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Role of C-reactive protein in complement-mediated hemolysis in Malaria

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Abstract Human C-reactive protein (CRP) is a clinically important classical acute phase protein. Although CRP has been reported to bind with many nucleated cells, the direct binding of CRP to erythrocytes in diseases remains largely unexplored. The main focus of the present study was to investigate the binding of disease-specific CRP to erythrocytes of same patients. Distinct molecular variant of disease-specific CRP was affinity purified from sera of malaria patients (CRP_{Mal}). This CRP showed strong binding with malaria erythrocytes (RBC_{Mal}) as confirmed by flow cytometric analysis (FACS), enzyme-linked immunosorbent assays (ELISA), and radio binding assays. Calcium and phosphoryl choline (PC) were found to be essential for this interaction. A 2.3-fold increased binding of induced CRP to RBC_{Mal} as compared to normal erythrocytes (RBC_N) confirmed disease-specificity. Preincubation of RBC_{Mal} with unconjugated CRP showed 3–5 fold inhibition. The association constant of CRP and RBC_{Mal} was 4.7×10^6 cpm/μg with the corresponding number of receptors/cell being 4.3×10^5. The effector function of CRP_{Mal} has been demonstrated by its potency to activate the complement pathway. An optimal dose of 10 μg/ml of CRP induced three-fold higher hemolysis of patient erythrocytes as compared to RBC_N. These studies provide direct evidence for an important phagocytic functional interaction of this acute-phase protein by triggering the CRP-complement pathway after the binding of CRP_{Mal} with RBC_{Mal}. Hemolysis as triggered by this pathway may be one of the causative factors of anemia, a common clinical manifestation of this disease.

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Abbreviations

CRP	C-reactive protein
CRP_{Mal}	Purified CRP from patients with malaria
PC	Phosphorylcholine
EDTA	Ethylene diamine tetra acetic acid
PAGE	Polyacrylamide gel electrophoresis
HRP	Horseradish peroxidase
SDS	Sodium Dodecyl sulphate
FITC	Fluorescein isothiocynate
FACS	Fluorescence activated cell sorter
IgG	Immunoglobulin G
RBC_{Mal}	Erythrocytes from Malaria
RBC_N	Erythrocytes from normal individual

Introduction

CRP is a major acute phase protein in human. The level of this conserved protein is low (0.1–0.5 μg/ml) under normal conditions, but increases up to a 1000-fold during inflammation or tissue injury [1]. CRP shows the ability to bind with pneumococcal cell wall polysaccharide through phosphoryl choline (PC) in a calcium dependent manner [2]. In addition to PC, we have recently demonstrated a new ligand, Protein-A, for human CRP, establishing its extended definition [3]. It is also capable of binding with a wide range of ligands including certain membrane phospholipids, polycations, chromatin and to various naturally occurring biological substrates [4,5]. In addition, binding of ligand-complexed CRP to human neutrophils and monocytes is well documented [6,7]. A number of biologically important functions have been

assigned to this phylogenetically ancient protein, though its precise physiological role still remains uncertain. Being an important component of the innate immune system, CRP is able to recognize damaged cells of the host to help with their elimination. It can activate the complement pathway as an opsonic protein or opsonization by binding with its ligand leading to complement-independent phagocytosis [7,8].

Recently, we have convincingly demonstrated that human CRPs are glycosylated and distinct molecular variants are induced in several pathological conditions [9]. This minute structural variation showed considerable impact on their lectin-binding interaction with different glycoprotein and antibodies [10].

The main aim of the present study was to investigate the binding nature of erythrocytes of patients with malaria (RBC_{Mal}) to CRP_{Mal}, which have been purified from the sera of these patients. As this aspect of direct binding remains largely unexplored, this study has been undertaken to evaluate the impact of complement on this binding. A correlation between triggering of complement cascade after RBC-CRP binding has been observed. Taken together, it may be envisioned that disease-specific CRP is possibly essential for proper *in vivo* activity, in favor of the host, by promoting the lysis of diseased RBC and establishing an urgent need for such modification. This study may further provide evidence in support of opsonization as a major beneficial role of CRP.

