Sickle Cell Trait (HbAS) is Associated with Increased Expression of Erythrocyte Complement Regulatory Proteins CR1 and CD55 Levels in Children

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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 MMO 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.

Research Article

ABSTRACT

Aims: Erythrocyte complement regulatory proteins, complement receptor 1 (CR1) and decay accelerating factor (CD55) protect red blood cells (RBCs) from complement mediated damage by controlling complement activation cascade and potentially protect RBCs from complement mediated damage that may occur when immune complexes are

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formed following malaria infection. Given the important role of RBCs in regulation of complement activation, we considered the competence of sickle cell trait RBCs in these functions.

Methods: Children (age 0-192 months; n=116) were enrolled in a nested case controlled study conducted in Kombewa Division, Kisumu west District between October and December 2004. Based on hemoglobin (Hb) type, children were stratified into those with 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. Circulating CR1 levels and CD levels were quantified using a FACScan cytometer under normal and reduced oxygen saturation.

Results: The mean CR1 copy numbers per RBC was comparable in the two groups. However, between the ages of 49-192 months, the mean CR1 copy numbers per erythrocyte was significantly higher in children who had HbAS compared to those with HbAA (*P*=0.0332). The mean CD55 levels were comparable between the two groups but after deoxygenation, the mean CD levels in RBCs of individuals with HbAS was significantly higher than in the HbAA (*P*=0.011).

Conclusion: The mean CR1 and CD55 copy numbers per RBC were comparable between the two groups under normal and reduced oxygen saturation. Beyond the age of 49 months, the CR1 copy numbers was higher in the HbAS compared to HbAA and this was also true for CD55 levels under deoxygenated conditions. Taken together, these results demonstrate that in the younger age groups, the protection afforded by HbAS against severe manifestations of malaria may be due to other factors other than complement regulatory proteins but beyond the age of 49 months, this protection may be partly due to the high CR1 copy numbers in the HbAS individuals.

1. BACKGROUND

The erythrocyte complement regulatory proteins, Complement Receptor-1 (CR1), Decay Accelerating Factor (DAF/CD55) and Membrane Inhibitor of Reactive Lysis (MIRL/CD59) are important in protecting RBCs from complement mediated damage and in controlling the complement activation cascade. Erythrocyte CR1 and CD55 are important determinants of malaria susceptibility [1-3].

Studies carried out in Western Kenya where malaria is holoendemic and malarial anaemia is the main manifestation of severe malaria in children have suggested that low CR1 and CD55 levels on red cells of severe malarial anaemia cases increase their susceptibility to phagocytosis and complement mediated lysis [2,3]. The CR1 and CD55 levels were shown to be correlated with treatment and transfusion and remained high long after the maximum expected lifespan of the donor RBCs suggesting that these changes were acquired [2,3]. In Thai patients with severe malaria, a significant higher frequency of the Low expression genotype [LL] was observed. The LL genotype is associated with decreased CR1 copy numbers per erythrocyte and this may result in decreased clearance rate of immune complexes from the circulation which then predisposes one to malaria through increased deposition of immune complexes on red cells [4].

There is epidemiologic evidence that children with sickle cell trait are protected from severe manifestations of malaria in malaria endemic areas [5-7]. HbAS has been shown to protect against severe malarial anaemia, high density parasitemia and to be associated with

reduced all cause mortality between the ages of 2 to 16 months [8]. This also happens to be the age when children have the highest episodes of severe malarial anaemia [8]. The balanced polymorphism in the HbS-malaria relationship is thought to be maintained by higher mortality risk of HbAAs due to malaria and high mortality risk of HbSSs caused by complications of HbSS [9]. The HbS allele apparently does not prevent infection but results in impaired entry and growth of the parasites during the erythrocytic stage of development [10-13], enhanced removal of the parasitized variant RBCs [14] and reduced rosette formation [15]. When heterozygote's are infected, the merozoites, which have a high metabolic rate, consume a lot of oxygen and this leads to sickling under low oxygen tension. The spleen removes these sickled cells before the micro-organisms have a chance to produce a large infectious population in the body. It is this selective heterozygote advantage that maintains HbS gene at a higher level in malarial than in non-malarial environments [9,14]. The prevalence of the sickle cell trait in Western Kenya is estimated to be approximately 17.4% [8]. This high level of sickle cell carrier state in this region is thought to be maintained by the high malaria prevalence [9].

