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Unraveling the C-reactive Protein Complement-Cascade in Destruction of Red Blood Cells: Potential Pathological Implications in *Plasmodium Falciparum* Malaria

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Key Words

C-reactive protein • Complement-regulatory proteins • Malaria • Red blood cells

Abstract

Background: Deficiencies of the complement-regulatory proteins on RBC (RBC_{Mal}) of patients with *Plasmodium falciparum* were reported. Here, we sought to determine the role of affinity-purified C-reactive protein from patients (CRP_{Mal}), in modulating the complement-regulatory proteins and downstream effect on complement-cascade. Methods: CRP_{Mal} was characterized by analytical ultracentrifuge and electrophoretic analysis. Surface plasmon resonance, Western blotting, co-immuno-precipitation, flow-cytometry and ELISA determined the binding of CRP_{Mal} with RBC_{Mal}. Modifications of membranes for RBC_{Mal}-CRP_{Mal} binding were explored by scanning electron microscopy, osmotic and turbulence fragility, hydrophobicity and oxyhemoglobin release. Flow-cytometry, ELISA, Western blotting and Scatchard analysis monitored the status of complement-regulatory proteins on RBC_{Mal}. Complement-activation via CRP_{Mal} was quantified by C3-deposition and hemolysis. Results: CRP_{Mal} binds specifically to RBC_{Mal} through distinct molecules. Such binding altered the normal discoid-shape of RBC_{Mal} with increased membrane fluidity and hydrophobicity. In the presence of CRP, RBC_{Mal} showed reduced complement-regulatory proteins (CR1 or CD35, CD55 and CD59) with decreased affinity. These changes caused enhanced C3-deposition and complement-mediated hemolysis. Conclusion: Taken together, we have established the contributory effect of CRP_{Mal} causing decreased complement-regulatory proteins, possibly providing a new mechanism of complement-fueled RBC_{Mal} destruction refractory to erythrophagocytosis and may account for pathogenesis of anemia.

Introduction

Malaria incidence ranged between 2-2.5 million cases annually in India [http://www.whoindia.org/EN/Section3/Section128/Section235.htm]. Despite a few reports [1-3], the pathology of *Plasmodium falciparum* (*Pf*) anemia remains to be defined. Complement-regulatory proteins on RBC include complement receptor 1 (CR1 or CD35), decay accelerating factor (DAF or CD55), and the membrane inhibitor of reactive lysis (CD59). CR1 promotes the inactivation and binding of

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C3b in immune complexes (ICs). CR1 and CD55 accelerate the degradation of C3b convertases and CD59 interfere with the assembly of the membrane attack complex C5b-9 [4-5]. *Plasmodium falciparum* exploits CR1 as an adhesion-ligand in the process of rosetting, the pathogenic clumping of erythrocytes [6]. Destruction of erythrocytes, however, cannot be solely explained by the direct effect of the parasite [7-8], or by deposition of parasite-derived antigen nor by its surface damage through IgG and complement-regulatory proteins or complement-fixation [9-10] or alternatively C3b-bearing ICs via CR1 reticulo-endothelial system [11-12].

C-reactive protein (CRP), an acute-phase reactant, is found in trace amounts in normal human serum (NHS) [13], markedly increased in inflammatory conditions [14-15]. Our laboratory convincingly demonstrated glycosylated molecular variants of human CRP induced in certain pathological conditions [16], which interact with several biological macromolecules [17-18]. CRP elicits a multitude of effects on different blood cells and can activate the complement-cascade by binding with phosphocholine (PC) containing substances [19-21]. Excess ICs in vasculature may deposit in tissues and complement is required in removing them. This evidence led us to examine the composite role of CRP and complement regulatory proteins in the pathogenesis of *Pf* anemia.

Accordingly, CRP_{Mal} was affinity-purified and characterized from the serum of freshly recruited new cohort of malaria patients. The specific-binding of CRP_{Mal} with RBC_{Mal} was demonstrated which altered the membrane characteristics resulting in the significant decrease of membrane-anchored CR1, CD55 and CD59 on RBC_{Mal}. This RBC-CRP_{Mal} complex leads to concomitant C3b-deposition on RBCs and its subsequent clearance from circulation through hemolysis. Our findings, for the first time, suggest a possible new mechanism in which RBC-CRP complex, being triggered with complement, promote the destruction and clearance of damaged RBCs in malaria.

Materials and Methods

Study design

Clinically confirmed individuals (mean age 35.14 ± 15.7 years, n=65) infected with asexual *Plasmodium falciparum* and age-matched normal healthy volunteers (n=45) were enrolled. The diagnosis was established on the basis of microscopic examination of Giemsa-stained thick and thin blood smears, DAT test, concurrent axillary temperature ≥37°C, headache, fever, vomiting, painful joints and shaking chills with a hemoglobin concentration of 4.5-7.8 g/dl. Individuals had no history of red cell disorder, autoimmune diseases and parasitic co-infections. The Institutional Human Ethical Committee had approved the study, and samples were taken with the consent of the donors, patients.

Clinical samples

Blood were collected in Alsever's solution and used within 2-4 hours. It was centrifuged, buffy coat removed. Washed RBC were suspended in Tris/HCl (20 mM)-NaCl (150 mM) (TBS, pH 7.5) containing 1% BSA (TBS-BSA) and used immediately. The RBC_{Mal} consisting of a mixture of infected (7.2-8.6 parasitemia/red cell) and uninfected (~90%) population were used for all the experiments. Fresh sera from patients (MS) and normal were obtained by clotting blood samples.

Purification and characterization of human CRP_{Mal}

Serum showing elevated levels of CRP by latex agglutination (Rhelax-CRP kit, Tulip, India) was selected for purification [16, 22]. Typically, serum samples from individual patients (n=10) were separately passed through an agarose column in Tris/HCl (50 mM) buffered saline (TBS, pH 7.5)-Ca^{+2}(5.0 mM). It was immediately applied to a Sepharose-PC matrix, washed with TBS-Ca^{+2} (10 mM) and bound protein was eluted with EDTA (10 mM)-Ca^{+2} (1.0 mM). Protein fractions were further purified through another Sepharose–PC column using TBS-Ca^{+2} (10 mM) and pure CRP was eluted with PC (2.0 mM) in TBS-Ca^{+2} (0.50 mM). The eluted protein was dialyzed against 20 mM Hepes with saline containing EDTA (2.0 mM), followed by same buffer without EDTA. The protein was estimated using the extinction coefficient, $E_{1\%}^{1cm}$ at 280 nm of 19.50 [23] and also by Lowry method [24]. The yield of purified CRP_{Mal} was 105-139 µg/ml corresponded to 210-278-fold induced above the normal level (0.5 µg/ml) confirming that CRP_{Mal} as an acute-phase protein [14]. A single band in SDS-PAGE [25], autoradiograph of $^{125}ICRP_{Mal}$ (Fig.1A) and analytical isoelectric focusing with a pI of 8.4 confirmed its purity (Fig.1B) [26]. CRP was >99% pure based on the reactivity with anti-human CRP and no binding with anti-human IgG antibodies in Western blotting [18, 27]. A commercially available CRP from Tulip (CRP_{Tulip}), purified from pooled human plasma, was used as control and showed similar subunit molecular weight like normal CRP (CRP_N) (23 kDa, Fig. 1A) [28]. However, the subunit molecular weight of CRP_{Mal} was different (27 kDa, Fig. 1A) [16-18]. A small change between these two CRPs confirmed the induction of disease-associated molecular variant. CRP_{Tulip} behaved differently than CRP_{Mal}. A homology to known human CRP sequences [29] suggested similar native pentraxin structures of CRP_{Mal} and CRP_{Tulip}.

The homogeneity of CRP_{Mal} was additionally confirmed by analyzing sedimentation velocity experiment (Fig. 1C). CRP_{Mal} with an absorbance 0.1-0.5 at 280 nm were loaded on a Beckman XL-A ultracentrifuge in an An60Ti rotor at 20°C at 4000 rpm along with buffer in reference compartment into 2-sector cells. Radial absorbance data were acquired at 280 nm in continuous mode for every 5 min for a period of 10 h with radial increments of 0.002 cm. Data were analyzed with SEDFIT using the C(S) or C(M) Lamm equation model [30-32]. Collections of 10-30 radial

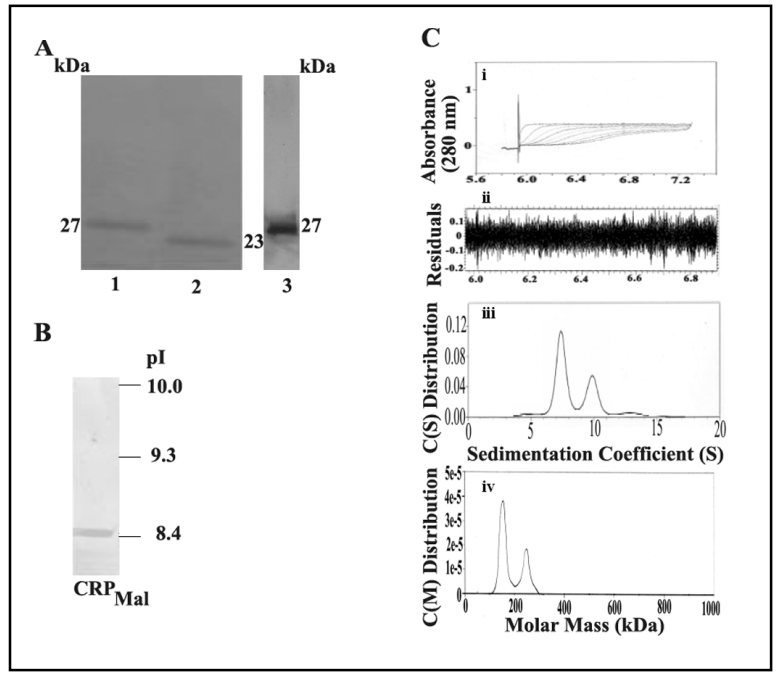

Fig. 1. Characterization of affinity-purified CRP_{Mal}: A. Electrophoresis. Equal amounts of affinity-purified CRP_{Mal} (1 µg, lane 1) was electrophoretically analyzed on a SDS-PAGE (10%) and silver stained. A commercially available CRP_{Tulip} was used as normal CRP (lane 2). Lane 3 is autoradiograph of $^{125}ICRP_{Mal}$. B. Isoelectric focusing. CRP_{Mal} (1.5 µg) was applied to ampholine polyacrylamide tube gel (4%) in a pH gradient (3.5-10.0) and stained with silver nitrate. C. Sedimentation velocity analysis. (i). Optical traces of CRP_{Mal} during ultracentrifugation. These traces were fitted to the Lamm equation by SEDFIT (ii) showed the fitting residuals. The fitting residuals were randomly distributed. The distribution of the pentameric and decameric form of CRP_{Mal} was analyzed by sedimentation velocity. (iii) Continuous sedimentation coefficient distribution C(S). (iv) Continuous molar mass distribution C(M) are shown.