Materials and methods

Study population

Blood samples ($n = 15$) from clinically confirmed and diagnosed individuals with malaria and normal healthy donors ($n = 10$) of either sex of different blood groups were included in this study. The hematological parameters were indicative of anemia in these malaria patients (Table 1). Patients' blood slides were microscopically confirmed to be positive for *Plasmodium falciparum* or *vivax*. The parasitic load per slide was 70–90%. However, these patients were devoid of any other red cell disorder. Sera of patients showing a detectable level of CRP by latex agglutination using the Rhelax-CRP Kit (Tulip, India) were used for purification of CRP. Venous blood was collected from individuals with informed consent and sent to the Indian Institute of Chemical Biology. As per the protocol of the Indian Council of Medical Research, the Institutional Human Ethical Committee approved the study.

Table 1 Clinical features of patients

	Patient	Normal
RBC count	0.8–2.4 $\times 10^6/\mu$l	4–6 $\times 10^6/\mu$l
Hb	4–7 g/dl	12 ± 2 g/dl
MCHC	28–31 g/dl	31–35 g/dl
Hct	40%	45–50%
Reticulocyte count	1–1.75%	0.2–1.5%
Bilirubin	1.8–2.2 mg/dl	>1 mg/dl
Spleen size	Just palpable	Not palpable

MCHC, mean cell hemoglobin concentration; Hb, hemoglobin, Hct: hematocrit

Preparation of erythrocytes

Blood was collected separately in the presence and absence of Alsevier's solution. Erythrocytes were isolated by centrifugation for 10 min at 3000 rpm in 4°C, washed three times with cold saline (0.9%) solution, suspended in Tris-buffered saline (TBS, 0.02 M Tris-HCl and 0.15 M NaCl, pH 7.5), and used immediately.

Affinity purification of CRP_{Mal}

Sera was collected from clotted blood and stored at −70°C until use. CRP_{Mal} was purified to apparent homogeneity from the sera following a standard protocol [11,12]. Typically, serum (∼4 ml) from a patient was passed through an Agarose column (Biorad) (1 × 10 cm) in presence of TBS (50 mM), pH 7.5 with $CaCl_2$ (5 mM) to remove serum amyloid P-component (SAP). Unbound fraction was immediately allowed to bind to Sepharose-PC (1 × 10 cm) affinity matrix [13], washed with TBS (20 mM) and $CaCl_2$ (10 mM) (TBS-Ca^{++}). Fractions eluted with EDTA were passed through another Sepharose-PC affinity matrix. The column was washed with TBS-Ca^{++} and, pure CRP was eluted with PC (2 mM) in TBS containing $CaCl_2$ (0.50 mM). The PC eluted fractions were sequentially dialyzed against HEPES (0.01 M) -saline buffer, pH 7.5 in presence and absence of EDTA. Protein content was estimated by Lowry method [14] and also by using molar absorption coefficient at 280 nm of 19.50 [15]. Purified protein was analyzed by SDS-PAGE (10%) and band was visualized by silver staining [16]. The purity was further checked by Western blot analysis [17] using murine anti-human CRP and probed with HRP-anti-murine IgG.

Flow-cytometric analysis (FACS) for determination of CRP-RBC binding

CRP was conjugated with FITC as described by Coligan *et al*. [18]. The binding of erythrocytes with CRP_{Mal} was established using FITC-CRP. In brief, cells (1×10^9) were incubated with FITC-CRP_{Mal} (0.05–1.0 μg) in the presence of PC (50 mM) and $CaCl_2$ (0.10 M) at 0°C for 1 h, washed twice with TBS-Ca^{++}, fixed with 1% para formaldehyde (Sigma), and analyzed on a Becton Dickinson FACSCalibur using CELL QUESTPRO software. Only cells, FITC-BSA or

unconjugated CRP (cells preincubated with unconjugated CRP followed by incubation with FITC-CRP), were used as different sets of controls. Different number of cells (10^6–10^9), PC concentration (10–60 mM), presence and absence of both PC and Ca^{++} as well as EDTA (0.25 M) were included for the optimization of the assay.

