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Variations in binding characteristics of glycosylated human C-reactive proteins in different pathological conditions

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C-reactive protein (CRP) is a clinically important classic acute phase pentameric protein. It is thought to play an important role in immunomodulation. Earlier reports convincingly demonstrated that human CRP is differentially glycosylated in different pathological conditions. Although CRP is considered to be a clinically important molecule, changes in binding characteristics with appropriate ligands with respect to glycosylation remain unexplored. In an effort to demonstrate that these glycosylated molecular variants are capable of modulating their binding activity with different ligands, CRPs were affinity purified from six different clinical samples. Variable amounts of linkage-specific sialic acid derivatives were found in these CRPs with varying tryptophan contents. Differential binding patterns with antibodies against human CRP, human IgG, and other ligands like fibronectin, fetuin, and asialofetuin indicated that the purified CRPs differed significantly in their lectin-like interactions. Thus, we have convincingly demonstrated that differentially induced CRPs exhibited variable binding characteristics. These results may have far reaching practical applications for understanding acute phase responses. ***Published in 2004.***

Keywords: **C-reactive protein, plasma glycoprotein binding, ligand binding, IgG binding, lectin-like property**

Abbreviations: **CRP, C-reactive protein; CPS, pneumococcal C polysaccharide; PC, phosphorylcholine; EDTA, ethylene diamine tetra acetic acid; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; HRP, horseradish peroxidase; ELISA, enzyme linked immunosorbent assay; ABTS, 2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) ammonium salt; VL, Visceral Leishmaniasis; ALL, Acute Lymphoblastic Leukemia; TB, Tuberculosis; SLE, Systemic Lupus Erythematosus; OS, Osteogenic Sarcoma.**

Introduction

C-reactive protein (CRP) is a phylogenetically ancient and highly conserved protein having a pentraxin structure. It is composed of five identical non-covalently linked subunits. CRP, a major acute phase protein, could precipitate the C-polysaccharidde (CPS) of *Streptococcus pneumonae* cell walls by its ability to bind phosphorylcholine (PC) and phospholipid constituents of damaged cells [1]. It is rapidly synthesized in liver in response to inflammatory cytokines, and serum levels increase up to 1000-fold from the basal levels (0.1–0.5 μg/ml) within a few hours [1], making CRP probably the single most useful molecule for monitoring acute phase reactions. However, the way in which CRP mediates in the acute phase reactions still remains unclear.

CRP is thought to be an important component of the innate immune system implicating its critical role in the host defense mechanism. In spite of *in vitro* demonstration of the involvement of CRP in multiple functions, namely, activation of the complement, opsonization, phagocytosis, binding to platelets, chromatin, histones, and small nuclear ribonucleoprotein particles [2], its precise physiological role is yet to be established.

We have recently reported that human CRPs are glycosylated and show variations in both their glycosylation patterns and amino acid sequences in different pathological conditions [3]. In the present investigation, we have designed experiments to demonstrate that these minute structural variations have considerable impact on their lectin-like interactions with different glycoproteins and antibodies, thereby providing information

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which may be beneficial to understand *in vivo* activities of CRP as acute-phase reactant and, accordingly, could be exploited as a potential biochemical marker to monitor the clinical status of patients.

