

## Anaemia & expression levels of CD35, CD55 & CD59 on red blood cells in *Plasmodium falciparum* malaria patients from India

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**Background & objectives:** Severe anaemia in *Plasmodium falciparum* (Pf) associated malaria is a leading cause of death despite low levels of parasitaemia. In an effort to understand the pathogenesis of anaemia we studied expression level of RBC complement regulatory proteins, CR1 (CD35), CD55 and CD59 with haemoglobin status in a group of malaria cases from Assam, Goa and Chennai, and in healthy controls.

**Methods:** Flowcytometry was used to study expression of CR1, CD55 and CD59 in 50 Pf cases and 30 normal healthy volunteers. Giemsa stained thick and thin blood films were used for microscopic detection and identification of malarial parasites and parasite count.

**Results:** No correlation was found between degree of expression of RBC surface receptors CR1, CD55 and CD59 with haemoglobin level. However, expression of CD55 was less in malaria cases than in healthy controls.

**Interpretation & conclusions:** The present findings indicate that malaria infection changes the expression profile of complement regulatory protein CD55 irrespective of severity status of anaemia. Further studies are needed to explore the pathophysiology of anaemia in malaria cases in Assam where expression of RBC complement receptors appears to be low even in normal healthy population.

**Key words** Anaemia - Assam - CD55 - CD59 - CR1 - Chennai - flowcytometry - Goa - India - *Plasmodium falciparum*

In India severe anaemia in patients suffering from *Plasmodium falciparum* (Pf) is an important concern<sup>1</sup>. Direct destruction of RBCs following Pf infection cannot account for the degree of anaemia observed during malaria infection instead it has been suggested that the destruction of uninfected RBCs is a major cause of haemoglobin loss<sup>2</sup>. Recent evidence suggests that RBC complement regulatory proteins are involved in malaria associated anaemia<sup>3,4</sup>. The complement cascade plays a key role in the modulation of inflammatory responses and its activation has been reported to be crucial to

the pathogenesis of various diseases<sup>5</sup>. Several key membrane complement regulatory proteins (MCRPs) regulate the activation of complement cascade, thus preventing damage to the self tissues and cells during an inflammatory reaction<sup>6</sup>. Decay accelerating factor (DAF, CD55) is a membrane bound regulatory protein that downregulates the complement cascade at the critical step of C3 activation<sup>7</sup>. Failure to regulate C3 and C5 convertases allows cytolytic membrane attack complex (MAC) to be generated on the surface of cells<sup>6</sup>. CD59, a membrane bound complement regulatory protein prevents MAC formation by

inhibiting the incorporation of C9<sup>8</sup>. Another membrane bound protein CRI (complement reception 1 or CD35) is very important for processing and clearing of complement opsonized immune complexes and acts as a negative regulator of the complement cascade, mediates immune adherence and phagocytosis and inhibits both classical and alternative pathways<sup>9</sup>.

In an effort to understand the pathogenesis of anaemia in Pf infection we studied the relationship between expression level of CD35, CD55 and CD59 with haemoglobin status in a group of malaria cases from three regions of India, namely Assam, Goa and Chennai.

### Material & Methods

Blood samples were collected from 50 consecutive *P. falciparum* malaria cases attending malaria clinics [Regional Medical Research Centre, Dibrugarh, Assam (33 cases); Goa and Chennai field units of National Institute of Malaria Research, New Delhi (14 and 3 cases respectively)] in three regions of India *viz.*, Assam (East), Goa (West) and Chennai (South) during 2007-2008. This study was approved by institutional ethics committee of Postgraduate Institute of Medical Education & Research, Chandigarh, and written informed consent was obtained from all the study subjects prior to collection of blood samples. Subjects were excluded from participation if there was evidence of other concomitant infections like TB, typhoid, history of haemolytic disorders, *etc.* or had a history of blood transfusion or antimalarial treatment 3 months before enrolment. To compare the results with normal population, 30 apparently healthy age matched individuals were included as controls from Assam. Giemsa stained thick and thin blood films were used for microscopic detection and identification of malarial parasites. Parasites were counted against 200 WBCs and the value converted to parasites per  $\mu$ l of peripheral blood. Approximately 5 ml of venous blood was also collected in EDTA vials and processed for flowcytometric study following the method of Waitumbi *et al*<sup>3</sup> with slight modifications. In brief, fluorescent staining was performed using monoclonal antibodies (Becton Dickinson, Biosciences, USA) against cell surface receptors [anti-human CR1 (clone E11; WS No.: III 204), CD55 (clone IA10; WS No.: V BP352, S031) and CD59 (clone p282 (H19); WS No.: V S006)]. For each sample 1  $\mu$ l (each) of whole blood was put into 5 sample tubes containing 100  $\mu$ l of staining buffer (PBS with 2% BSA); 20  $\mu$ l of anti-

