Glycoconjugate Journal 16, 741–750, 1999

Lectin like properties and differential sugar binding characteristics of C-reactive proteins purified from sera of normal and pollutant induced *Labeo rohita*

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Different forms of C-reactive proteins (CRPs) have been purified to electrophoretic homogeneity from the sera of *Labeo rohita* confined in freshwater (CRP_N) and water polluted with nonlethal doses of cadmium (CRP_{Cd}) or mercury (CRP_{Hg}). CRP_N, CRP_{Cd}, and CRP_{Hg} show remarkable differences in their electrophoretic mobility but exhibit strong immunological cross reactivity. All these CRPs exhibit variable agglutination properties with erythrocytes from diverse sources in presence of Ca^{+2}, which could be inhibited by a variety of sugars showing specificity for galactose. Inhibition results show that the potency of galactose as an inhibitor increases about 4 fold in the process of transformation of CRP_N to CRP_{Cd} and CRP_{Hg}. In case of CRP_N, Gal β(1 → 1) Gal and oNO_2 phenyl β-Gal show highest inhibitory potency while oNO_2-phenyl β-Gal is the most potent inhibitor for CRP_{Cd} and CRP_{Hg} but the potency of Gal β(1 → 1) Gal reduced drastically. 6-phosphate D-Gal and stachyose are 20 times weaker inhibitors than D-Gal for induced CRP mediated agglutination, in contrast, these sugars are only 6 times weaker for CRP_N. Dissociation constants of the binding of CRP_N with phosphoryl choline (PC) and galactose are about 9 mM and PC binding causes a change in the α and β conformations of these CRPs.

Keywords: **C-reactive protein, pollutant, agglutinin, galactose binding lectin, fish, *Labeo rohita***

Abbreviations: **PC, phosphorylcholine chloride; Galβ(1 → 1)Gal, β-Galactopyranosyl β-D-Galactopyranoside; o-NO_2-phenyl β-Gal, o-nitrophenyl-β-D-galactopyranoside; 4NH_2 phenylβ-Gal, 4-amino-phenyl-β-D-galactoside; Galα(1 → 6)Glc, 6-O-α-D-galactopyranosyl D-glucopyranose (meliblose); Phenyl β-Gal, phenyl-β-D-galactoside; Galβ(1 → 1)S-Gal, D-Galactopyranosyl-1-thio-β-Galactopyranoside; 2-O-CH_3-Gal, 2-O-methyl-Galactoside; 3-O-CH_3-Gal, 3-O-methyl-Galactoside; α-D-Gal, D(+)Galactose; α-D-Fuc, D(+)fucose; 2-Deoxy-Gal, 2-deoxy-D-Galactose; p-NO_2phenyl-Galβ(1 → 4)Glc, p-nitrophenyl-β-D-lactopyranoside; Galβ(1 → 4)Glc, 4-O-β-D-galactopyranosyl-D-glucopyranose (lactose); oNo_2-phenylGal-NAc, o-nitrophenyl-N-acetyl-α-D-Galactosamine; 6-phosphate-Gal, D-Galactose-6-phosphate; GalNAc, N-acetyl-D-Galactosamine; Man, D(+)mannose; Glc, D(+)glucose; GlcNAc, N-acetyl-D-glucosamine; HGM, hog gastric mucin; BSM, bovine submaxillary mucin; EGTA, ethylene glycol bis-tetra acetic acid; EDTA, ethylene diamine tetra acetic acid; *L. polyphemus, Limlus polyphemus; L. rohita, labeo rohita; C. catla, Catla catla*; pneumococcal C polysaccharide (CPS).**

Introduction

C-reactive proteins (CRP) are highly conserved acute phase macromolecules and thought to play an important role in immunomodulation by virtue of their specificity for PC. CRPs have been characterized by their Ca^{+2} dependent ligand binding affinity for the PC moiety of pneumococcal C-polysaccharides (CPS). In spite of *in vitro* demonstration of their involvement in a variety of biological events, their normal physiological role is yet to be established. CRPs are normal serum constituents in a wide variety of species including *Limulus polyphemus* [1–5]. The increased presence of CRP in invertebrates and fishes suggests that they may well have an important role in host defense mechanism. In contrast, CRPs are present in trace amounts in almost all mammals in which they have been sought [1,6]. Their serum levels are elevated immediately after chemical trauma, intoxication, infection, injury and certain chemical stress mainly as a result of accelerated rates of transcription of respective genes in the liver [1,6], making CRPs probably the single most class of useful molecules for monitoring acute reactions.

The reactivity of human CRP with galactose containing polysaccharides including agarose [7], snail galactans, depyruvylated *Streptococcus pneumoniae* type-4 polysaccharides [8],

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C-carbohydrates and bacteria [9] had prompted the speculation that CRP has lectin like binding activity [10]. CRPs from *Limulus polyphemus* [2], eel [11] and *Achatina fulica* snail [3] have been found to behave as agglutinins, which could be inhibited by sialic acid, mannose and galactose respectively.