Materials and methods

Purification of human CRP

Clinical samples showing detectable levels of CRP by Rhelax-CRP kit, (Tulip, Mumbai, India) were selected. Informed consent was taken from all individuals and the study was carried out in accordance with the Institutional Human Ethical Clearance Committee. CRPs were essentially purified from serum of several samples of five different clinical conditions, e.g. VL, ALL, TB, SLE and OS named as CRP_{VL}, CRP_{ALL}, CRP_{TB}, CRP_{SLE}, and CRP_{OS} respectively [3]. CRP was also purified from pleural fluid of OS ($CRP_{OS(p)}$). In brief, an unbound fraction of samples from agarose beads (BioRad) column was allowed to bind with Sepharose-PC [4]. EDTA eluted fractions from a Sepharose-PC column were passed through another Sepharose-PC affinity matrix, and the PC eluted fraction was dialyzed sequentially against Hepes (0.01 M)-saline buffer, pH 7.5 in the presence or absence of EDTA and electrophoretically analyzed by native-PAGE (7.5%) [5]. The purity of CRP was checked by SDS-PAGE [6] and western blot analysis [7]. A commercially available human CRP from Tulip (CRP_{Tulip}) was included as a standard. The protein was estimated using the extinction coefficient, $E^{1cm}_{1\%}$ at 280 nm of 19.50 [8], and by Lowry method [9]. Types of sialic acids and their linkage specificity were qualitatively measured with the help of digoxigenin (DIG) enzyme-immuno assay using DIG-glycan detection and differentiation kits according to the manufacturer's instructions (Cat no: 1142372 & 1210238 respectively, Roche Molecular Biochemicals).

Fluorescence spectroscopic analysis

CRP_{VL} CRP_{ALL}, CRP_{SLE} (50 μg/ml in Hepes-saline) were analyzed in a spectrofluorimeter (Hitachi F-4010, Japan) at 25°C with excitation at 280 nm, and emission wavelengths were recorded over the range of 300–400 nm.

Antibody binding with CRPs

BALB/c mice were immunized with a mixture of all five CRPs (30 μg/injection) with Freund's complete adjuvant followed by three booster injections in Freund's incomplete adjuvant at intervals of 10 days [10]. IgG fractions were precipitated by saturated ammonium sulfate precipitation (33%) and used for binding assay. Each CRP (500 ng/well) was coated on a microtitre plate separately, washed, blocked with BSA, and incubated with murine anti-human CRP (dilution 1:5000). Following washing, the antigen-antibody complex was detected by incubating HRP-anti-murine Ig (Cappel, dilution 1:10000) for 30 min at 37°C, washed and the bound complex monitored using ABTS and read at 405 nm by an ELISA reader (Win Read V.2.1, Anthos Labtec, U.K.).

Binding of CRPs with human IgG, other plasma glycoproteins and sugars

The extent of binding of human IgG with CRPs was assessed [11,12] by immobilizing them (200 ng/100 μl in 25 mM Hepes-saline with 0.5 mM $CaCl_2$, pH 8.2/well) on microtiter plates overnight at 4°C. Plates were washed with 20 mM tris-buffered saline, 0.5 mM $CaCl_2$, pH 6.0 (TBS-Ca^{+2})-Tween-20 (0.05%, v/v), blocked with TBS-Ca^{+2}-10% BSA, and different concentrations of adsorbed human IgG on latex beads (Polysciences, Germany) were added, shaken gently for 2 to 3 h at 25°C and incubated overnight at 4°C. The CRP bound IgG complex was detected with HRP-anti-human IgG (1:5000) as stated above. The nonspecific binding was determined from CRP-free wells. CRP_{Tulip} served as internal control. Binding of CRPs to different plasma glycoproteins was similarly quantitated by immobilizing fibronectin, fetuin, and asialofetuin (500 ng/100 μl TBS-Ca^{+2}, pH 7.5/well) separately on ELISA plate. After washing and blocking, different concentrations (5 ng – 1 μg, 100 μl/well) of CRPs were added and processed using HRP-anti-murine Ig as second antibodies.

In parallel, binding of CRP with simple sugars was also monitored by incubating equal amounts (50 ng) of ^{125}I-CRPs [13] with agarose-bound GalNAc and lactose (20 μl) overnight at 4°C. After washing, bound radioactivity was determined in a gamma counter. All experiments were done in triplicate.