human FITC conjugate of CR1 or CD55 or CD59 or unstained control were put separately in the sample tubes and incubated at room temperature in dark for 20-30 min. After incubation, RBCs were washed in 2 ml of staining buffer and re-suspended in 500  $\mu$ l of staining buffer and analyzed in flowcytometer. The FACScan flowcytometer (Becton Dickinson, USA) which was used for the measurement of expression studies was optimized using standard fluorescent beads. For acquisition and analysis RBCs were gated using logarithmic amplification of their forward and side scatter characteristics. FITC fluorescence was measured by FL1 detector using logarithm amplification.

*Statistical analysis:* Statistical analysis was carried out using SPSS v11.0 (Spss Inc., Chicago, IL, USA). Correlation between different variables like expression levels of RBC surface receptors, haemoglobin levels, age of patients, level of parasitemia, *etc.* were studied using Spearman's correlation coefficient. Analysis of Covariance (ANCOVA) was used to compare mean expression of CD35, CD55 and CD59 between malaria cases or controls and between malaria cases with haemoglobin less than 7 g/dl versus malaria cases with haemoglobin more than 7 g/dl using age as a covariate.

### Results & Discussion

A total of 50 Pf malaria patients ranging in age from 1.5 to 65 yr (average age 21.7 yr) were included in this study. The intensity of infection in peripheral blood of patients ranged from 319-2743 (mean = 901.29) asexual parasites/ 200 WBC. The mean fluorescence intensities (MFIs) of the expression of CD35 (mean  $\pm$  SD 2.11  $\pm$  0.43 vs 2.05  $\pm$  0.34) and CD59 (40.65  $\pm$  SD 7.6 vs 35.16  $\pm$  4.59) in malaria patients and healthy controls was not statistically different. On the other hand, there was a statistically significant decrease in expression of CD55 in malaria cases than controls (3.64  $\pm$  1.88 vs 5.36  $\pm$  1.32,  $P < 0.01$ ). However, the MFI of CD55 on RBCs

**Table.** Characteristics of malaria cases and healthy controls

Characteristics	Malaria cases (n=50)	Healthy controls (n=30)
Female sex (%)	16	46.7
Mean age, yr (95% CI)	23.4 (19.5-27.2)	23.7 (13.2-24.1)
Mean Hb, g/dl (95% CI)	7.59 (7.19 to 7.99)	8.43 (7.93 to 8.93)
Parasite density, mean (Range)	901.29 $\mu$ l (319-2743)	-

of patients having haemoglobin level below 7 g/dl was not significantly different from malaria patients having haemoglobin level above 7 g/dl ( $5.5 \pm 4.75$  vs  $6.7 \pm 4.85$ ). Waitumbi *et al*<sup>3</sup> reported decrease in expression of CR1 and CD55 on RBCs from children with severe anaemia as compared to age matched controls. However, our study did not find any correlation between expression level of RBC surface receptors CR1, CD55, CD59 and haemoglobin level in malaria patients. As in the present study Helegbe *et al*<sup>10</sup>, 2007 also did not find any relationship between the severity of anaemia and levels of complement receptor 1 or decay accelerating factor (CD 55) in Ghanaian children with Pf malaria. The present study revealed that expression of decay accelerating factor (CD55) and membrane inhibitor of reactive lysis (CD59) was significantly lower in Pf cases from Assam (mean CD55 = 4.15, CD59=42.69) as compared to Pf cases from Goa (mean CD55 = 11.85, CD59=70.59) or Chennai (mean CD55 = 5.58, CD59=70.26). Interestingly, the expression of CD55 and CD59 was significantly ( $P<0.05$ ) low in healthy control population from Assam (mean CD55 = 8.44, CD59=44.16) as compared to malaria cases from Goa or Chennai. This difference in expression level of RBC complement receptors may probably be due to genetic polymorphisms as has been suggested earlier<sup>11,12</sup>. Further elaborate studies are needed to explore the pathophysiology of anaemia in malaria cases in Assam where expression of RBC complement receptors appeared to be low even in the normal healthy populations.

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