scans were used for analysis and 200 sedimentation coefficients between 1.2 and 20 S were employed for calculation. The position of the meniscus and cell bottom were determined and refined in the final fit. Analysis resulted in high-quality-data fits (Fig. 1Ci) with randomly distributed residuals and root-mean-square deviations below 0.1% (Fig. 1Cii). The calculated sedimentation coefficient distributions revealed well-resolved peaks corresponding to the different aggregation states of the CRP (Fig. 1Ciii). Pentameric CRP_{Mal} appears to be the major macroscopic form (75%) with a peak at 7.5S, whereas by intermolecular association, the solution also contained the decameric (9.8S) form, whose share hardly reached 15% (Fig. 1Civ). Depending on conditions of microenvironments, CRP may exist as monomer, pentamer or multimer each of them showing preferential ligand binding with differential biological relevance [33].

Examination of RBC-CRP binding

CRP_{Mal} or CRP_{Tulip}, at physiological concentration (10 µg/ml) was incubated in TBS-Ca^{+2} (2.0 mM, pH 7.5)-PC (1.0 mM); designated as CRP-PC-Ca^{+2}-complex and used for following experiments. Membranes were prepared by re-suspending packed RBCs in ice-cold buffer containing digitonin (1.0 mg/ml) for 20 mins (0°C) [34], centrifuged and washed ghosts were solubilized in equal volume of extraction buffer (20 mM Tris-HCl, pH 7.5; 1.0%Triton X-100; 0.1% SDS; 200 mM NaCl) for 1 h at 0°C and centrifuged. The supernatant containing membrane proteins was quantified [24].

Surface plasmon resonance (SPR)

SPR analysis of the interaction between CRP_{Mal} and RBC_{Mal} membrane proteins was performed using a BIACORE 3000 instrument. CRP_{Mal} (0.5-10 µg/ml) in sodium acetate buffer (10 mM, pH 4.5) was immobilized onto the carboxymethylated dextran surface of a CM5 sensor chip. All flow cells of CM5 were activated at 25°C, each with a mixture (20 µl) of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (0.20 M) and *N*-hydroxy-sulfosuccinimide (0.05 M) at a flow rate 5 µl/min, subsequently CRP_{Mal} was injected. Unreacted groups were blocked with ethanolamine (20 µl, 1 M, pH 8.5). Flow buffer was Hepes (10 mM)-NaCl (150 mM)-Ca^{+2} (2 mM)-PC (1.0 mM)-Tween-20 (0.005 %), pH 7.4. Activated and blocked flow cell served as blank sensograms for substraction of the bulk refractive index background. The sample was first injected over the negative control surface and then over immobilized CRP. RBC membrane (5-50 µg/ml) were passed at a flow rate of 30 µl/min. Association and dissociation times were 5 and 3 min respectively and the regeneration consisted of a 30 µl injection of 2 M NaCl. Sensograms were analyzed with the Biaevaluation software version 4.1. Resonance responses in arbitrary units (RU) were plotted against the varying concentrations of RBC membrane protein and the slopes of the linear plots were calculated. Such analysis with RBC_N membrane proteins was performed for comparison.

Analysis of membrane alterations induced on RBC due to binding with CRP

RBC_{Mal} or RBC_N (1x 10^9 cells/tube, n = 15) were incubated with CRP_{Mal}-PC-Ca^{+2} complex for 1 h on ice and washed with TBS-Ca^{+2} and following experiments were done [35-36].

A) Osmofragility assay. RBC-CRP_{Mal}-PC-Ca^{+2} complex was exposed to different NaCl concentrations for another 1 h at 37°C. Only cells in TBS-Ca^{+2} served as control. Lysis of RBC in distilled water was taken as 100%. The hemolysis (%), as a measure of osmotic fragility was analyzed spectrophotometrically before and after binding with $CRP_{Mal.}$

Hemolysis (%) = OD_{412} nm at particular saline concentration/ OD_{412} nm with distilled water X 100.

B)Hydrophobicity assay. 8-anilino-1-napthalenesulfonic acid (ANS, 5.0 µl, 1.0 mM in TBS) was allowed to bind with RBC-CRP_{Mal}-PC-Ca^{+2} complex for 5 min at 37°C. Fluorescence spectra were scanned in a spectrophotometer with an excitation wavelength of 365 nm. The excitation and emission band passes were of 5 nm [35-36]. Only RBCs served as controls.

C) Oxyhemoglobin release. The RBC-CRP_{Mal}-PC-Ca^{+2} complex were lysed with distilled water, osmotically balanced with TBS and exposed to superoxide radical generated from a pyrogallol autooxidation system by adding freshly prepared pyrogallol solution in water (10 µl, 0.02 M) for 20 min at 30°C. The absorbance (A) of released oxyhemoglobin was measured by Winterbourn spectrophotometric method [37] using following equation: [oxyhemoglobin] = $119A_{577} - 39A_{630} - 89A_{560}$.

D) Turbulence fragility. To exploit the mechanical strength of the membranes, packed RBC (0.5 ml) were suspended in TBS (10 ml) and shaked vigorously using an orbital shaker at 2000 rpm for 4 h. A portion (0.5 ml) of suspension was withdrawn during 0-4 h, centrifuged and the absorbance of the supernatant was determined spectrophotometrically at 540 nm. The hemoglobin release (%) was determined in reference to a completely lysed cell suspension [38].

E) Scanning electron microscopy (SEM). The morphological changes of RBC_{Mal}-CRP_{Mal}-PC-Ca^{+2} complexes were evaluated by SEM (Jeol, JSM-5200). Cells were initially fixed with 2.5% glutaraldehyde and subsequently in 1% osmium tetraoxide in TBS, pH 7.2 for overnight. The suspensions were dehydrated in an ethanol series (70-100%) and by the critical point drying; subsequently gold-coated in a sputter device and examined. Only RBCs served as controls.

Assessment of complement-regulatory proteins on RBC after binding with CRP_{Mal}

A) Flow-cytometry. To understand the status of complement-regulatory proteins due to binding of CRP, RBC_{Mal}-FITC-CRP-PC-Ca^{+2}-complex was incubated with PE-anti-CD35, PE-anti-CD55 and PE-anti-CD59 mAbs (1:100) separately for 1h on ice. Washed cells were fixed in 1% paraformaldehyde, acquired in Becton Dickinson FACSCalibur and analyzed by CELLQUESTPRO software. Specific-binding was determined by pre-incubation of 50-fold excess of unlabeled antibodies with RBC_{Mal}-CRP-PC-Ca^{+2}-complex. Binding of mAbs with complement-regulatory proteins on $RBC_{Mal,}$ in absence of CRP, was similarly processed.

The number of complement-regulatory proteins/cell was also determined [39] in which the median values from histograms plot were converted to arbitrary units in a linear scale (1-1024 channels). The values for CD35, CD55, and CD59 were normalized to the mean of the median fluorescence of the red cell standard using following equation

$$Fsc = Fs\ (Mean\ Fc/Fc)$$

where Fsc and Fs are the corrected and uncorrected median fluorescent channel of the sample. Fc is the median fluorescent channel of the control stained in parallel with the sample. Mean Fc is the mean of all the median values of the standard control. Only RBC or isotype control served as different negative controls.

FITC was conjugated to CRP [40]. The binding of RBC with FITC-CRP was determined. To distinguish complementary-regulatory proteins from the CRP-binding molecules present on the surface of RBC, cells were pre-incubated with un-conjugated anti-CD35, CD55 and CD59 mAbs (1:100) separately for 1 h on ice and allowed to bind with FITC-CRP_{Mal} (10 µg/ml). RBC_N was similarly used for comparison. The binding of RBC_N with FITC-CRP_{Tulip}-PC-Ca^{+2}-complex was also monitored. Only RBC or isotype controls were used.

B)ELISA. Ghost membranes from RBC_{Mal} (n=15) were immobilized (2.0 µg/well/100 µl) on 96-well microtitre plates (Nunc, USA) for overnight at 4°C, washed with TBS-Tween, blocked (TBS-2% BSA), and further incubated with murine anti-human CD35 (Clone E11), anti-human CD55 (Clone IA10), and anti-human CD59 (Clone H19) mAbs (BD Biosciences) (1:1000) separately. The bound complex (ghost membrane protein-anti-complement-regulatory protein-CRP_{Mal}-PC-Ca^{+2}) was detected by horseradish peroxidase (HRP)-goat-anti-murine IgG (1:8000, Cappel) using azino-bis-thiosulphonic acid (ABTS) as substrate and quantified by an ELISA Reader (Thermo Electron Corporation) at 405 nm.

In order to distinguish the complement-regulatory proteins and binding sites for CRP on RBC surface, a competitive ELISA was designed. Anti-CD35 mAbs (1:1000) and CRP_{Mal} (10 µg/ml)-PC-Ca^{+2} were added onto the immobilized ghost membrane protein. Bound complex was detected as described above.