Binding of CRP with erythrocyte membrane protein by Enzyme-linked immunoabsorbent assay (ELISA)

Erythrocytes ghost membrane protein was prepared [19] and the extent of binding of CRP_{Mal} was assessed by immobilizing membrane protein (2 μg/well/100 μl) in carbonate buffer (50 mM, pH 9.5) on 96-well flat-bottomed polystyrene microtitre plates (Nunc-Immunoplate, USA) by incubating overnight at 4°C. The wells were washed with TBS-Tween (0.01%), blocked with TBS containing 2% BSA (TBS-BSA) and incubated overnight at 4°C with different concentrations of CRP_{Mal} (0.25–1.0 μg) which was preincubated with PC (50 mM) and $CaCl_2$ (0.10 M). Following washing with TBS-Tween, RBC-CRP complex was incubated with murine anti-human CRP (1:2000) overnight at 4°C, washed and binding was assayed colorimetrically by incubating with HRP-conjugated anti-murine IgG (Cappel, dilution 1:8000) for 45 min at 37°C. The bound complex was monitored using azino-bis-thiosulphonic acid (ABTS) and read by an ELISA Reader (Thermo Electron Corporation) at 405 nm. Experiments were done in duplicates.

Radio-binding assay of I^{125}-CRP_{Mal} and erythrocytes

CRP_{Mal} was iodinated with Na[^{125}I] using the Chloramine T method [20]. RBC_{Mal} (1×10^9) was incubated for 1 h at 0°C with increasing amounts of ^{125}I-CRP (specific activity - 4.7×10^6 cpm/μg) in presence of PC (50 mM) and $CaCl_2$ (0.10 M). After extensive washing with TBS-Ca^{++} to remove non-specific binding, bound radioactivity in the cell pellet was quantified in a Gamma Counter (Electronic Corporation of India). For evaluating the specific nature of binding, a 50-fold excess of unlabeled CRP was added. Measurements were done in triplicate.

Activation of complement cascade after RBC-CRP binding

Erythrocytes (1×10^9 cells) were incubated with 0.1–10 μg/ml of CRP_{Mal} (preincubated with 50 mM PC containing $CaCl_2$) for 30 min at 0°C and washed with ice-cold Gelatin-Veronal-buffered saline (GVB) containing Ca^{++} (0.15 M) and Mg^{++} (0.02 M) [21]. An equal volume of normal human serum (NHS) diluted (1:50) in GVB buffer was added as a source of complement and incubated for 1 h at 37°C to allow complement binding. Unbound complex was removed by washing three times by centrifugation at 3000 rpm for 5 min at 4°C with cold GVB buffer (1 ml). The extent of hemolysis in the supernatant was recorded at 412 nm. Cells incubated with EDTA, decomplement serum or patients' serum served as different controls. Cells only in distilled water (1 ml) were taken as 100% lysis. In all cases the average of duplicate tubes was determined. The percent-lysis was determined as follows:

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

Statistical analysis

For individual sets of experiments, results are expressed as mean ± SD and are representative of 2 or 3 experiments.

Results

Purification and molecular characterization of CRP_{Mal}

CRP purified from sera of malaria patients eluted as a single peak from affinity column. The induced CRP levels varied from 100–123 μg/ml of serum corresponded to 200–246 folds increase above the normal level (0.5 μg/ml) confirming that CRP is an acute phase reactant in all these patients. SDS-PAGE analysis (Fig. 1) of crude serum (lane 1) and SAP-free sample (lane 2) showed a number of major and minor bands. However, only three bands were visible in EDTA eluate from the first PC column (lane 3). A single band in PC elution from the second PC-column (lane 4) was taken as pure CRP corresponding to 27 kDa. Its purity was further demonstrated by

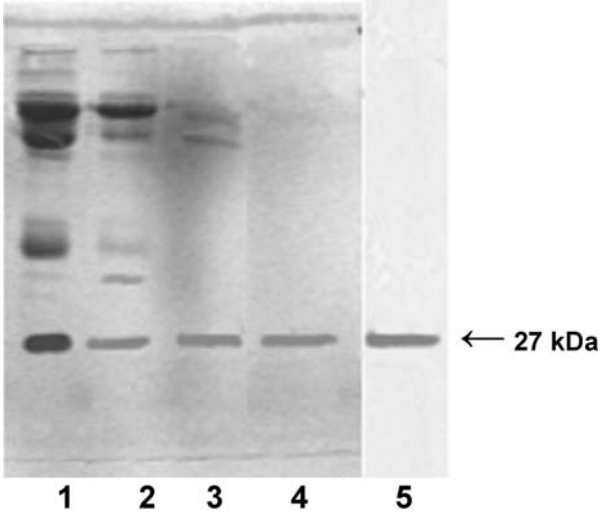


Fig. 1 Purification and characterization of CRP_{Mal} SDS-PAGE analysis. Lane 1–4 are crude patient serum, SAP-free serum, EDTA and PC eluted fractions respectively. Lane 5 is western blot analysis of purified CRP probed by murine anti-human CRP and HRP-anti-murine IgG respectively