1.1 Malaria, Sickle Cell Trait and the Complement System

Erythrocyte complement regulatory proteins, CR1, CD55, and CD59 may protect RBCs from complement mediated damage that occurs when immune complexes are formed during malaria infection [3,16]. Children with severe malaria associated anaemia have been shown to have reduced immune complex binding capacity and increased susceptibility of their red cells to immune complex deposition and this may predispose their erythrocytes to complement mediated damage and phagocytosis [17]. On the other hand, HbAS individuals have been shown to have fewer episodes of severe malaria associated anaemia, reduced risk of anaemia episodes and reduced risk of high density parasitemia than their counterparts with HbAA [8].

Severe *P. falciparum* malaria leads to the formation of immune complexes in the peripheral circulation and this increased circulation of immune complexes has been associated with the development of severe anaemia [2,18]. CR1 and CD55 protect RBCs from complement mediated damage that can occur when immune complexes are formed during malaria infection [16].

Given the important role of RBCs in removal of immune complexes from circulation and in regulation of complement activation, it is therefore important to consider the competence of sickle cell trait RBCs in these functions. So far, the level of CR1 and CD55, the immune complex binding capacity and the susceptibility to complement in heterozygous carriers of the sickle cell gene has not been studied. There is strong evidence that the complement regulatory system machinery is important in the protection from the severe manifestations of malaria. The aim of this study was therefore, to study the same machinery in sickle cell trait to determine whether this could explain the protection from severe malaria.

Erythrocytes are normally protected from autologous complement attack through the action of various complement regulatory proteins, such as complement receptor 1 (CR1/CD35), decay accelerating factor (DAF/CD55) and membrane inhibitor of reactive lysis (CD59) expressed on these cells [19]. Recent studies have demonstrated age-related changes in the expression levels of the erythrocyte complement regulatory proteins; CR1 and CD55 [20] and associated deficiencies of these molecules with SMA [2]. Although complement regulatory proteins play an important role in preventing complement mediated erythrocyte destruction, they have not been investigated in association with protection of sickle cell traits

against SMA. In this study, it was hypothesized that erythrocytes of individuals with sickle cell trait may have increased CR1 per erythrocyte and CD55 (DAF) which in turn causes increased rate of immune complexes clearance from the circulation and hence resistance to complement attack. To test this hypothesis, RBCs of individuals with HbAS and those with HbAA were examined for differences in CR1 and CD55 expression levels under both normal and reduced oxygen saturation from a cohort of individuals living in a malaria endemic region of western Kenya.

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 [21,22]. The rest of the site details and study design have been previously reported elsewhere [23,24].

2.2 Study Population

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 [2,3,25-28] 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 [23,24].

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 RBCs for Assay

An equal amount of RBCs in wash buffer was added to freshly prepared disodium hydrogen phosphate (Na_2HPO_4, FW 142g) 0.114M and sodium dithionite ($Na_2S_2O_4$ FW 174.1g) 0.114M at a ratio of 2:3, filter sterilized through a 0.22um filter [15]. The disodium hydrogen phosphate was prepared from a stock solution while the sodium dithionite was prepared fresh every day. The RBCs 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 RBCs. This procedure was done to see the effect of reduced oxygen saturation on the parameters under investigation

2.5 Staining of Red Blood Cells for CR1 and CD55

Venous blood (2.5 mL) was collected in ethylenediaminetetraacetic acid (EDTA) vacutainers (Becton Dickinson, USA). A 1:50 dilution of RBC pellet in Alsevers buffer was made from EDTA anticoagulated blood samples, centrifuged at x500g for five minutes and a portion resuspended in the same volume of buffer for storage at 4º C until used. The remaining pellet was washed with ten volumes of phosphate buffered saline pH 7.4 (PBS) and resuspended in the glycerolyte (50% glycerol, 16g/L sodium lactate, 300mg/L KCL, 25mM sodium phosphate pH 6.8) for cryo-preservation [29], stored at -70º C freezer overnight then transferred to liquid nitrogen until use.