Additionally, bound complex was also detected by murine anti-CRP antibodies and processed as above. The RBC-CRP binding, in absence of complement-regulatory protein detected with murine anti-CRP antibodies served as positive control. The control wells were devoid of either CRP or membrane protein. The competitive ELISA was repeated by varying concentrations (1:4000,1:2000,1:1000,1:500,1:100) of anti-complementary-regulatory protein mAbs with the fixed concentration of CRP_{Mal}. The experiment was repeated using anti-CD55 and anti-CD59 mAbs and also RBC_N.

IgG is known to bind with RBC *in vivo* [9]. To explore the binding site of IgG different from that for CRP_{Mal}, RBC_{Mal} were co-incubated with human IgG (5-20 mg/ml; 100 µl) and CRP_{Mal}-PC-Ca^{+2} complex (10 µg/ml; 100 µl) and then probed with HRP-anti-human IgG. The binding of RBC with IgG was determined in absence of CRP. Similarly, the RBC_{Mal}-CRP_{Mal} in absence and presence of IgG (10mg/ml) was determined by anti-CRP antibodies.

C) Western blot anlysis. Ghost membrane proteins from RBC_{Mal} or RBC_N (n=15) were electrophoresed (50 µg/ lane) separately on SDS-PAGE gradient gel (4-12%). The separated proteins were transferred to nitrocellulose membranes [41], blocked (TBS-2%BSA) and incubated with CRP_{Mal}-PC-Ca^{+2} complex. Membranes were washed with TBS-Ca^{+2}-Tween, incubated with murine anti-human CRP (1:500) for overnight at 4°C and followed by HRP-rabbit anti-murine IgG (1:1000) in TBS-BSA, washed and visualized by 3, 3-diaminobenzidine. Similarly, CD35 antigen present on RBC_{Mal} membrane was visualized with anti-CD35 mAb (overnight, 4°C; 1:100) followed by HRP-goat-anti-murine IgG and processed as above.

In parallel experiment, ghost membrane protein from malaria was co-incubated with a mixture of CRP_{Mal}-PC-Ca^{+2}

complex and anti-CD35 and analyzed separately using murine anti-human CRP antibodies or HRP-goat-anti-murine IgG and processed as above. Pre-stained markers were used as standard to calculate R_f values. RBC_N was used as control. Similar experiments were carried out with RBC_N membrane proteins and CRP_{Tulip}-PC-Ca^{+2} complex.

Immunoprecipated protein was separated on SDS-PAGE, transferred and the blots were probed with CRP_{Mal}-PC-Ca^{+2} and processed as above. The experiment was repeated with RBC_N using CRP_{Tulip}-PC-Ca^{+2}.

RBC membrane proteins (50 µg/25 µl) from malaria and normal individuals were incubated separately with CRP_{Mal}-PC-Ca^{+2} (5.0 µg) in TBS-BSA for overnight at 4°C, and incubated with polyclonal rabbit anti-human CRP (1:100/100 µl) for overnight (4°C) followed by addition of 5 µl of protein A–Sepharose 4B (6 mg/ml, Sigma). After incubation for overnight at 4°C, the Sepharose bound protein complexes were washed with cold TBS and protein samples were boiled with 4X Laemmli sample buffer for 10 mins and centrifuged. The supernatant was further incubated with 5 µl of protein A–Sepharose 4B for overnight, centrifuged. Supernatant was boiled again with Laemmli sample buffer and loaded on each lane and proteins were separated on SDS-PAGE followed by electrophoretic transfer to nitrocellulose membrane. The blots were probed with CRP_{Mal}-PC-Ca^{+2} and subsequently were incubated with polyclonal murine anti-human CRP (1:500) and processed as above.

The experiment was repeated with RBC_N using CRP_{Tulip}-PC-Ca^{+2}. In parallel experiment, ghost membrane protein from malaria was co-incubated with a mixture of CRP_{Mal}-PC-Ca^{+2} complex and anti-CD35 and analysed separately using polyclonal rabbit anti-human CRP antibodies and processed as above.

D) Scatchard analysis. To quantify the complement-regulatory proteins on RBC due to binding with CRP, purified murine anti-human CD35, CD55, and CD59 mAb were iodinated separately using $Na^{125}I$ [42]. The specific activity was ~$2x10^6$ cpm/µg protein. Approximately 99% of the total radioactivity was observed in ice-cold TCA precipitation.

RBC_{Mal} ($1x10^9$cells/tube, n = 25) in TBS-BSA were incubated with CRP_{Mal}-PC-Ca^{+2} complex and subsequently incubated with increasing doses of ^{125}Ianti-CD35 for 1 h on ice in the presence or absence of a 50-fold excess of unlabeled anti-CD35 mAb. ^{125}I-anti-CD35 bound cells were washed and separated by centrifugation. Bound reactivity in the cell pellet was measured by a Gamma counter (Electronic Corporation of India). The number of CD35 molecules per cell was extrapolated by the intersection of the curve with the χ axis, and the association constant was obtained by dividing the number of receptors per cell by the bound/free ratio at the *y* axis intercepts [43]. Measurements were done in triplicates and represented data are means of three independent experiments. Quantification of CD55 and CD59 on RBCs was done similarly. In absence of CRP, RBC_{Mal} were probed with ^{125}Ianti-CD35, anti-CD55 and anti-CD59 separately and processed as above for comparison. RBC_N was similarly included in all the sets of experiment.

Analysis of activation of the CRP-complement pathway

A) Quantitation of C3-deposition on RBC. Purified anti-human C3α chain mAb, SIM 27-49 [44], was iodinated and used for measuring C3-deposition on RBC_{Mal} (n = 25) due to triggering of the CRP-complement pathway. RBC_{Mal}-CRP_{Mal}-PC-Ca^{+2} (5-20 µg/ml) complex were incubated with NHS (diluted 1:50 in TBS, 100 µl), as a source of complement, for 30 min at 37°C and washed twice in cold TBS, and the amount of C3-deposition on the RBC_{Mal} was quantified by incubating further with ^{125}I-anti-C3 mAb (3×10^5 counts/minute) for 1 h on ice. After washing, the radioactivity in C3–anti-C3-complexes was determined by Gamma counter. For comparison, CRP-mediated C3-deposition on RBC_N was also determined.

Anti-human CRP antibodies was used to block the binding of CRP with RBC_{Mal}. Reaction was also performed in absence of CRP under identical condition. EDTA (10 mM) was used to chelate Ca^{+2} required for CRP-PC-Ca^{+2}-complex essential for binding with RBC. In all these experiments NHS or MS (25%) or CRP-depleted MS were used as sources of complement. De-complemented NHS served as control.

To quantitate the C3-deposition on RBC_{Mal}-CRP_{Mal}-PC-Ca^{+2} (5-20 µg/ml) complex, cells were incubated with increasing doses of ^{125}I-anti-C3 mAb in the presence or absence of a 50-folds excess of unlabeled anti-C3 mAb and processed similarly. The association constant was calculated from Scatchard plot.

RBC of anemic patients showed increased surface IgG [39], accordingly, to block the C3-deposition through these IgG, RBC_{Mal} were pre-incubated with anti-human IgG and reaction was continued in presence of NHS under identical condition.

C3b-containing-immune complex is known to bind with CR1 (CD35) on RBC [45]. In order to block this binding, RBC_{Mal} were pre-incubated with anti-CD35 mAb, subsequently allowed these cells to bind with CRP_{Mal}-PC-Ca^{+2} (5-20 µg/ml) complex and NHS. The amount of C3-deposition on these cells was quantified as described above. Each set was repeated thrice.

B) Evaluation of CRP-complement pathway mediated hemolysis. RBC_{Mal}(n=25)-CRP_{Mal}(10 µg/ml)-PC-Ca^{+2} complex washed with ice-cold gelatin-veronal-buffered (GVB) saline supplemented with $MgCl_2$ (2 mM) and $CaCl_2$ (0.15 mM) and incubated for 30 mins at 37°C in the presence of NHS (diluted 1:50 in GVB buffer, 100 µl). The reaction was terminated with cold GVB buffer (1.0 ml), centrifuged and the absorbance (412 nm) in the supernatant was determined.

In parallel, hemolysis was also measured in all conditions as described for C3-deposition experiments. EGTA was used as activator of alternative pathway. EDTA was used as the blocker of all complement-pathways. Equal amount of RBC suspension in distilled water was set to 100%. Experiments were done in triplicates and reported as mean ± SD. For comparison, CRP-complement pathway mediated hemolysis was also carried out with RBC_N.

Statistical analysis

Statistical analyses were performed using the Graph-Pad Prism statistics software (San Diego). Results were reported as mean ± SD and values are 2-tailed, with $p < 0.05$ considered statistically significant.