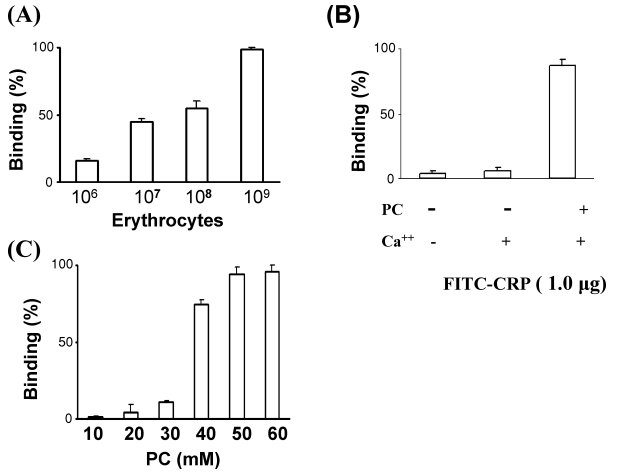


Fig. 2 Optimization of RBC-CRP binding assay by Flow cytometric analysis. (A) Different dilutions of erythrocytes (1×10^6–1×10^9) were incubated with fixed concentration of FITC-CRP (1.0 μg) in presence of PC and Ca^{++} as described in materials and methods. (B) The binding of RBC (1×10^9 cells) with FITC-CRP_{Mal} (1.0 μg) in absence of PC or Ca^{++} or both and presence of both PC and Ca^{++} as described in materials and methods. (C) PC concentration was varied from 10–60 mM in the binding of RBC (1×10^9 cells) with FITC-CRP_{Mal} (1.0 μg) as described in materials and methods

Western Blot analysis using murine anti-human CRP probed with HRP-anti-murine IgG (lane 5).

Optimization of binding of CRP_{Mal} with RBC_{Mal} by Flow cytometry

To optimize the cell concentration, increasing concentrations of RBC_{Mal} (10^6–10^9) was allowed to bind with CRP_{Mal}. Maximal binding at saturation level (99%) was observed with 10^9 cells (Fig. 2A). Both calcium and PC play very important roles in the binding of CRP_{Mal} with RBC_{Mal}. Absence of PC or Ca^{++} or both PC and Ca^{++} or presence of EDTA gave negligible binding (Fig. 2B). FITC-CRP (1.0 μg) showed maximum binding with RBC_{Mal} (10^9 cells) in the presence of 50 mM PC (Fig. 2C) and $CaCl_2$ (0.1 M). Accordingly, these combinations were used in all subsequent experiments.

Flow cytometric analysis confirmed the binding of RBC_{Mal} with FITC-CRP_{Mal}

The binding of FITC-CRP_{Mal} with RBC_{Mal} (1×10^9) increased in a dose-dependent manner (Fig. 3A). As compared to RBC_N, 80 ± 5% of RBC_{Mal} showed positive binding with CRP_{Mal} (1.0 μg). Under similar conditions, binding of CRP_{Mal} with RBC_N was much lower (27 ± 5%). Mean Fluorescence Intensity increased with increasing concentration of FITC-CRP; MFI being 49, 163, 177, 200, 213 and 247 at concentration of 0.05, 0.10, 0.166, 0.33, 0.50, and 1.0 μg of FITC-CRP_{Mal} respectively, with respect to negative control. At a fixed concentration of FITC-CRP_{Mal}, the binding with a fixed number of RBC_{Mal} was ~2.3-fold higher as compared to RBC_N (Fig. 3B). The specificity of this binding was demonstrated by preincubating RBC_{Mal} with unconjugated CRP_{Mal} and binding was monitored. A reduction of 3–5 fold binding reflected the binding specificity towards RBC_{Mal} (Fig. 3B). A representative profile of flow cytometric analysis to compare the percentage of binding with FITC-CRP is shown in Fig. 3B, inset.