Results

Purification and characterization of CRPs

CRP_{VL}, CRP_{ALL}, CRP_{TB}, CRP_{SLE}, CRP_{OS}, and $CRP_{OS(p)}$ have been affinity purified to apparent homogeneity. The crude serum showed 5 prominent bands with several minor bands (Figure 1, lane 1). After removal of serum amyloid-P component by an agarose column (lane 2), four bands were visible in an EDTA-eluted fraction (lane 3). However, the PC eluted fraction from the 2nd Sepharose-PC column appeared as a single band (lane 4) corresponding to CRP_{Tulip}, (lane 5).

A higher degree of sialylation was demonstrated in five out of six CRPs (Figure 2A, spots 1–5). Amongst six clinical samples, CRP_{OS} showed no reactivity indicating the absence of sialic acid (spot 6). More importantly, the intensities of the spots differed. Quantification by densitometric scanning of these blots revealed a three-fold increase in the degree of sialylation in CRP_{SLE} *vs* $CRP_{OS(p)}$. The extent of sialylation was approximately similar in CRP_{VL}, CRP_{ALL}, CRP_{TB}, and two fold more sialylation was observed as compared to $CRP_{OS(p)}$.

The presence of α2,3-linked sialic acid has been demonstrated in CRP_{VL}, CRP_{ALL}, CRP_{TB}, and CRP_{SLE} based on their strong but differential reactivity with MAA. Quantification by densitometric scanning of this blot revealed a 2.3-fold

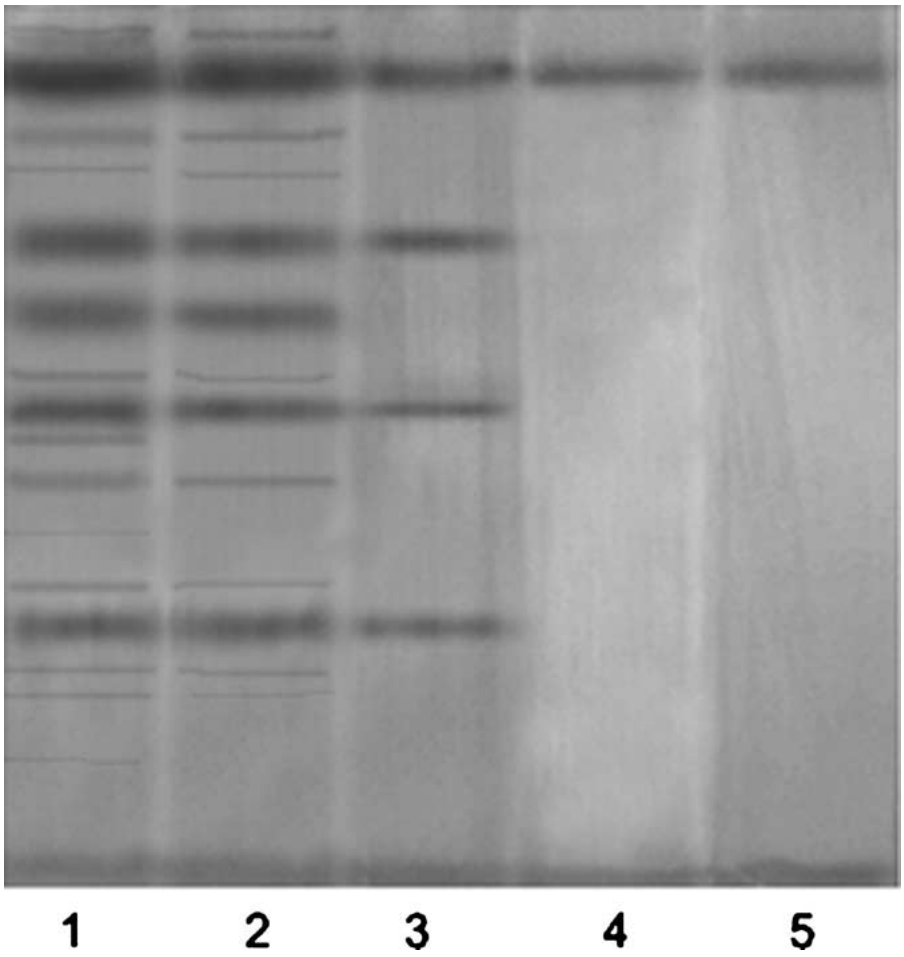


Figure 1. Native PAGE analysis. Equal amounts of proteins (2 μg/lane) were analyzed on native PAGE. Lanes 1–5 are crude serum, pass through from agarose column, EDTA and PC eluted fractions, and CRP_{Tulip} respectively.