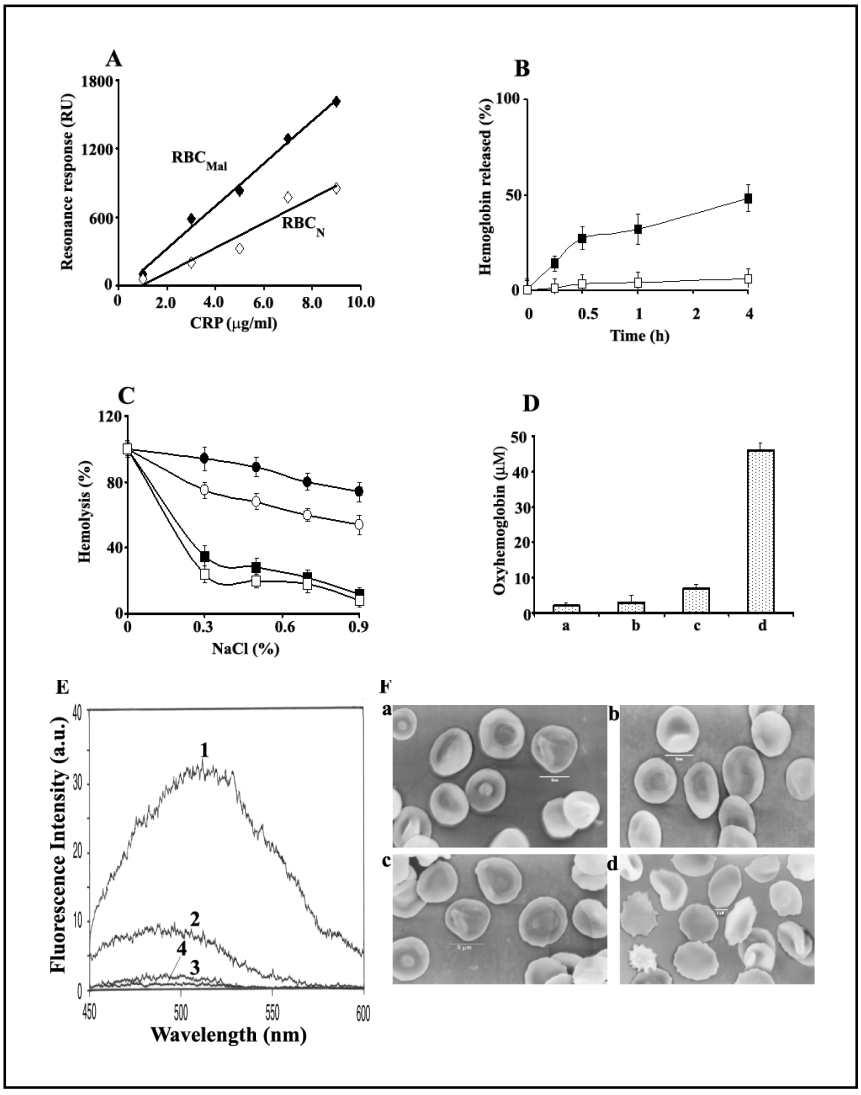

Fig. 2. Analysis of RBC-CRP binding: A. Standard curve of the RBC-CRP binding by surface plasmon resonance (SPR). Varying concentrations of CRP_{Mal} were immobilized onto the dextran CM5 chip. Membrane protein (50 μg/ml) from RBC_{Mal} (-◆-) or RBC_N (-◇-) was used as analyte. RBC-CRP binding was represented in arbitrary resonance response (RU). Fig 2B-F. Alteration of membrane parameters due to binding of RBC with CRP. B. Turbulence fragility. The turbulence fragility of RBC_{Mal} (-■-) was compared with normal RBC (-□-) by measuring the amount of released hemoglobin (%) with time (h). Absorbance was determined at $O.D_{412nm}$. C. Osmotic fragility. Fragility of RBC_{Mal} before (-○-) and after (-●-) binding of CRP_{Mal} compared to RBC_N before (-□-) and after CRP_{Mal} binding (-■-) was determined by incubating RBCs at different NaCl (%) as described in materials and methods. Data are represented in hemolysis (%) and expressed as mean ± SD of 4-5 independent experiments. D. Oxyhemoglobin concentration in the supernatant of (a) RBC_N, (b) RBC_{Mal}, (c) RBC_N-CRP_{Mal} and (d) RBC_{Mal}-CRP_{Mal} complex after exposure to superoxide radicals as described in materials and methods. Values are expressed as mean ± SD. E. Decreased hydrophobicity of RBC_{Mal} membrane due to CRP binding, RBC_{Mal} or RBC_N were incubated separately with CRP_{Mal}-PC-Ca^{+2} complex, washed and subsequently ANS were allowed to bind to the hydrophobic sites of RBC as described in materials and methods. The binding of ANS to RBC_{Mal}-CRP_{Mal} complex (curve 1), RBC_{Mal} (curve 2) and RBC_N (curve 3) and TBS buffer (curve 4) is shown as a measure of hydrophobicity. The graphs represent results from one of four independent experiments. F. Morphological changes of RBC after binding with CRP_{Mal} by SEM. Pictures of micrographs of RBC_N (a), RBC_{Mal} (c), RBC_N-CRP_{Mal} (b) and RBC_{Mal}-CRP_{Mal} (d) are shown. They were taken at magnification of 3500 and about 200 cells were observed.

Results

Higher binding of disease-associated CRP_{Mal} with RBC_{Mal} than RBC_N

Affinity-purified and well-characterized CRP_{Mal} (Fig. 1A-C) was used in all subsequent experiments. CRP_{Mal} that gave 3000 RU was immobilized on the CM5 chip. The association and dissociation phases of binding, along with representative sensograms were recorded. A concentration–dependant increase of RU value of CRP_{Mal}-RBC_{Mal} membrane protein binding was observed by immobilizing CRP_{Mal} (0-10 μg/ml) with a fixed concentration of RBC_{Mal} membrane protein (50 μg/ml)(Fig.2A). In contrast, RU for RBC_N membrane protein was lower. Significant difference between the slopes (140.5 RU/μg/ml) of CRP_{Mal}-RBC_{Mal} membrane protein binding as compared to CRP_{Mal}-RBC_N membrane protein (83.3 RU/μg/ml) indicated higher binding of CRP_{Mal} with membrane protein of RBC_{Mal} than RBC_N. The kinetic curves of binding of CRP_{Mal}-RBC_{Mal} membrane protein showed nearly 2.0-fold higher RU pick as compared CRP_{Mal}-RBC_N membrane protein (5-50 μg/ml) binding.

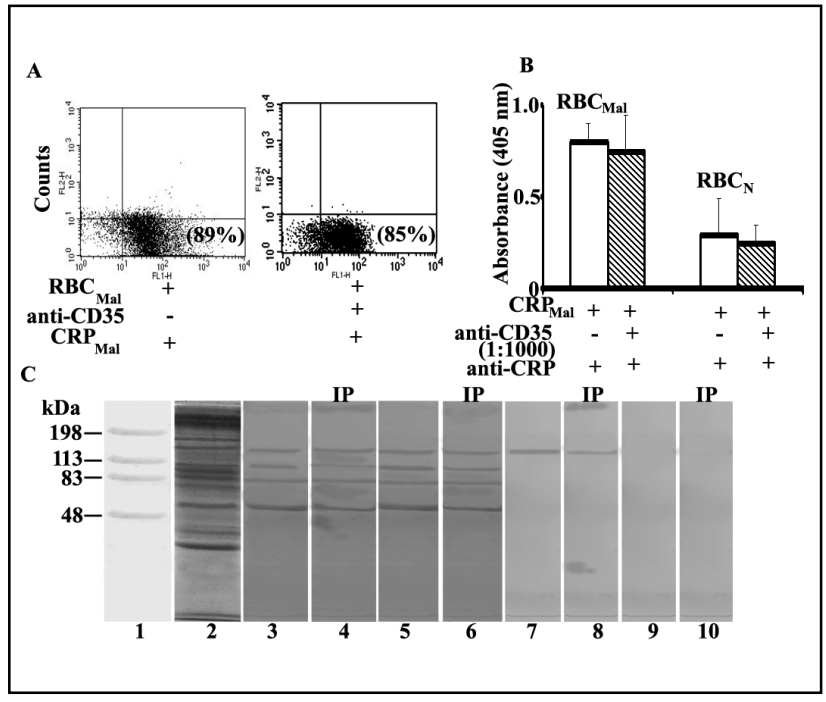

Fig. 3. The binding site for CRP on RBC is different from complement-regulatory proteins: A. Flow-cytometric analysis. The binding of RBC_{Mal} with FITC-CRP_{Mal} in absence and presence of anti-CD35 mAb was determined as described in methods. A representative dot-plot of binding is shown. B. ELISA. Equal amount of membrane proteins (2 µg/well/100 µl) from RBC_{Mal} or RBC_N were coated on 96-well microtitre plates separately, blocked and incubated with CRP_{Mal}-PC-Ca^{+2} complex. In parallel set, anti-CD35 mAbs (1:1000) and CRP_{Mal}(10 µg/ml)-PC-Ca^{+2} were co-incubated with immobilized ghost membrane protein. Bound complexes were probed with anti-CRP antibodies as described in materials and methods. C. SDS-PAGE, Western blotting and immunoprecipitation profile of RBC membrane protein after binding with CRP. Ghost membrane protein from RBC_{Mal} was electrophoresed (50 µg/lane) on SDS-PAGE (lane 2). The separated proteins were transferred to nitrocellulose membranes, blocked and incubated with CRP_{Mal}(10.0 µg/ml)-PC-Ca^{+2} complex. Washed membranes were subsequently, probed with anti-CRP antibodies and processed as described in materials and methods (lane 3). In parallel, CRP_{Mal}-PC-Ca^{+2} complex and anti-CD35 mAbs was simultaneously co-incubated to RBC_{Mal} and probed as above (lane 5). Ghost membrane proteins from a representative RBC_N (lanes 7) were electrophoresed and transferred to nitrocellulose membranes, blocked and incubated with either CRP_{Mal}- (lane 7) or CRP_{Tulip}-PC-Ca^{+2} complex (lane 9) and processed similarly. Lane 1: Pre-stained molecular weights marker. Lane 2-6: Membrane proteins from a representative RBC_{Mal}. Lane 2: visualized by Coomassie stain. Lane 3: Western blotting of RBC_{Mal} with CRP_{Mal}. Lane 4 Immunoprecipitation of RBC_{Mal} followed by binding with CRP_{Mal}. Lane 5: Western blotting of RBC_{Mal} with simultaneous co-incubation of CRP_{Mal}-PC-Ca^{+2} and anti-CD35 mAbs. Lanes 6: Immunoprecipitation of RBC_{Mal} with both CRP_{Mal}-PC-Ca^{+2} and anti-CD35 mAbs. Lane 7-10: Membrane proteins from a representative RBC_N. Lane 7: Western blotting of RBC_N with $CRP_{Mal.}$. Lane 8: Immunoprecipitation of RBC_N with CRP_{Mal}. Lane 9: Western blotting of RBC_N with CRP_{Tulip}. Lanes 10: Immunoprecipitation of RBC_N with CRP_{Tulip}.

Binding of CRP_{Mal} altered membrane parameters on RBC_{Mal}

In order to investigate any changes on the membranes due to the enhanced binding of CRP_{Mal} with RBC_{Mal}, different parameters were investigated. RBC_{Mal} showed nearly 40-50% of hemoglobin release as compared to 5-15% on RBC_N after 4h indicating less resistance of the membrane of patient against vigorous turbulent flow (Fig. 2B).