CRP_{Mal} showed specific binding to erythrocyte membrane protein as confirmed by ELISA

A dose-dependent binding of membrane protein from RBC_{Mal} (□) with CRP_{Mal} was further corroborated by ELISA. The binding of RBC_{Mal} with CRP_{Mal} (1.0 μg) was ~3-fold higher than RBC_N (⊡) under similar conditions; O.D. 405 nm being 0.91 ± 0.12 vs 0.27 ± 0.04 (Fig. 3C). This assay also confirmed the importance of PC and Ca^{++} in this binding interaction. Absence of PC and Ca^{++} showed negligible binding at 405 nm close to negative control (not shown in figure).

Specific receptors for CRP are expressed on RBC_{Mal}

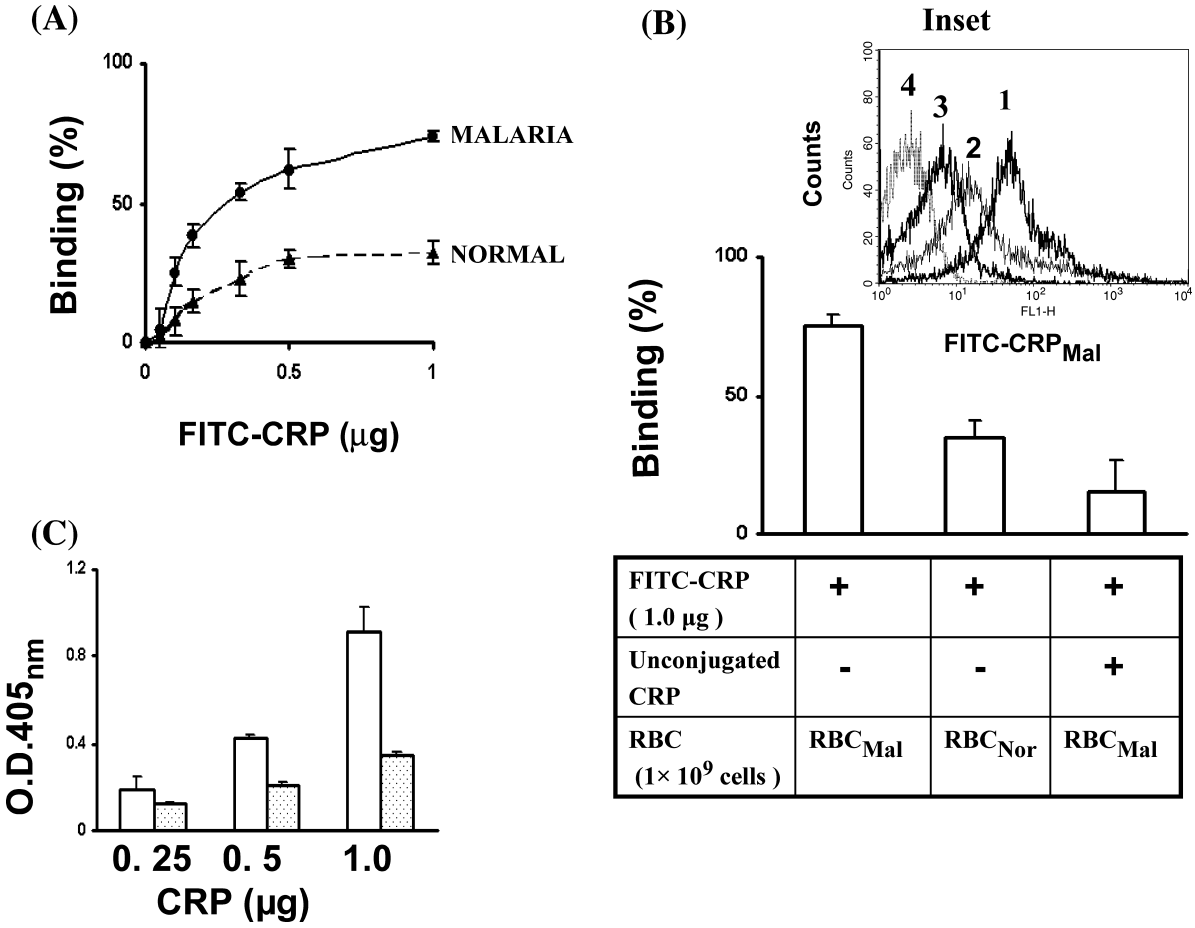
The dose-dependent binding of $^{125}ICRP_{Mal}$ with a fixed number of RBC_{Mal} in presence of PC and Ca^{++} and the corresponding Scatchard Plot are shown in Fig. 4. The plot demonstrated that binding reached almost to saturation at ~16 μl (Fig. 4A). Apparent binding constants (K_a) of ^{125}I-CRP_{Mal} with RBC_{Mal}, as calculated from the slopes of Scatchard plots are found to be 4.7×10^6 cpm/μg, with the corresponding number of receptors/cell are 4.3×10^5 (Fig. 4B). The binding of $^{125}ICRP_{Mal}$ with RBC_{Mal} could be inhibited by 50-fold excess unconjugated CRP (Fig. 4A).