Since our preliminary experiments reported no significant effect of freezing on the levels of erythrocyte CR1 and CD55, current investigations were carried out using cryo-preserved samples. For the determination of CR1 and CD55, the following primary antibodies were used in dilution of 1:20: anti-CR1 clone E11, anti-CD55 clone IA10, and isotype controls for each (Becton-Dickinson, Belgium). A secondary FITC-conjugated goat anti-mouse IgG (Becton-Dickinson, Belgium) was used at a dilution of 1:50. Ten μL of thawed erythrocyte pellet was washed twice in 1mL of Alsevers buffer and resuspended in the same volume of buffer. The rest of the procedures were done as previously described [3,20]

2.6 Flow Cytometric Analysis

Flow cytometry was carried out using a FACScan cytometer (Becton-Dickinson, San Jose, CA, USA). Analysis was carried out using FCS Express v2.5 (De Novo Software, Los Angeles, CA). Red cells were gated on the basis of their forward and side scatter characteristics using logarithmic amplification. As an internal measure of consistency, all assays were done in parallel to RBCs from a healthy aparasitemic subject who acted as a positive control for complement regulatory proteins. The mean fluorescence intensity (MFI) values for CR1 and CD55 were normalized to the mean of the MFI of the red cell standard using the formula:

$$CorrMFIs = MFIs \ x \ MFIcmean/MFIc,$$

Where "CorrMFIs and MFIs are the corrected and uncorrected sample MFI respectively, 'MFIcmean" is the mean of all the MFI values of the standard control, and "MFIc" is the MFI of the control obtained in parallel with the sample.

The number of molecules of CR1 per red cell was derived from a fluorescence standard curve created using cells with known CR1 numbers. Red cell anti-CD55 antibody binding capacities (ABC) were derived from a standard curve created using beads of known ABC (Bangs Lab, Fishers, IN) [30].

2.7 Statistical Analysis

Statistical analyses were performed using SPSS for windows version 16.0 software (SPSS Inc, Chicago, IL, USA). The mean CR1 copy numbers per red cell and mean CD55 levels per red cell data 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 ($\chi2$) 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 CR1 copy numbers per erythrocyte and mean CD55 levels per red cell between HbAS and HbAA .The General Linear Method (GLM) was used to test between subject effects. The Chi-square ($\chi2$) test was used to compare proportions across groups. All tests were two-sided with α ≤ 0.05.

3. RESULTS

3.1 Demographics

Forty seven (47) and 69 children aged 0 to 192 months were enrolled in the HbAS and HbAA arms respectively as shown in Table 1. There was no significant difference in gender, mean age in months and mean haemoglobin level between the two groups (*P*=0.447, *P*=0.059 and *P*=0.548 respectively). The mean CR1 level and mean antibody binding capacity under both normal and reduced oxygen saturation were comparable (*P* =0.2432, 0.3651, 0.8155 and 0.1700 respectively).

3.2 Differences between Mean CR1 Copy Numbers between HbAS and HbAA Cells under normal and reduced Oxygen Saturation

The mean CR1 copy numbers before and after treatment with sodium dithionite are presented as box-and-whisker plots. The mean CR1 copy numbers per red blood cell was higher (509, [SD=177]) in the HbAS group than in the HbAA group (471 [SD=165]), this difference was statistically not significant (*P*=0.243). When RBCs were treated with sodium dithionite, the mean CR1 copy numbers per erythrocyte was higher for HbAA (605, [SD=184] compared to HbAS (576, [SD=149] and but this was statistically not significant, (*P*=0.365) (Fig. 1).

3.3 Differences in CR1 Copy Numbers between HbAS and HbAA Red Cells in each Age Cohort

The CR1 copy numbers per erythrocyte (Fig. 2) was observed to be significantly higher in the 49-192 months age cohort, HbAS [mean=596.6, SD 168] and HbAA [mean=468.6, SD=99], *P* = 0.033. Overall, significant positive correlation was observed between the CR1 copy numbers per erythrocyte with age for all individuals in the HbAS and HbAA groups, r(116)=0.190, *P*=0.041 as well as CD55 levels: r(116)=0.417, *P*=0.000 and CD 55 levels after deoxygenation: r(116).=0.451, *P*=0.000.