At normal physiological saline concentration, RBC_{Mal} was 3.36 ± 0.05-folds more osmotically fragile than RBC_N (Fig. 2C). However, after binding with CRP_{Mal}, additional 1.37 ± 0.14-folds increased osmofragility of RBC_{Mal} ($p<0.05$) was observed, signifying CRP-mediated additional membrane fragility of RBC_{Mal}. As expected, enhanced hemolysis was more pronounced at lower concentration (0.1-0.3%) of NaCl. Negligible differences in RBC_N were observed before and after binding with CRP_{Mal} suggesting minimal binding between CRP_{Mal} with RBC_N.

RBC-CRP complex were exposed to superoxide radicals generated by pyrogallol auto-oxidation. A ~6-folds more release of oxyhemoglobin was detected in RBC_{Mal}-CRP_{Mal} compared to RBC_N-CRP_{Mal}; being 45.6 ± 2 µM vs. 7.43 ± 0.25 µM respectively (Fig. 2D, $p<0.001$). Negligible release of oxyhemoglobin both by RBC_{Mal} (4.4 ± 2.5 µM) and RBC_N (1.12 ± 0.35 µM) was observed in the absence of CRP.

A 4-folds increased binding of ANS (expressed as arbitrary fluorescence intensity, a.u., Fig. 2E) with RBC_{Mal} (8 ± 3 a.u.) along with a resultant red shift from 490nm to 515nm of the emission maximum was observed after RBC_{Mal}-CRP_{Mal} (32 ± 5 a.u.) binding. It suggests a significant alteration in hydrophobicity, indicating asymmetry in the phospholipid membrane of RBC_{Mal}. Binding of ANS with RBC_N was negligible before (2 ± 1 a.u.) and after binding with CRP (3 ± 2 a.u.).

Membrane alteration induced by CRP was further corroborated from the ultrastructural morphological changes analyzed by SEM (Fig. 2F). RBC_{Mal} after binding

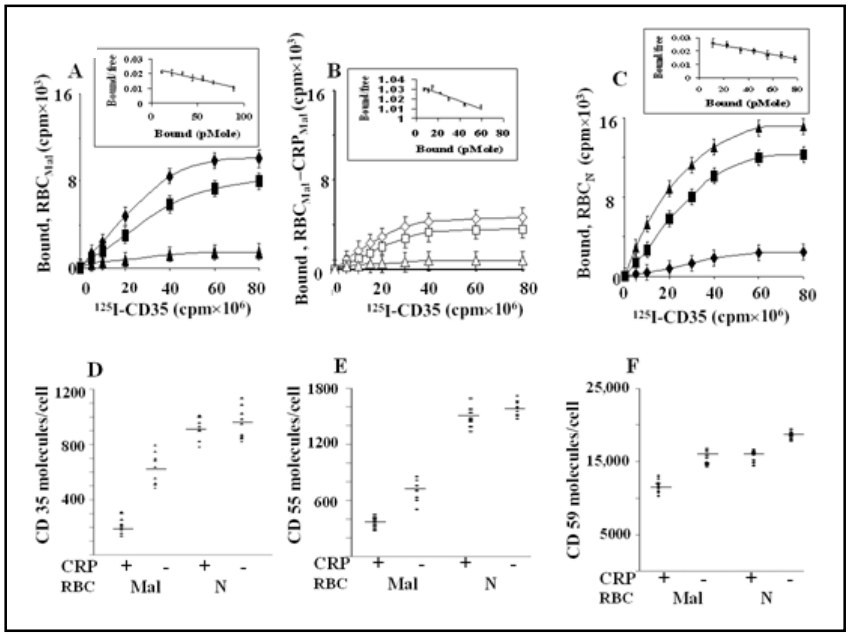

Fig. 4. Differential expression of complement-regulatory proteins on RBC by Scatchard analysis: A. Binding of ^{125}I-anti-CD35 to $RBC_{Mal.}$ RBC_{Mal} (1x10^9/tube) in TBS-BSA were incubated for 1 h on ice with increasing doses of ^{125}I-anti-CD35. The bound and unbound anti-CD35 was separated at 4°C and the bound radioactivity was determined as described in materials and methods and plotted against added ^{125}Ianti-CD35. To evaluate the specific nature of binding, a 50-folds excess of unlabeled antibodies was added. Specific-binding (-■-) was determined by calculating the difference between total binding (-◆-) and nonspecific binding (-▲-). Results are expressed as mean ± SD of data from triplicate experiments. Inset: Association constant (K_a) and the number of CD35 molecules per RBC_{Mal} were determined by Scatchard plots of the binding data. A representative Scatchard plot showing bound/free against the various amounts of bound ^{125}I-anti-CD35 is shown. B. Reduced complement-regulatory proteins on RBC_{Mal} in presence of $CRP_{Mal.}$ After binding of CRP_{Mal} with $RBC_{Mal,}$ the number of CD35 molecules/RBC_{Mal} were determined using ^{125}I-anti-CD35 as described in materials and methods. Specific-binding (-□-) was obtained by the difference between total binding (-◇-) and binding in presence of unlabeled anti-CD35 (-△-). Inset: Association constant of the binding of ^{125}I-anti-CD35 with RBC_{Mal}-CRP_{Mal} complex was determined from the Scatchard plot. C. Complement-regulatory proteins on RBC_N . Scatchard analysis was repeated similarly using RBC_N. Specific-binding (-■-) was calculated by the difference between total binding (-▲-) and binding in presence of unlabeled CD35 (-◆-). Results are expressed as mean ± SD of data from triplicate experiments. Inset: Representative Scatchard plot of the binding of ^{125}ICD35 to RBC_N in which bound/free was plotted against the various amounts of bound anti-CD35. D-F. Differential expressions of CD35 (D), CD55 (E) and CD59 (F) molecules on RBC from 10 individual patients, before or after exposure to $CRP_{Mal,}$ were shown. Similar studies with RBC_N (N=10) are presented before or after exposure to CRP. $p<0.001$ for the number of CD55 molecules on RBC_{Mal} before and after exposure to CRP, paired t test. $p<0.004$ for the number of CD35 molecules on RBC_{Mal} before and after exposure to CRP, paired t test. $p<0.07$ for the number of CD59 molecules on RBC_{Mal} before and after exposure to CRP, paired t test.

with CRP_{Mal} induced a drastic morphological change comprising different stages of biconcave, spherocyte, stomatocyte, echinocyte and irregular-shaped cells (Fig. 2F d). Nearly 55 ± 6 % deformed cells were found after binding with CRP_{Mal} as compared to only 10 ± 3% in CRP-untreated RBC_{Mal}. Only RBC_{Mal} showed some structural changes as compared to normal discoid-shape indicating partial damage in diseased condition (Fig. 2F c). RBC_N, on the other hand, before (Fig 2F a) and after (Fig 2F b) CRP-exposure retained the normal biconcave shape. These observations established that erythrocytes undergo considerable structural changes after interacting CRP.

Complement-regulatory proteins were different from binding sites for CRP_{Mal} on RBC_{Mal}

The status of complement-regulatory proteins and binding sites for CRP on the cell surface of RBC_{Mal} was investigated by flow cytometry. FITC-CRP_{Mal} showed strong binding with RBC_{Mal} both in presence and absence of anti-complement-regulatory proteins indicating that binding sites for CRP on RBC was different than complement-regulatory protein molecules (Fig. 3A). Only RBC or isotype controls gives no binding suggesting the binding was specific for CRP. The binding of FITC-CRP_{Tulip} with RBC_N was only 4 ± 1% (figure not shown).

Above observation was corroborated in a competitive ELISA, wherein CRP_{Mal} and anti-complement-regulatory proteins were simultaneously co-incubated with immobilized RBC_{Mal} membrane protein. Anti-human CRP antibodies was used to detect the bound complexes. The binding of RBC_{Mal} with CRP_{Mal} was comparable ($O.D_{405nm}$ being 0.84 ± 0.04) in presence and absence of anti-CD35 antibodies (Fig. 3B) suggesting no apparent competition between CD35 molecule and binding site for CRP on RBC_{Mal}. Probing with anti-CRP antibodies,

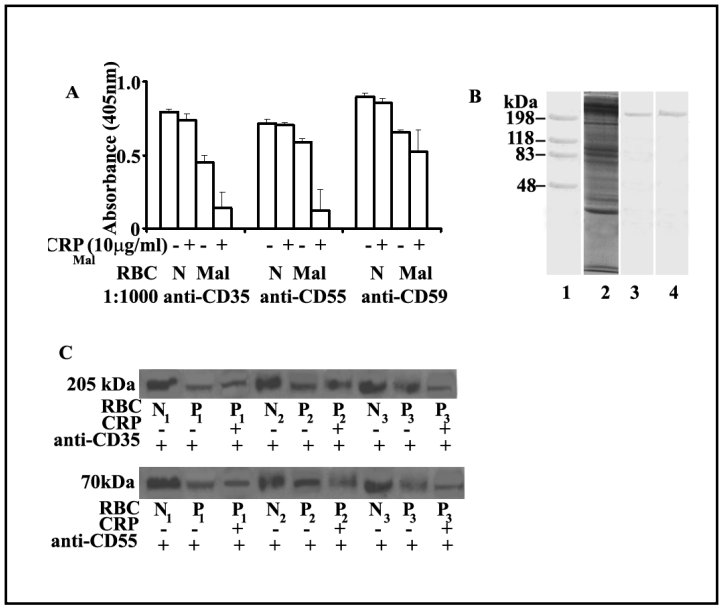

Fig. 5. Down regulation of complement-regulatory proteins on RBC_{Mal} after binding with CRP_{Mal}: A. ELISA. RBC_{Mal} and/or RBC_N (2 μg/well/100 μl) were coated on 96-well microtitre plate. The number of CD35, CD55 and CD59 were quantified in absence and presence of CRP_{Mal}-PC-Ca^{+2} using anti-complementary regulatory protein antibodies and detected using HRP-anti-murine IgG as described in methods. Data are expressed as mean ± SD. B-C. Western blot analysis. B. RBC_{Mal} were electrophoresed on SDS-PAGE and visualized by Coomassie stain (lane 2). The protein was transblotted onto nitrocellulose. The blot was probed with purified anti-CD35 mAb (lane 3) In parallel, transblotted RBC_{Mal} was co-incubated with CRP_{Mal}(10.0 μg/ml)-PC-Ca^{+2} complex followed by addition of anti-CD35 mAbs (lane 4). Bound complex in both the blots were detected with HRP-goat-anti-murine IgG antibodies as described in materials and methods. Lane1: Molecular weight markers. C. RBC_N from three individuals (N_1, N_2 and N_3) probed with either purified anti-CD35 mAb or anti-CD55 mAb. In parallel, transblotted RBC_{Mal} from three patients (P_1, P_2 and P_3) with anti-CD35 mAbs or anti-CD55 mAb separately were shown. In parallel, transblotted RBC_{Mal} (P_1, P_2 and P_3) was co-incubated with CRP_{Mal}(10.0 μg/ml)-PC-Ca^{+2} complex and anti-CD35 mAbs or anti-CD55 mAb separately. Bound complex in both the blots were detected with HRP-goat-anti-murine IgG antibodies as described in materials and methods.