CRP_{Mal} differentially triggers complement-dependent hemolysis of erythrocytes in malaria

CRP_{Mal} (10.0 μg) triggered 80 ± 3% hemolysis of RBC_{Mal} that was nearly 2.8 ± 0.02-fold higher than that of RBC_N using normal human serum as a source of complement; O.D. 412 nm being 0.42 ± 0.04 vs 0.14 ± 0.02 (Fig. 5A). Both patients' serum and NHS, as a source of complement, showed comparable lysis. The percentage of lysis was inhibited by 50% in presence EDTA (Fig. 5B).

Discussion

The CRP has long been linked to natural immunity and host defense. It plays a role in the *in vivo* clearance of



FITC-CRP (1.0 μg)	+	+	+
Unconjugated CRP	-	-	+
RBC (1×10^9 cells)	RBC_{Mal}	RBC_{Nor}	RBC_{Mal}

Fig. 3 RBC-CRP binding assay by Flow cytometric analysis. (A) The binding of RBC_{Mal} in comparison to RBC_N with increasing concentration of FITC-CRP (μg). (B) Binding of a fixed amount of FITC-CRP_{Mal} (1.0 μg) with (1) RBC_{Mal}, (2) RBC_N, (3) preincubation of RBC_{Mal} with unconjugated CRP_{Mal}. Inset: A representative profile of flow cytometric analysis to compare the binding of FITC-CRP_{Mal} with (1) RBC_{Mal} (2) RBC_N and (3) preincubation of RBC_{Mal} with unconjugated CRP_{Mal} and (4) represents corresponding negative control. (C) Differential binding of erythrocyte membrane from $RBC_{Mal/N}$ with increasing concentration of CRP_{Mal} (0.25–1.0 μg) as detected by ELISA, as described in materials and methods

cells to which it is bound [6]. Although the capacity of human CRP to alter the site of organ sequestration of murine erythrocytes was reported [22], to date, the binding nature of CRP_{Mal} with erythrocytes has not been explored in details.

The major achievement of the current investigation includes (i) affinity purification of malaria specific CRP to introspect the RBC-CRP interaction (Fig. 1), (ii) confirmation of a significant role of Ca^{++} and PC (Fig. 2), (iii) demonstration of specific binding of CRP_{Mal} to RBC_{Mal} in comparison to RBC_N (Figs. 2–4), (iv) establishment of specific receptors on RBC_{Mal} for the binding of CRP_{Mal} and (v) finally, clearance of diseased erythrocytes through complement-triggered hemolysis mediated by the specific binding of RBC_{Mal} with CRP_{Mal}.