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 CR1 copy #/Erythrocyte (95% CI)	471.6 (431.9-511.4)	509.4 (457.4-561.4)	0.346 [b]
Mean CR1 copy #/Erythrocyte Deoxygenated (95% CI)	605.3 (561.0-649.6)	575.9 (532.1-619.7)	0.321 [b]
Mean CD55 ABC/Erythrocyte (95% CI)	2520.02 (2331.36-2708.68)	2671.4 (2388.0-2954.8)	0.813 [b]
Mean CD55 ABC/Erythrocyte Deoxygenated (95% CI)	2590.8 (2373.2-2808.4)	2934.2 (2609.1-3259.2)	0.169 [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.
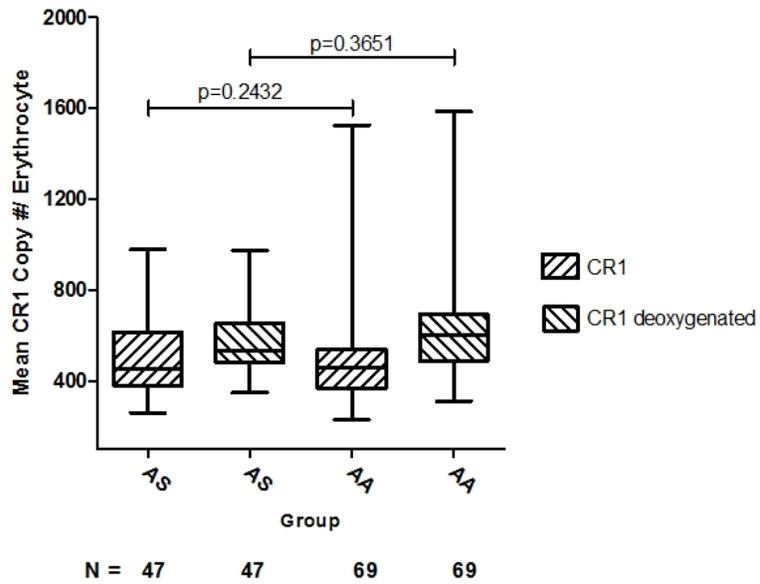
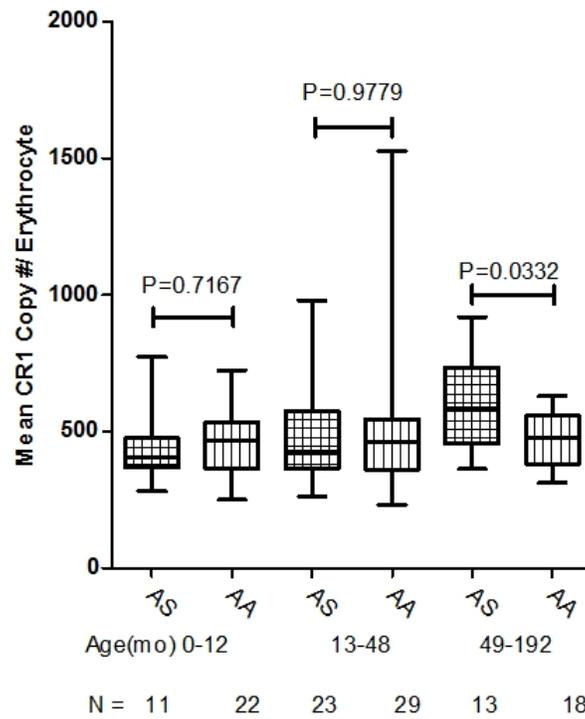**Fig. 1. CR1 copy numbers between HbAS and HbAA red cells under normal and reduced oxygen saturation**

Fig. 2. Mean CR1 copy numbers for HbAS and HbAA red cells across the various age cohorts

3.4 Mean CR1 Copy Numbers per Erythrocyte for the various Age Cohorts by Haemoglobin Electrophoresis Results under reduced Oxygen Saturation

The CR1 copy numbers per erythrocyte was observed to vary with age for both HbAS and HbAA groups (Fig. 3). The mean CR1 copy numbers per erythrocyte after treatment with sodium dithionite for the various age was observed to be higher in the HbAA compared to the HbAS. For the 0-12 months age cohort, the mean CR1 copy numbers per erythrocyte was 531 (SD 71) for the HbAS and 596.7 (SD 133.5) for the HbAA . Between the ages of 13-48 months, the mean CR1 copy numbers per erythrocyte was 55.4 (SD 157.2) and 629.7 (SD 240.7) for the HbAS and HbAA respectively. After the age of 96 months, the mean CR1 copy numbers per erythrocyte was higher in the HbAS (mean 652 [SD 163.7]) compared to the HbAA (mean 576.7 [128.9]) respectively but again, this was not statistically significant.
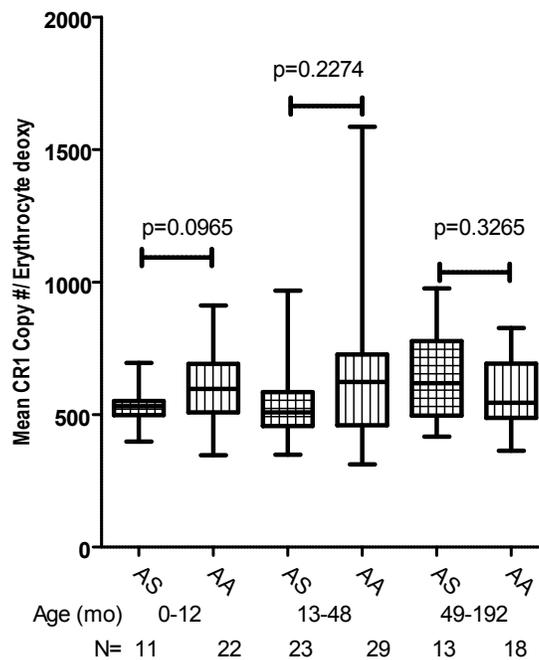