no significant variations in the absorbance were observed with increasing concentration of anti-CD35 antibodies (1:4000 to 1:100), implying no competition for the binding of anti-CD35 and CRP on RBC_{Mal}. Similar result was observed in presence of anti-CD55 and anti-CD59 antibodies.

The molecular identity of the specific protein on the RBC membrane that reacts with CRP_{Mal}, revealed four distinct bands corresponding to 112, 82,149 and 57 kDa on RBC_{Mal} by Western blotting analysis (Fig. 3C, lane 3). In parallel, when the RBC_{Mal} membrane blot was co-incubated with both CRP_{Mal} and anti-human CD35 antibodies similar four band profiles were observed (lane 5).

To check whether CRP_{Mal} can bind with the native structure of RBC membrane protein, an immunoprecipitation experiment was designed. The specific molecules present on the membrane that reacts with CRP_{Mal} were immunoprecipitated and analyzed by Western blotting. Immunocomplex of RBC_{Mal} membrane proteins with CRP_{Mal} showed same four bands (lane 4). Co-immunoprecipitated RBC_{Mal} membrane proteins after co-incubating with both CRP_{Mal} and anti-CD35 antibodies also showed similar four proteins (lane 6).

In contrast, only a 149kDa protein was observed on membrane of RBC_N reacting with CRP_{Mal} (lane 7). Similar band on RBC_N membrane proteins was immuno-precipitated, which reacted with CRP_{Mal} (lane 8). This common band was found also on RBC_{Mal}. Thus, this may not be a disease-associated molecule. No band was detected after reacting RBC_N membrane with CRP_{Tulip} before (lane 9) and after (lane 10) immunoprecipitation.

Decrease in complement-regulatory proteins on RBC_{Mal} after binding with CRP_{Mal}

The number of complement-regulatory proteins was always less on RBC_{Mal} compared to RBC_N ($p<0.05$) (Fig. 4-5). Next, we have explored the possible role of CRP-induced perturbed membrane structure of $RBC_{Mal,}$ to assess the status of CD35, CD55 and CD59 molecules using anti-CD35/CD55/CD59 antibodies as respective probes by Scatchard analysis (Fig. 4), flow-cytometry, Western blot (Fig. 5B-C) and ELISA (Fig. 5A), Accordingly, after specific-binding of RBC_{Mal} with $CRP_{Mal,}$ the status of complement-regulatory proteins on RBC_{Mal} was monitored.

After binding with $CRP_{Mal,}$ the presence of less number of CD35/RBC_{Mal} was reduced from 630 ±124 to 255 ± 58 as revealed by Scatchard analysis (Fig. 4A-B, $p<0.001$). Similarly, decreased number of CD55/RBC_{Mal} was observed after binding with CRP_{Mal}; reduction being from 700 ± 185 to 363 ± 194. The association constant (Ka) of the binding of ^{125}Ianti-CD35 or ^{125}Ianti-CD55 or ^{125}Ianti-CD59 with respective CD35 or CD55 or CD59

Association constant Ka (M^{-1})	RBC_{Mal}		RBC_N	
	CRP_{Mal}			
	+	-	+	-
^{125}I anti-CD35	$2.7 \pm 0.21X\ 10^3$	$4.79 \pm 0.45X\ 10^6$	$2.84 \pm 0.12X\ 10^7$	$3.65 \pm 0.85X\ 10^7$
^{125}I anti-CD55	$1.63 \pm 0.65X\ 10^4$	$2.24 \pm 0.72X\ 10^6$	$3.85 \pm 0.28X\ 10^7$	$4.99 \pm 0.31X\ 10^7$
^{125}I anti-CD59	$4.45 \pm 0.58X\ 10^6$	$8.15 \pm 0.64X\ 10^7$	$5.21 \pm 0.74X\ 10^7$	$5.79 \pm 0.47X\ 10^7$

Table 1. Association constants of complement-regulatory proteins /RBC_{Mal} after binding with CRP. RBC was incubated with radioactive anti-complement-regulatory proteins mAbs. A 50-folds excess of unlabeled mAbs was used to evaluate specific nature of binding. Bound radioactivity was measured. The apparent Ka values and numbers of complement-regulatory proteins per cell were calculated from Scatchard analysis.

molecules on RBC were shown in absence and presence of CRP (Table 1). In presence of CRP, the Ka of these three molecules for their respective antibodies was reduced probably due to altered membrane parameters of RBC_{Mal}.

However, the number of CD59/RBC_{Mal} did not change appreciably even after binding with CRP_{Mal} as corroborated by earlier reports [39]. Similar patterns were observed in all the patients included in this study ($p<0.01$), as shown for 10 individual patients, before and after CRP binding, along with 10 normal volunteers (Fig. 4D-F). The number of complement-regulatory molecules/cell on RBC_N in presence and absence of CRP was more or less constant (Fig. 4C).

The observation was further corroborated by FACS analysis. A decrease in the $CD35^+RBC_{Mal}$ from $60 \pm 4\%$ to $20 \pm 5\%$ was observed after sensitizing with CRP_{Mal}. Similar decrease in the $CD55^+RBC_{Mal}$ cells from $78 \pm 3\%$ to $17 \pm 4\%$ was always observed on RBC_{Mal}. In contrast, decrease in $CD59^+$ cells was low from $86 \pm 3\%$ to $60 \pm 5\%$.

The decrease of number of CD35 molecules/cell before and after CRP-treatment was 599 ± 124 to 215 ± 101 whereas the decrease of CD55 molecule/cell was from 685 ± 154 to 363 ± 126. However, no significant change in CD59 molecules/cell was observed; values being $16{,}218 \pm 2214$ and $14{,}881 \pm 1762$ respectively. In contrast, insignificant loss of complement-regulatory proteins was observed on RBC_N in presence of CRP_{Mal}.

Similar trend was further corroborated by an inhibition-ELISA (Fig. 5A) wherein anti-CD35 and CRP_{Mal} were added simultaneously on immobilized RBC_{Mal} membrane proteins. Bound complexes were detected using secondary antibody specific for the measurement of complement-regulatory proteins. Binding of anti-CD35 with RBC_{Mal} was significantly reduced in presence of CRP as reflected in the decreased $O.D_{405nm}$ of 0.139 ± 0.035 from 0.449 ± 0.057 in absence of CRP demonstrating lowering of these molecules due to RBC_{Mal}-CRP binding (Fig. 5A). Decrease in CD55 molecules were also observed in presence of CRP. Small differences in absorbance both in presence and absence of CRP signify less reduction of CD59 molecules. In contrast, no major changes in the absorbance were observed before and after binding of CRP_{Mal} with RBC_N (Fig. 5A).

In order to pinpoint the effect of CRP, the ELISA was repeated with varying concentration of anti-CD35 antibody (1:100 to 1:4000) with a fixed concentration of CRP. A decrease in the absorbance was observed with increase in dilution and reached a plateau after 1:1000. Similar result was obtained using anti-CD55 antibody. In contrast, no major changes in absorbance were observed with anti-CD59 antibody. In a competitive ELISA, where anti-CD35, CD55 and CD59 antibody concentration were titrated, keeping the concentration of CRP constant, the binding of RBC decreases with decreasing concentration of anti-complement-regulatory proteins. Therefore, it may be envisaged that there was no apparent steric hindrance created by bound CRP. However, the possibility of steric hindrance could not be ruled out.

Western blot analysis using anti-CD35 antibodies, as an analytical probe, showed a single band (205 kDa) on RBC_{Mal} in presence and absence of CRP, consistent with the reported molecular weight of CD35 on RBC_N (Fig. 5B). Therefore, CRP_{Mal} is possibly not competing to bind with CD35 molecules on RBC. Similar result was observed with anti-CD55 and anti-CD59 antibodies.

Similar patterns were observed in all the patients included in this study. A representative profile of three patients, before and after CRP binding, along with three normal volunteers has been shown (Fig. 5C). Normal individuals showed no major differences before and after CRP-treatment. The complement-regulatory molecules/cell on RBC_N in presence and absence of CRP was stronger than on RBC_{Mal}.