increase in the degree of sialylation with α2,3 linkage in CRP_{SLE} vs. CRP_{VL}. In contrast, no reactivity was observed with SNA (Figure 2B).

Differences in tryptophan content of CRPs

Fluorescence emission measurement of all three CRPs gave the emission maxima at around 340 nm. Using equal concentrations, the intensities at the maxima differed in the following order: $CRP_{VL} \gg CRP_{ALL} \ggg CRP_{SLE}$ (Figure 3A).

Diversity in binding with CRP antibodies

The differences in glycosylation and tryptophan content of CRPs were found to be critical for interaction with antibodies (Figure 3B). Equal amounts of antibodies (raised against a mixture of CRP_{VL}, CRP_{ALL}, CRP_{TB}, CRP_{SLE}, and CRP_{OS}) showed wide variations in binding with CRPs ($O.D._{405nm}$ 0.46-1.97). A maximum of 4.3-fold elevation in binding was observed with CRP_{ALL} as compared to CRP_{SLE}/CRP_{TB}/CRP_{OS}. The degree of binding differed in the following order: $CRP_{ALL} > CRP_{VL} \gg CRP_{OS} > CRP_{TB}$/$CRP_{SLE}$.

Differential binding with human IgG

The interaction between CRPs and human IgG was also modulated due to the minute structural differences in CRPs induced in different pathological conditions. IgG displayed significant variations in binding with five different CRPs (Figure 4A). The binding was concentration-dependent and approached saturation at higher concentrations of antibody with a half maximal point at 10, 13.2, 11.5, 23.1 and 13.2 nM for CRP_{VL}, CRP_{ALL}, CRP_{TB}, CRP_{SLE}, and CRP_{Tulip}, respectively. It may be noted that the binding curves are different for different CRP revealing their intrinsic differences in binding affinity.

Modulation of binding with other plasma glycoproteins

The extent of interaction of plasma fibronectin with various molecular variants of five CRPs was studied (Figure 4B). Steady increase in binding was observed with an increase in CRP concentration and a fixed concentration of fibronectin. The degree of binding differed among CRPs. CRP_{VL} (1000 ng) showed the maximum binding. A 2.1-fold enhancement in the binding was observed as compared to CRP_{ALL}. In