The molecular weight of purified CRP_{Mal} (27 kDa) is distinctly different as compared to CRP purified from normal human volunteers (23 kDa) as determined by electrospray mass spectrometry [23] suggesting a new molecular variant specifically induced in malaria. The binding of CRP_{Mal} to RBC_{Mal} has been evidenced by several approaches. Binding parameters using FITC-CRP (Fig. 2 and 3) and ^{125}I-CRP (Fig. 4) with whole red blood cells as well as erythrocyte membrane protein (Fig. 3C) convincingly demonstrated their specific interactions. The specificity of this interaction was reconfirmed by inhibition of binding using FITC or radiolabeled conjugated CRP pre-incubated with excess unlabeled CRP both by FACS (Fig. 3B) and by radio-binding assay (Fig. 4A). Calcium and PC are absolutely essential for this binding (Fig. 2B). The presence of specific receptors on diseased erythrocytes further confirmed the interaction of CRP_{Mal} with RBC_{Mal} (Fig. 4). Notably, CRP_{Mal} showed significantly low binding with RBC_N as compared to RBC_{Mal}, the respective percent of positive cells being 80 $\pm$ 5% vs. 27 $\pm$ 5% suggesting disease-specific interaction. As malaria-infected erythrocytes are reported to display significant alteration

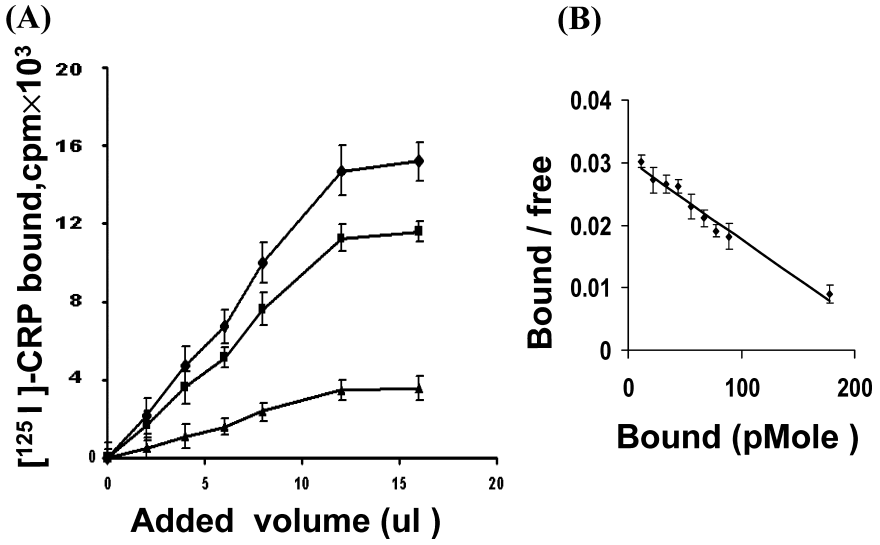


Fig. 4 Scatchard analysis of binding of CRP_{Mal} with RBC_{Mal} (A) Fixed amount of RBC_{Mal} (1×10^9 cells) was incubated with increasing volume of (μl) of $^{125}ICRP_{Mal}$ (4.7×10^6 cpm/μg). For evaluating the specific nature of binding, 50-fold excess of unlabeled CRP was added. The bound radioactivity was measured as described in Materials and method and plotted against added $^{125}ICRP_{Mal}$. Specific binding (–■–) was determined by calculating the difference between total binding (–●–) and non-specific binding (–▲–). Results are expressed as mean ± SD of data from triplicate experiments. (B) The apparent binding constants (K_a) of ^{125}I-CRP_{Mal} with RBC_{Mal} were calculated from the slopes of Scatchard plots in which bound/free was plotted against the various amount of bound CRP

in their shape and membrane [24] as compared to normal erythrocytes, low binding of malaria-specific CRP with RBC_N is quite obvious (Fig. 3).

The vital question arises regarding the fate of RBC_{Mal} after binding with CRP_{Mal}. What role is CRP playing after binding with RBC? Is it playing any protective role? A definite and substantiated answer to this question is the triggering of the complement pathway. As compared to $RBC_{N,}$ the complement experiment gave definite proof of the lysis of diseased erythrocytes when it specifically binds with CRP_{Mal} following activation of the complement cascade (Fig. 5). Thereby, it may be concluded that this binding opens up a new avenue for identifying the protective role of CRP, an important innate immune component, by clearing diseased erythrocytes through complement-mediated cell lysis (Fig. 5). A similar percentage of lysis using both patients' serum and NHS as a source of complement suggests undisturbed complement components in the diseased state. In control sets, both EDTA and decomplemented serum consistently demonstrated an inability to block complete lysis. To pin point, whether the lysis was due to binding of CRP to erythrocytes, CRP-RBC binding were carried out in the presence of EDTA (0.50 M). Although, this binding was completely inhibited in presence of EDTA, a certain percentage of complement mediated lysis of erythrocytes was observed suggesting a mechanism which may be independent of in-