Fig. 3. Mean CR1 copy numbers for HbAS and HbAA red cells across the various Age cohorts under reduced oxygen saturation

3.5 Differences in Mean CD 55 levels between HbAS and HbAA Red Cells under normal and reduced Oxygen Saturation

There were no significant differences in the mean CD 55 levels between red cells of individuals with HbAS and those with HbAA. Mean CD 55 levels of HbAS cells (2671, [SD 149]) was higher than HbAA cells (2541, [SD 777]), However, this difference was not statistically significant P = 0.816. Under reduced oxygen saturation, the mean CD 55 levels for the HbAS (2934, [SD 1107] was higher than for HbAA group (2591, [SD 906]). This again did not reach statistical significance (P = 0.1700) (Fig. 4)
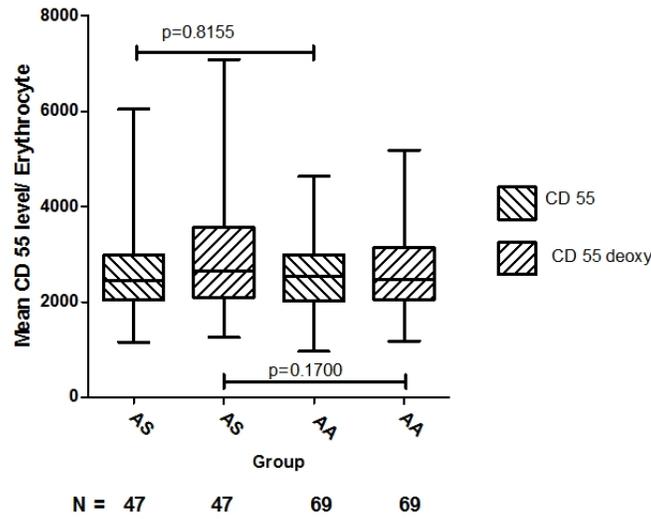
141

Fig. 4. Mean CD 55 levels of HbAS and HbAA red cells under normal and reduced oxygen saturation

3.5.1 Differences in Mean CD 55 levels of HbAS and HbAA Red Cells in each Age Cohort

There was no statistically significant difference between the mean CD55 level per erythrocytes between HbAS and HbAA in the 0-12, 13-48 and 49-192 months age cohorts (P= 0.6028, 0.7746 and 0.2539 respectively) (Fig. 5).
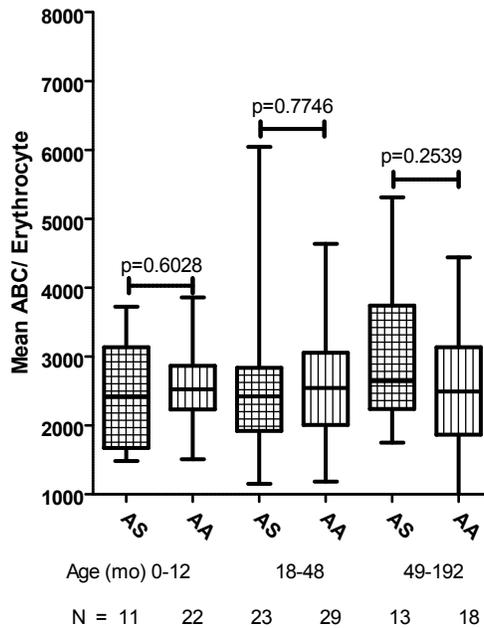