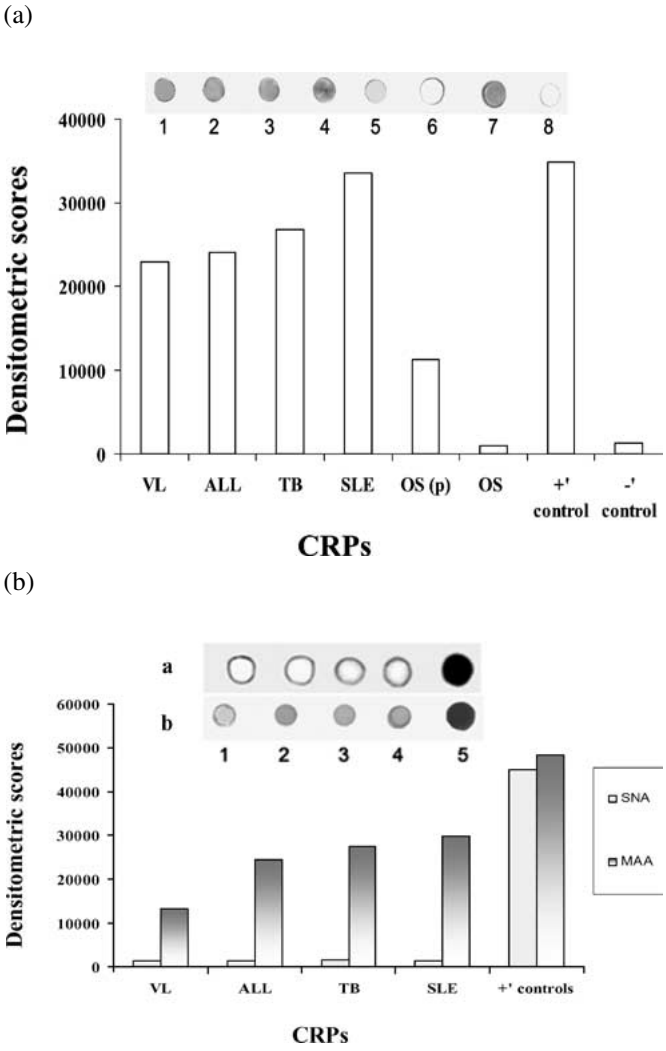


Figure 2. Detection of linkage specific sialic acids by DIG glycan kits (a.) Densitometric scoring of sialylation profiles. Inset: Dot blot analysis of sialylation profile. Spots 1–8 represent CRP_{VL}, CRP_{ALL}, CRP_{TB}, CRP_{SLE}, CRP_{OS}, and $CRP_{OS(p)}$. Transferrin and creatinase are positive and negative controls respectively (spots 7–8). (b.) Presence of linkage-specific sialic acids was scored by densitometer scanning of blots using SNA (□) and MAA (▭). Inset: Panels a and b represent reactivity of CRP_{VL}, CRP_{ALL} CRP_{TB} and CRP_{SLE}, with SNA and MAA respectively. Column 5 of both the panels is the respective positive controls.

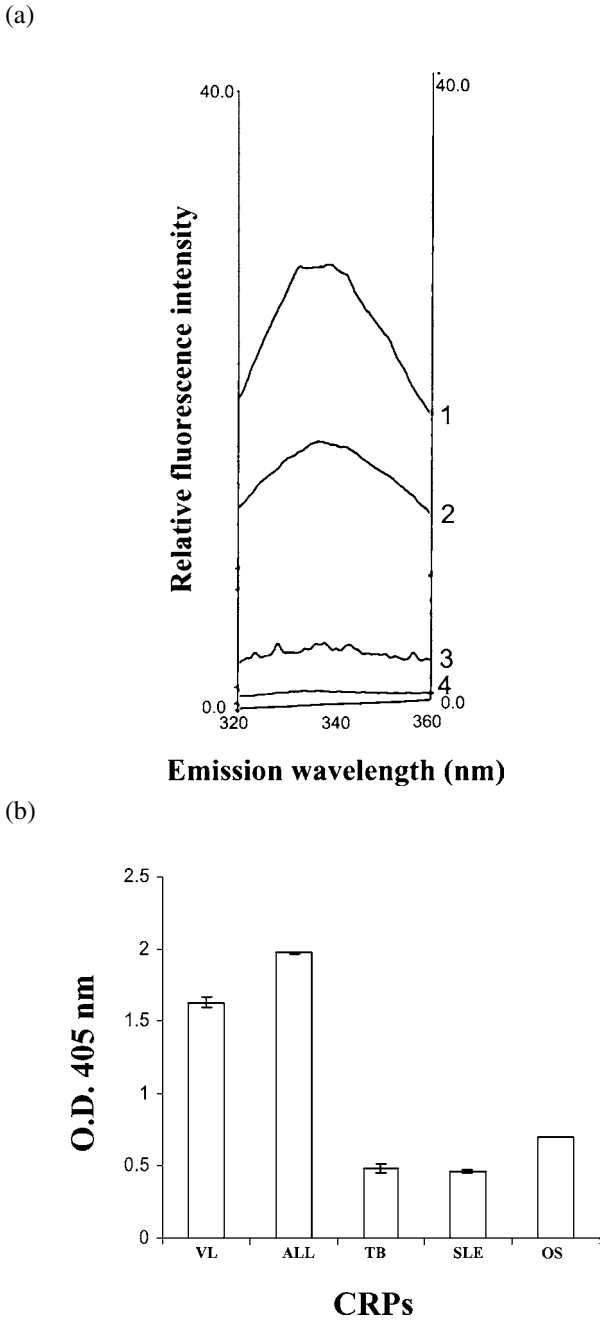
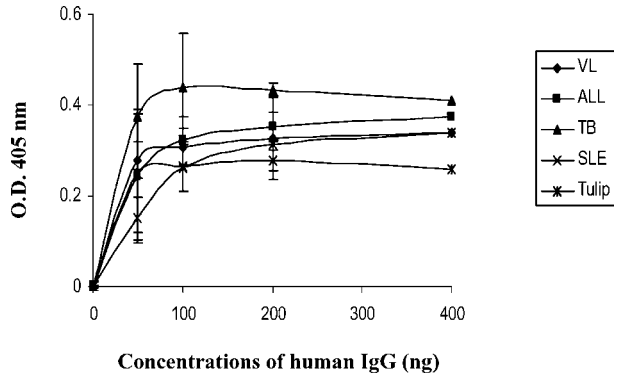