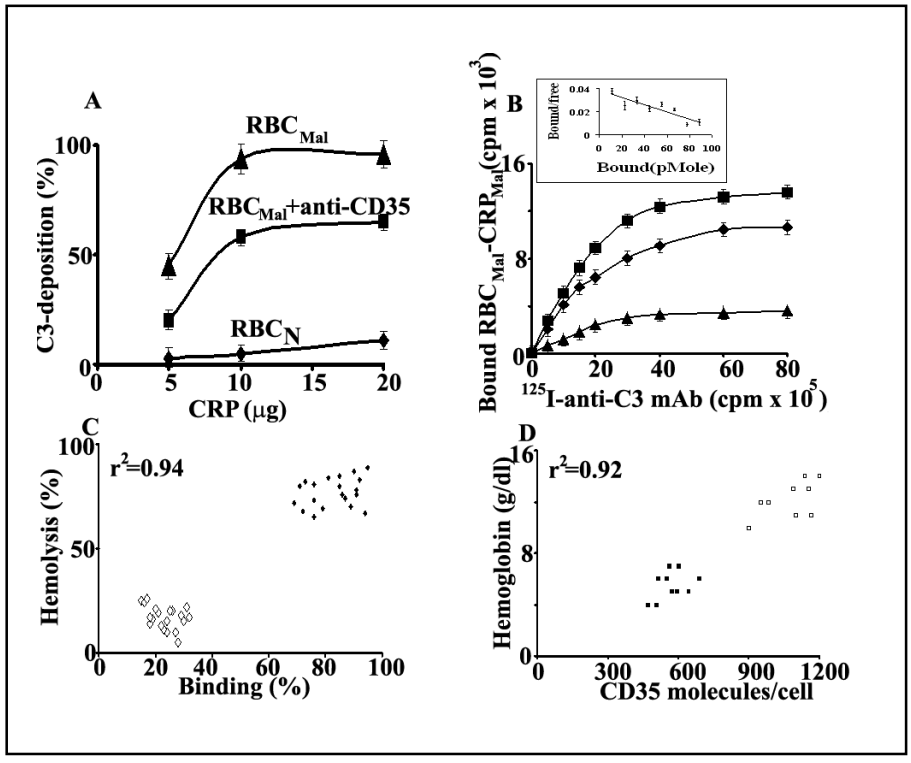

Fig. 6. Activation of the CRP-complement pathway: A. Estimation of C3-deposition. RBC-CRP$_{Mal}$-PC-Ca^{+2} complex (-▲-) was incubated at 37°C for 30 min with NHS as source of complement. Activation of CRP-mediated complement pathway was monitored by C3-deposition on RBC using ^{125}I-anti-C3 mAb as described in materials and methods. C3-deposition is represented as percentage of specific-binding of anti-C3 mAb (mean ± SD of triplicate values). Additionally, RBC$_{Mal}$ of same patient was initially incubated with purified anti-CD35 followed by binding with CRP$_{Mal}$-PC-Ca^{+2} complex (-■-) and C3-deposition was compared in presence of NHS. Deposition of the C3 on RBC$_N$ (-◆-) without pre-incubation with CD35 is shown. B. Scatchard analysis of deposited C3-molecules on RBC: RBC$_{Mal}$ (1x10^9)-CRP$_{Mal}$(10.0 μg/ml)-PC-Ca^{+2} complex was incubated with NHS at 37°C for 30 min. Activation of CRP-complement pathway was monitored by C3-deposition on RBC surface using increasing amounts of ^{125}I-anti-C3 mAb as described in materials and methods (mean ± SD of data from triplicate experiments). Specific-binding (-◆-) was calculated by the difference between total binding (-■-) and binding in presence of unlabeled anti-C3 mAb (-▲-). Inset: Scatchard plot of the binding of ^{125}I-anti-C3 mAb to RBC$_{Mal}$. C. CRP-complement-mediated hemolysis. Correlation (r^2=0.94) between CRP-complement pathway-mediated hemolysis (%) and binding (%) of RBC from 10 individual patients with CRP$_{Mal}$ (-◆-) are shown. Similar parameters were shown with RBC$_N$ (-◇-). Results are expressed as mean of ± SD of data from different experiments. D. Correlation (r^2=0.92) between hemoglobin concentration (g/dl) and number of CD35 molecules on RBC$_{Mal}$ from 10 individual patients (-■-, n=10). Similar parameters with RBC$_N$ (-□-) are shown for comparison.

CRP triggered complement-cascade with concomitant C3-deposition

The potential of CRP$_{Mal}$ to activate the complement pathway was investigated in the presence of NHS, by quantifying C3-deposition using ^{125}Ianti-C3 mAbs. A gradual increase in C3-deposition on RBC$_{Mal}$ was observed with increasing concentrations of CRP$_{Mal}$ (Fig. 6A). At physiological concentration (10 μg/ml), CRP is capable of inducing maximum C3-deposition (90 ± 6%) on RBC$_{Mal}$. A 50-folds excess unlabelled anti-C3 mAbs competed with the binding of ^{125}Ianti-C3 mAbs (Fig. 6B). The apparent association constant (Ka) for C3-deposition triggered by CRP was 1.13 ± 0.89x10^7 M^{-1} and the number of C3-molecules bound per cell was 2.5 ± 0.45x10^6 as determined by Scatchard analysis. On the other hand, significant low C3-deposition was observed on RBC$_N$ (10±2%), possibly due to low binding of CRP$_{Mal}$ and as a result less activation of complement.

To further demonstrate the role of CRP$_{Mal}$, RBC$_{Mal}$-CRP$_{Mal}$ binding was initially blocked by using either anti-CRP antibodies or EDTA separately and subsequently their effect on C3-deposition was investigated. Preincubation of CRP$_{Mal}$ with anti-CRP antibodies showed reduced C3-deposition from 90 ± 6% to 49 ± 8%. Similarly, EDTA was used to inhibit the Ca^{2+} dependent binding of CRP$_{Mal}$ with RBC$_{Mal}$ resulting only 50 ± 6% C3-deposition. In absence of CRP$_{Mal}$, C3-deposition through pathways other than CRP was only 52 ± 4%, suggesting ~42-45% of C3-deposition possibly through CRP$_{Mal}$-complement pathway.

CRP is able to activate the classical complement pathway serving as a potential link between complement activation and innate immune system [20]. However, CRP is unable to trigger the alternate pathway [46]. As EGTA is known to trigger alternate pathway, C3-deposition through this pathways was measured, being only 23 ± 3%. However, even in presence of EGTA, CRP could not trigger C3-deposition (S1). However, even in presence of EGTA, CRP could not trigger alternate pathway mediated C3-deposition, being 2 ± 1%.

When serum from normal (NHS) and malaria patients (MS) were used as sources of complements separately, comparable C3-deposition in presence of CRP_{Mal} was observed on RBC_{Mal} being 92±2% and 90±6% respectively. This suggested undisturbed complement components in diseased state, as observed by our previous reports in other diseases [46-47]. Similar result was observed in presence of CRP-depleted patient's sera (91±5%). In contrast, as expected, heat inactivated NHS unable to deposit C3-molecules on RBC_{Mal} both in presence (4±2%) and absence of CRP_{Mal} (2±1%). Only buffer showed negligible reading.

Additionally, to explore the role of CD35 in CRP-mediated C3-deposition, RBC_{Mal} was initially incubated with anti-CD35 and subsequently with CRP_{Mal} (Fig. 6A). C3-deposition was reduced from 90 ± 6% to 70 ± 5%. Both NHS and MS, used as source of complements separately, showed comparable reading. Anti-CD35 antibodies possibly acted as a surrogate for C3-deposition by binding with C3-bound Immune complexes (ICs). Approximately 20-22% of the C3-deposition may be considered to be through CR1-mediated erythrophagocytosis and anti-CD35 antibodies.

CRP-mediated complement-pathway triggered enhanced hemolysis of RBC_{Mal}

Though the interplay of a several pathways has been reported for hemolysis of RBCs (S2), our aim was to dissect the CRP-mediated complement-cascade. Accordingly, the fate of RBC_{Mal}, after CRP-mediated C3-deposition, was investigated, using NHS as complement source (Fig. 6C). A significantly higher CRP-mediated hemolysis of RBC_{Mal} was observed compared to RBC_N; being 85 ± 6% vs.13 ± 8% ($p<0.001$). Higher binding of RBC_{Mal} with CRP, even at normal physiological concentration, is possibly responsible for such enhanced hemolysis. A good correlation was observed between the binding of CRP_{Mal} with RBC_{Mal} and their respective hemolysis ($r^2=0.94$).

Comparable hemolysis was observed using patients' serum (87 ± 4%), CRP-depleted serum (82 ± 7%) and NHS as sources of complement, signifying the *in vivo* scenario. As CRP-mediated complement-activation requires Ca^{+2}, addition of EDTA to chelate Ca^{+2} causes drastic reduction of hemolysis (24 ± 7%). Reduced hemolysis (30 ± 8%) was also observed in presence of anti-CRP antibodies establishing the direct role of CRP. De-complemented serum, as expected, showed complete abolition of hemolysis (3 ± 1%). In contrast, in absence of CRP, when all other pathways are unblocked, only 26 ± 4% hemolysis of RBC_{Mal} was observed under identical condition.

Additionally, alternative pathway-mediated hemolysis was 20 ± 3% as triggered by EGTA. However, in presence of EGTA, CRP could not triggered alternate pathway-mediated hemolysis (2 ± 1%). Negligible hemolysis (4 ± 2%) was observed when EDTA was used suggesting blocking all complement pathways. Taken together, we have conclusively demonstrated a new mechanism of CRP-mediated complement pathway, for the clearance of damaged erythrocytes in malaria (S1).

Role of CRP in IgG-mediated reduction of complement-regulatory proteins

Human CRP bears functional similarity with human IgG [29, 45]. IgG after its deposition on RBC_{Mal} is reported to reduce the levels of complement-regulatory proteins [39]. Therefore, we wanted to explore the role of CRP in IgG-mediated reduction of complement-regulatory proteins. Accordingly, we have checked the binding of CRP_{Mal} with RBC_{Mal} using anti-CRP antibodies as detecting probe. Comparable absorbance in presence (0.91 ± 0.05) and absence (0.87 ± 0.04) of physiological concentration of IgG (10 mg/ml) was observed. The binding IgG with RBC_{Mal} in presence (0.83 ± 0.03) and absence (0.77 ± 0.06) of CRP was comparable as detected by anti-human IgG. As RBC_{Mal}-CRP_{Mal} binding could not be inhibited by IgG, it may be envisaged that binding pocket for IgG is different from that for CRP on RBC_{Mal}.

