varied haemoglobin S content which could potentially impact on the complement C3b deposition. The HPLC would have given us the percent Hb S content of the cells but due to limitations with finances, this was not possible. It would have been interesting to correlate the Hb S content with complement deposition.

5. CONCLUSION

In conclusion, we report for the first time that the mean %C3b copy numbers per erythrocyte is higher in the HbAS than HbAA individuals both under normal and under deoxygenated conditions. When the study volunteers were grouped by age cohorts into 0-12, 13-48 and 49-192 months, it was noted that in general; the mean complement C3b positive red cells was higher in the HbAS compared to HbAA but this was not statistically significant. Under deoxygenated conditions, the trend was the same but between the ages of 49-192 months, this difference was statistically significant. These results are in agreement with earlier results which found that the level of CD55 was greater in HbAS RBC than in HbAA RBC between 49-192 months of age. [11,15]. All these could apparently have an effect on the immune complex binding capacity of these cells and may partially explain the protection afforded by the HbAS cells against severe manifestations of malaria. These results taken together may translate into protection of the HbAS individuals against severe manifestations of malaria.

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DISCLAIMER

The opinions or assertions contained herein are the private views of the authors, and are not to be construed as official, or as reflecting true views of the Department of Defense.

COMPETING INTERESTS

There is no conflict of interest for any of the authors of the manuscript due to commercial or other affiliations. The study was approved by the

ethical and scientific review committees at the Kenya Medical Research Institute and the institutional review board at the Walter Reed Army Institute of Research.

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