Fig. 5. Mean CD 55 levels for the various age cohorts

3.5.2 Mean CD 55 levels for the various Age Cohorts under Reduced Oxygen saturation

Reduced oxygen saturation resulted in greater increase in the mean CD 55 levels for the HbAS erythrocytes between the ages of 49-192 months (Fig. 6). The mean CD 55 levels for the HbAS was 2934 (SD 1107) compared to HbAA which was 2591 (SD 906). This difference was statistically significant (*P*=0.011).
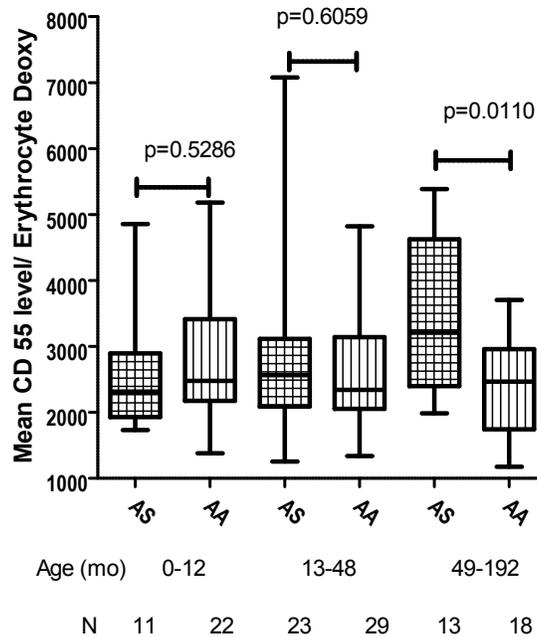

Fig. 6. Mean CD levels for the various age cohorts under reduced oxygen saturation

4. DISCUSSION

This study has shown that the mean CR1 copy numbers per erythrocyte was significantly higher in the HbAS than in the HbAA individuals only in the 49-192 months age cohort (*P* = 0.0332) under normal oxygenation. It is possible that beyond the age of 49 months, the high mean CR1 copy numbers per erythrocyte could translate to higher immune complex binding capacity and decreased immune complex deposition on the red blood cells and this could protect the red cells from immune complex mediated destruction. Although the mean CD55 levels for the HbAS and HbAA were comparable in the various age cohorts under normal oxygenation, the mean CD55 levels for the HbAS in the 49-192 months age cohort after deoxygenation was much higher in the HbAS (mean 3466 [SD1092]) than in the HbAA (mean 2373 [SD752]). This difference was statistically significant (*P*=0.011). We postulate that the apparent increase in CD55 under low oxygenation may be due to conformational changes in CD55 and this may result in decreased complement susceptibility of these cells. It is also possible that the high mean CR1 copy numbers per erythrocyte in the 49-192 months age cohort under normal oxygenation is complemented by the high mean CD55 levels for the HbAS under reduced oxygen saturation. Both of these could play a role in the

protection of HbAS cells against immune complex mediated destruction of HbAS cells during malaria infection.

Natural resistance to malaria based on the inheritance of selected genes that confer protection has been observed in a few individuals living in malaria endemic areas [31,32]. The relative protection of the sickle cell trait against clinical malaria increases throughout the first 10 years of life, returning thereafter to baseline [33]. Transgenic mice expressing sickle cell haemoglobin (HbS) are protected from rodent malaria [13] and rodent cerebral malaria [34]. In humans HbS provides selective protection to heterozygous sickle cell trait individuals (HbAS) against death from *Plasmodium falciparum* malaria while homozygous sickle cell trait individuals (HbSS) suffer severe clinical effects [9,24,35,36]. Studies carried out in The Gambia [11] and Kenya [37,38] have indicated that HbAS provides more than 90% protection from both cerebral malaria and severe malarial anemia in children. The mechanism of malaria protection by HbAS is not well understood but has been suggested to be due to enhancement of acquired immunity to the parasite [33,39] and innate factors such as impaired parasite growth in erythrocytes containing HbAS attributable to conditions of low oxygen tension [40]. A recent study [23] in the same population has shown that the protection afforded by HbAS against severe manifestations of malaria may be partly due to higher immune complex binding capacity of the HbAS compared to the HbAA red cells and this could result in protection of these cells against malaria. This high immune complex binding capacity could be partly due to the high CR1 levels and the high CD 55 levels when these cells undergo deoxygenation

5. CONCLUSIONS

In conclusion, we report for the first time that the mean CR1 copy numbers per erythrocyte is higher in the HbAS than HbAA individuals between the ages of 49-192 months. When the cells were subjected to deoxygenation, the level of CD55 was greater in HbAS RBCs than in HbAA RBCs between 49-192 months of age. This is most likely due to conformational changes of the CD55 molecule leading to increased availability of the epitope. This apparently has 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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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.

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.

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