IgG is known to mediate C3-deposition on RBC [45]. C3-deposition was 90 ± 6% in presence both CRP and IgG. To block IgG mediated C3-deposition, RBC_{Mal} was pre-incubated with anti-human IgG and subsequently incubated with CRP_{Mal}. C3-deposition was decreased from 90 ± 6% to 80 ± 8% indicating only 8-11% C3-deposition through human IgG. However, in absence of both CRP and IgG, hemolysis was 25 ± 4%. Thus contribution of CRP, IgG, CD35 and alternate pathway in C3-depositions was 42-45%, 8-11%, 20-22% and 20-23% respectively; 7-10% may be accounted for other unidentified pathways.

Discussion

The pathogenesis of malarial anemia is multi-factorial. The patients, included in this study, had low hemoglobin concentration (4.5-7.8 g/dl). Our aim was to address for clues of immunologic mechanisms and to identify some

key-players accounting for premature clearance of RBC contributing towards hemolysis resulting anemia. Surface-membrane alterations on RBC_{Mal} suggested occurrence of damaged cell surface (Fig. 2B-F). This may be responsible for higher binding of damaged RBC_{Mal} with CRP_{Mal} (Fig. 2A), which causes further alterations of these membranes (Fig. 2B-F). Additionally, we have dissected a number of pathways regulating RBC destruction which possibly contributing towards anemia due to higher binding of damaged RBC_{Mal} with CRP (S2).

To protect against complement-mediated cell lysis, RBC_N, in general, expresses more complement-regulatory proteins on their surface [39]. The expressions of CD35 molecules on RBC_{Mal} were significantly low as compared to RBC_N ($p<0.001$). The reduction was further enhanced due to specific-binding of CRP_{Mal} with RBC_{Mal}. A good correlation ($r^2=0.92$) between hemoglobin concentration and CD35 molecules/cell on RBC_{Mal} (Fig. 6D) suggested that the RBC_{Mal} are more prone to hemolysis and probably may crave the pathway to anemia in the milieu of malaria infection. Thus, it conclusively supports the idea that CRP_{Mal} possibly is an important contributor to the accelerated destruction of RBCs and may thus help to remove damaged RBC in malaria.

The presence of damaged RBCs in malaria compared to normal (Fig. 2B-F) may be due to reactive oxygen species (ROS) [49] or stress or disease-induced stimulators or key functioning of some enzymes in inflamed condition [50] with externalization of phosphatidylserine (PS). It may also be exposed following RBC interaction with leucotriens of activated neutrophils. This damage leads to molecular modifications of its outer cell membrane causing a flip-flop between the membrane bilayer, resulting in enrichment of the outer leaflet in phosphatidylserine and phosphatidylethanolamine and/or band 3. Thus the membrane phospholipids become susceptible to hydrolysis by secretory phospholipase, generating lysophospholipids and lysolecithin.

At sites of inflammation and tissue necrosis, CRP binds to cell membranes of damaged and necrotic cells, but not with normal cells. In biological membranes, PC is present in the outer layer as the polar head group of lecithin and sphingomyelin. Due to the presence of lysolecithin within the outer layer, more CRP can bind to the PC of lecithin [51-52]. Such binding required the addition of submicellar concentrations of lysolecithin, establishing requirement for cell damage for binding of the protein to cell membranes at inflammatory sites. PC might however, be altered, and exists in a different pattern in cell membrane.

The previous study from our laboratory conclusively demonstrated glycosylated molecular variants of human CRP in different pathological conditions including malaria [16, 27]. CRP is not only produced from hepatic cells but also from local inflammatory cells [16, 53]. It is suggested that upregulation/downregulation of some enzymes or cytokines or their modulators or other extra-cellular signaling molecules in local inflammatory cells possibly responsible for induction of altered CRP during malaria infection (S1). The possibility of induction of some post-translational enzyme regulation cannot be ruled out as preferential expression of certain genes in the presence of toxic materials, released by parasites, may facilitate the changes in CRP_{Mal} during post-translational modification. The small changes observed in CRP_{Mal} (Fig. 1) are possibly crucial for specific biological functions *in vivo*. The minute changes observed in CRP_{Mal} as compared to CRP_{Tulip} make these two molecules different but their native pentraxin structures remained almost the same (Fig. 1).

Circulating IgG could modify RBC membrane [39]. As CRP reported to share many functional properties with IgG, therefore, it was worthwhile to identify the probable role of CRP_{Mal} in modulating the surface membrane of RBC_{Mal}. Significant alterations of their membranes were conclusively demonstrated (Fig. 2) wherein a few specific molecules (112, 82,149 and 57 kDa) on RBC_{Mal} were seen after binding with CRP_{Mal} (Fig. 3). A common protein (149 kDa) both on RBC_{Mal} and RBC_N may not be a disease-associated molecule. These CRP-binding molecules are distinct from complement-regulatory proteins on RBC (Fig. 3).

Surface deposition of parasite antigen on RBC_{Mal} [49] and the binding of CRP with parasite antigens [54] suggested that a few of these proteins might be parasite-derived protein (Fig. 3C). The RBC_{Mal}, used in our experiment, comprises both infected and uninfected population, which is to some extent damaged due to altered membrane parameters in RBC_{Mal} as compared to normal (Fig. 2). Due to damage imposed on this RBC_{Mal}, probably the conformation or mosaics of distribution of CRP-binding molecules are altered. Further investigation on the nature of these CRP-binding molecules is underway.

Our observation supports the idea that the surface abnormalities affect both infected and uninfected RBCs because the fluorescence histograms were unimodal and not bimodal. We were unable to detect two separate populations of cells with drastically different fluorescence from each other. Furthermore, surface changes observed in SEM are unlikely to come from the sole contribution of

only 7-8% infected red cells. The small percentage of infected cells could not contribute towards such lowering of complement-regulatory proteins after CRP binding. As RBC_{Mal} are both stressed and damaged, they are possibly quite different from RBC_N. Therefore, it is quite possible that the major causes of anemia are the destruction of large number of uninfected along with infected RBCs, which enhanced in the presence of CRP_{Mal}.

CRP behaving as a lectin [55] may affect the membrane features such as fluidity. When RBC_{Mal} were exposed to superoxide radicals, they released ~10-folds more of oxyhemoglobin (Fig. 2D) in presence of CRP_{Mal} as compared to its absence suggesting enhanced susceptibility of RBC_{Mal} by superoxide damage. Still other mechanism of the changes in membrane components might be connected with the production of oxygen free radical species [56].

RBC_N are protected from autologous complement-mediated lysis by membrane-anchored complement-regulatory proteins. Deficiencies of these proteins on RBC are common in hemolytic anemia [57], paroxysmal nocturnal hemoglobinuria [58], systemic lupus erythematosus and other autoimmune diseases [59]. We also found similar deficiencies in malaria patients, at diagnosis, which were more pronounced in presence of CRP_{Mal} (Fig. 4-5). However, in contrary to 2-4-folds reduction of both the CD35 and CD55 in presence of CRP, the changes of CD59 were marginal (Fig. 4-5) as corroborated by earlier reports [39]. The mosaics of distribution of these molecules on cell surface are altered with a possible slight change in their conformation resulting in decrease in affinity. So, evidently in presence of CRP, the Ka of these molecules is lowered. Also the possibility of steric hindrance created by CRP molecule could not be ruled out. However, many theories are underway to propose for the reduction of complement-regulatory proteins. These molecules are decreased possibly due to partial or complete cleavage by proteases and/or phospholipases released by schizonts [39]. Whatever may be the mechanisms responsible for these changes, reduction of these molecules are quite common in *Pf* infection which clearly demonstrated CRP-mediated enhanced reduction of complement-regulatory proteins elucidating an underway mechanism for the pathology of anemia. However, role of bone-marrow dysfunction, genetic disorder and other factors may not be ruled out.

CR1 has recently implicated in rosette formation [6]. CRP, by lowering its number, may prevent the adhesion of RBC_{Mal}, which is possibly initiated by the parasites. As CR1 is known to bind with C3b to opsonize RBC, thus the contribution of CR1 was analyzed in CRP-triggered C3-deposition and subsequent hemolysis (S1). Destruction of RBC may occur via different mechanisms involving the interaction with parasite antigens, anti-parasite antibodies, immune complexes (ICs) and complement degradation products present in circulation, by erythrophagocytosis, complement-attack, or the removal of RBC-bound antibodies or ICs [S2, 60-65].

Initial study hinting CRP-mediated hemolysis [27], led us to relate its induced level and postulate a mechanism of CRP-mediated fate of RBCs. In conclusion, binding of CRP_{Mal} through a few specific molecules on RBC_{Mal} lowered the number of complement-regulatory proteins and thus accelerates the removal of the damaged RBCs from circulation by initiating complement-cascade. To the best of our knowledge, this is the first report to correlate CRP and complement-regulatory proteins with the pathology of Pf anemia.

Abbreviations

BSA (Bovine serum albumin); CRP (C-reactive protein); CRP_{Mal} (Purified CRP from patients with malaria); ELISA (Enzyme-linked immunosorbent assay); EDTA (Ethylene diamine tetra acetic acid); EGTA (Ethylene Glycol tetra acetic acid); FACS (Fluorescence activated cell sorter); FITC (Fluorescein isothiocyanate); HRP (Horseradish peroxidase); IgG (Immunoglobulin G); MS (Serum from malaria patients); mAb (Monoclonal antibody); Mal (Malaria); NHS (Normal human serum); *Pf (Plasmodium falciparum)*; PC (Phosphocholine); PAGE (Polyacrylamide gel electrophoresis); PE (Phycoerythrin); RBC (Red blood cells); RBC_{Mal} (Red blood cells from malaria); RBC_N (Red blood cells from normal individuals); RBC-CRP complex (RBC were complexed with CRP_{Mal}); SDS (Sodium dodecyl sulphate); SEM (Scanning Electron Microscopy); SPR (Surface Plasmon Resonance); TCA (Trichloroacetic acid).

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