Figure 3. (a.) Analysis of CRPs by fluorescence emission spectra. Fluorescence spectra of CRP_{VL}(1), CRP_{ALL}(2), CRP_{SLE} (3), (50 μg/ml) and buffer (4) were measured with excitation (280 nm) and emission wavelength 320–360 nm. (b.) Differential reactivity of CRP with anti-CRP. Murine anti-CRP was allowed to bind on CRP-coated ELISA plate. Each bar represents reactivity ($O.D._{405\ nm}$) of CRPs from VL, ALL, TB. SLE and OS.

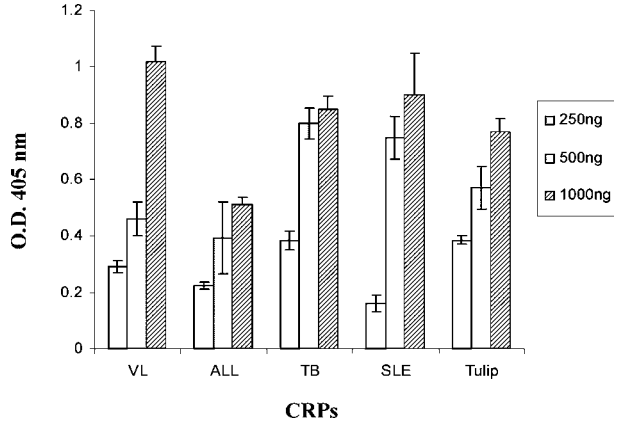
contrast, CRP_{SLE} and CRP_{TB} displayed almost similar binding. The pattern of binding at lower concentration of CRP (250 ng) was a little different. Highest binding was observed with CRP_{TB}. CRP_{SLE} showed 2.4-fold less binding as compared to CRP_{TB}.

Although the binding of fetuin and asialofetuin with CRP was monitored using a wide range of concentrations (5–500 ng), the variations were minimal and showed saturation with 10 ng (Figure 4C). As expected, due to their specificity towards the galactosyl moiety, all CRPs showed stronger binding with asialofetuin, which exhibits terminal galactosyl residues. On the other hand, fetuin having terminal sialic acids and the galactosyl residues are masked resulting in 50% less binding. However, no significant variation in sugar-binding properties using simple sugars, *e.g.* GalNAc and lactose, was observed.