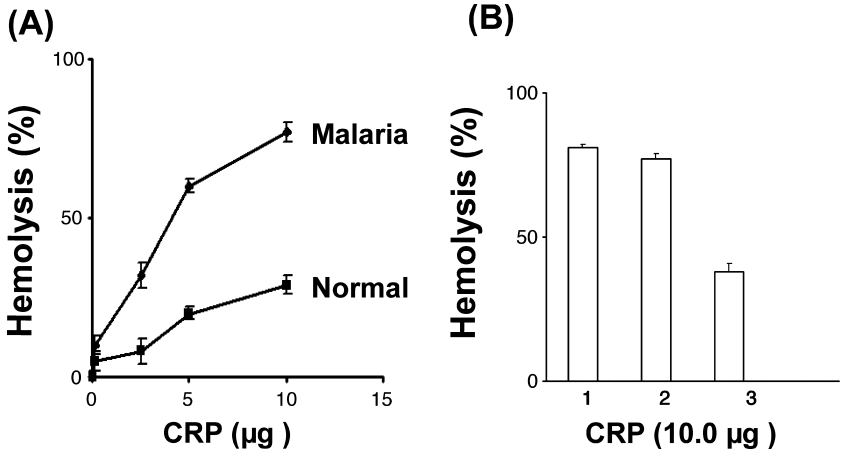


Fig. 5 Triggering of RBC-CRP complex by complement (A) Dose-dependent hemolysis of RBC_{Mal}, in comparison to RBC_N, when NHS was added, as a source of complement, to the mixture of CRP_{Mal} (0.1–10 μg) – erythrocyte complex. (B) At fixed concentration of CRP_{Mal} (10.0 μg), hemolysis of $RBC_{malaria}$ was compared using patient's sera (panel 1), NHS (panel 2) as a source of complement. Preincubation of reaction mixture with EDTA (0.50 M) reduced this hemolysis (panel 3)

volvement of CRP. Similarly, a 50% of lysis, even in the absence of complement, may suggest another additional possible pathway of C independent clearance. Taken together, 200–246-fold increase in the concentration of CRP along with a disease-specific molecular variant; absence of this variant in a normal individual suggests the absence of lysis of normal erythrocytes.

Our results support the hypothesis that these diseased erythrocytes are lysed as they may have some altered membrane characteristics like fragility, rigidity, or other parameters as compared to normal erythrocytes [24,25]. These observations are helpful in understanding the beneficial role of CRP in the recognition of damaged cells and their clearance from these patients.

Binding of RBC_{Mal} with disease-induced CRP may open up a new avenue in the field of CRP research. It may be envisioned that new molecular variants of CRP induced in patients with malaria may be due to the urgent need for clearance of the damaged erythrocytes. This may pave the way for major achievement of this current investigation including the significant role of CRP in modulating the RBC-CRP-binding. CRP_{Mal} may possibly endorse the RBC lysis and essentially favors the host *in vivo*, establishing an imperative need for such modification after the binding with diseased RBC. These findings clearly suggest that triggering of a complement surge followed by the binding of disease-specific CRP to erythrocytes. It would be interesting to build molecular model of the complex of CRP with an RBC component to identify the exact site of this binding. These observations help to understand the pathophysiological role of CRP in the recognition, clearance, and destruction of diseased RBC, again confirming the protective role of CRP in host-immune system.

Lysis of diseased erythrocytes may cause anemia, which is a common clinical feature in malaria. It may be hypothesized that a combined interplay of binding and subsequent lysis can contribute to the anemic condition in malaria. To the best of our knowledge, this is the first report describing the unique ability of the disease-induced molecular variant of CRP to bind differentially with diseased erythrocytes and to clear them from circulation as compared to normal, thus implicating its protective role. Knowledge of the interrelated functioning of all the features may be envisaged to have far fetched implications in biology-based therapy for better management of the disease and remains the future goal of the current study.

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