(a)



(b)



(c)

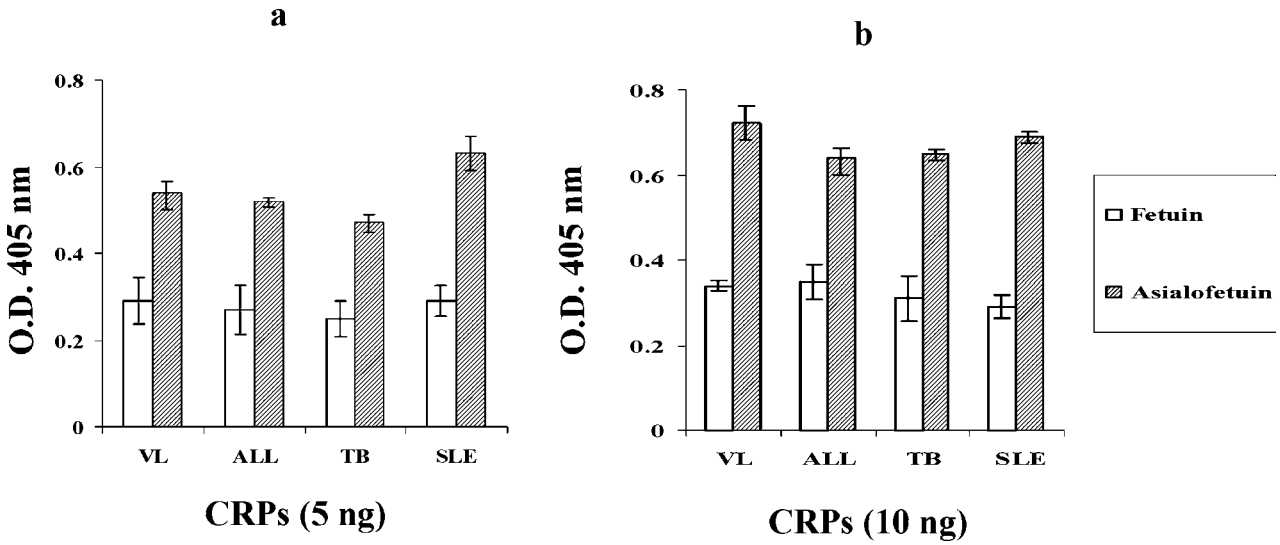


Figure 4. Differential binding of CRPs with plasma proteins. (a.) Latex-coupled human IgG (50-400 ng) was incubated with immobilized CRP (200 ng/well) and bound-complex was detected by anti–human IgG as described in Materials and Methods. Extent of binding of CRP_{VL} ( ), CRP_{ALL} ( ), CRP_{TB} ( ), CRP_{SLE} ( ) and CRP_{Tulip} ( ) are presented here. (b.) CRPs from VL, ALL, TB, SLE, and Tulip were incubated separately with fibronectin, fetuin and asialofetuin (500 ng/well) coated microtiter plate and bound complex detected by murine anti–human CRP. The binding of fibronectin with five CRPs 250 ng (▨), 500 ng (▨) and 1 μg (□) are plotted against $O.D.405_{nm}$. (c.) Binding of CRPs, 5 ng (a) and 10 ng (b) with immobilized fetuin (□) and asialofetuin (▨).

Discussion

Glycosylated molecular variants of human CRP induced in different diseases have been purified to electrophoretic homogeneity using the same PC affinity matrix based on their common property of calcium-dependent PC-binding. The present investigation revealed that the induced structural differences in CRPs, as reflected in their tryptophan contents (Figure 3A) together with glycosylation [3] and specific sialylation (Figure 2A-B), played a contributory role in their binding characteristics, *e.g.* with antibodies (Figure 3B) and different plasma glycoproteins (Figure 4A-C).

All these CRPs not only differed by their sialic acid content, the linkage specificity with sub-terminal sugars also varied as confirmed by binding with two plant lectins namely MAA and SNA, known to have the specificity toward α2,3- and α2,6-linked sialic acid, respectively. All four CRPs from VL, ALL, TB, and SLE contain sialic acids in α2,3 linkages (Figure 2B). In contrast, CRPs from Cushing's syndrome and $CRP_{OS}(p)$ showed SNA-specific binding [3] indicating the presence of α2-6 linked sialic acid. Thus, the linkages of sialic acid derivatives in CRPs varied in different disease conditions.

These induced structural variations of CRPs make them distinct from each other in their binding interactions. In order to understand the nature of these differential-binding patterns, we have investigated the binding characteristics of six CRPs with plasma glycoproteins and also with antibodies. Several points may be made regarding CRP functions in relation to their structural modifications and possible roles in acute-phase reaction from the binding profile of (i) anti-CRP antibody, (ii) human IgG, (iii) fibronectin, (iv) fetuin, and (v) asialofetuin.

Although immunochemical identity of five CRPs by immunodiffusion indicates the antigenic similarity among themselves suggesting the presence of common epitopes with sequence homology, a quantitative difference in the reactivity of the same antibody is evident from color intensities implying differences in the extent of binding (Figure 3B). These variations clearly suggest that the different heterogeneous forms comprise distinct immunodominant epitopes, possibly appearing exclusively on CRP due to specific pathological condition. These can only be resolved by developing monoclonal antibodies specific to these distinct epitopes. If specific epitopes could be identified, it would lead to detection of unique CRP induced under specific pathological conditions. Thus, structural variations, induced in different pathological conditions, producing distinct molecular variants, considerably affected the extent of their antibody binding. These observations may pave the way for overcoming many important obstacles for the clinical use of CRP and for new diagnostic approaches using CRP as a potential biochemical marker. Such studies are in progress.

Interestingly, significant variations in binding of these CRP with human IgG and fibronectin were consistently observed (Figure 4A and B). Although the biological significance of the interaction of CRP with fibronectin is still unclear, it may be envisioned that, at sites of tissue damage, CRP is selectively deposited along with plasma fibronectin and that they act together. Thus CRP can modulate early events of tissue repair by altering cell-binding activity. The observed differences in CRP-fibronectin binding may be due to different molecular variants influencing the activities of CRP at the sites of tissue damage, thus assigning distinct physiological functions. The binding is possibly not a lectin-ligand interaction as it occurs through the phosphate group present in fibronectin [14,15].

So far opinions differ as to the possible function of human CRP as lectin. Though interactions of CRP with D-galactose-containing ligands has been reported earlier [11], the anti-galactan reactivity of human CRP is not based on galactose-binding properties and may be attributed to the antiphosphate/anti-PC specificity. This finding may question the proposed lectin function of CRP [16]. However, human CRP upon surface immobilization behaves as a galactose-specific lectin, and this lectin function is masked by terminal sialic acid [11]. This corroborated our observation regarding binding studies with fetuin and asialofetuin; the terminal sialic acids present on ligands inhibit the binding and, when it is removed a two-fold increase in the degree of binding is observed (Figure 4C). This differential reactivity indicates the possibility of different *in vivo* action. All these CRPs, despite their structural variations, showed almost similar profiles of binding with fetuin, asialofetuin, and simple sugars, in contrast to fish CRP [17].

Our results provide a new direction in understanding the role of induction of unique glycosylated molecular variants of CRP in different clinical conditions. Minor differences induced by post-translational modification, may possibly be essential for proper *in vivo* activity of CRP, thereby establishing an urgent need for such modification as observed earlier with two species of sweet water fish [10,17–20]. Therefore, routine clinical use of CRP, simply by detecting quantitative alterations of its level [21], will dramatically change, if qualitative alterations of this molecule could be used as a unique marker for diagnosis and monitoring of an acute-phase response to inflammation.

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