We have reported earlier that poisonous heavy metals like mercury and cadmium can induce CRP in the sera of fresh water fishes [4,5]. Different forms of CRPs has been purified to electrophoretic homogeneity from the sera of fishes confined in freshwater (CRP_N) and water polluted with nonlethal doses of cadmium (CRP_{Cd}) or mercury (CRP_{Hg}) [4,5]. It has been reported that CRP undergoes molecular transformation in response to metal pollution. To the best of our knowledge it has been shown for the first time that during the acute phase response distinct molecular variants of fish CRP appear in a specific time dependent manner and appear as a single entity [5]. The molecular variants appear due to differences in their glycan part [4,5]. This observation prompted us to investigate whether these molecular differences in CRPs also influence their lectin like property. Here we report experiments designed to characterize and compare the detail carbohydrate binding properties of CRP_N, CRP_{Cd} and CRP_{Hg}.

The aim of the present study is (i) to characterize different forms of CRPs induced by pollutants in the aquatic environment, (ii) to demonstrate the lectin like activity in normal and induced CRPs, (iii) to search for the differences in agglutination and the carbohydrate binding properties of the different forms of CRPs and (iv) to identify important characteristics of lectin–ligand interaction which might provide new information for understanding the physiological role of these acute phase proteins.

Materials and methods

Sephadex G-200 and Sepharose-4B were from Pharmacia Biotech Inc. p-nitrophenyl phosphorylcholine, PC chloride, all the sugars and glycoproteins were from Sigma. The Sepharose-PC affinity matrix was prepared as previously described [4]. Pneumococcal C-polysaccharide (CPS) was a kind gift from Prof. S. Mookerjea of Memorial University of New Foundland, Canada. All other chemicals were of analytical grade.

Animals and treatment

Labeo rohita fishes were maintained under standard light, temperature and food in an aquarium. One set was kept in fresh water and other sets were separately exposed to nonlethal doses of cadmium chloride ($CdCl_2$, 60 ppm) and mercuric chloride ($HgCl_2$, 0.11 ppm) polluted water for 24 h and 12 h respectively (peak point for their induction).

Purification of CRPs

The purification of CRPs from the sera of normal (CRP_N), $CdCl_2$ (CRP_{Cd}) and $HgCl_2$ (CRP_{Hg}) treated fishes was accomplished as follows: Fish serum (9 ml) following a 70% saturated ammonium sulfate precipitation was fractionated using Sephadex G-200 (80 × 1.5 cm) column pre-equilibrated with TBS (0.05 M Tris, 0.15 M NaCl, pH 7.8). Each peak was tested by agglutination using rabbit erythrocytes. Peak 2 (having highest agglutination titre) was applied to a Sepharose-PC affinity matrix (10 × 1.5 cm) in TBS buffer with 10 mM $CaCl_2$ (Buffer A). After extensive washing to remove nonspecific proteins, the bound protein was eluted using 0.1 M EDTA in TBS at 25–30°C and assayed for absorption at 280 nm. The specific fraction was dialyzed against TBS, pooled, concentrated and stored in aliquots at −20°C. The purity was also checked by HPLC using a gel filtration column.

Protein was estimated by the method of Lowry *et al*. [12] using crystalline bovine serum albumin as standard. Homogeneity of purified CRP was analyzed by native [13] and SDS-PAGE [14]. Gels were fixed and stained for protein with Coomassie Brilliant Blue R-250. Purified CRPs (20 µl, 200 µg/ml) were also tested against CPS (10 mg/ml) by precipitin reaction.

Immunological studies

Polyclonal rabbit antisera were prepared by injecting purified CRP_N (250 µg) emulsified in complete Freund's adjuvant (DIFCO), subcutaneously into New Zealand white female rabbit followed by two injections (350 µg) emulsified in incomplete Freund's adjuvant at an interval of 10 days. Blood was drawn after 7 days of last injection and the antisera were stored at −20°C. Immunological crossreactivity among different forms of CRP was studied using the following techniques:

(a) *Double immunodiffusion studies*—Immunodiffusion [15] was carried out with all CRPs (20 µl, 200 µg/ml) and antiserum.
(b) *ELISA*—Three CRPs in varying amounts (0.01–6 µg) were coated separately on to the plates. After washing and blocking steps, they were probed with polyclonal rabbit anti-CRP_N antisera and binding assayed by the horseradish peroxidase (HRP) conjugated goat anti-rabbit lgG (diluted 1 : 10,000) with a chromogenic substrate. The absorbance was read at 405 nm by a microtiter plate spectrophotometer (model Lab systems multiscan MS).
(c) *Western blot analysis*—Purified CRPs were electrophoresed on native PAGE, electroblotted onto nitrocellulose and incubated with anti-CRP_N (diluted 1 : 200) in TBS-tween 20 (pH 7.8). The antigen antibody complex was detected by HRP color development system.

Binding of $^{45}Ca^{+2}$ with CRP

Affinity labeling of CRP by $^{45}Ca^{+2}$—Purified CRP_N was electrophoresed on SDS-PAGE (12%) under reducing condition. After electro blotting, it was incubated with $^{45}Ca^{+2}$ (1.5×10^6 CPM/2 ml of TBS) at 25°C and autoradiographed [16].

Quantitative analysis of the binding of CRP_N with $^{45}Ca^{+2}$ was carried out using Sephadex G-25 (1 ml) packed in a 1 ml syringe as a spun column. Reaction mixture (0.01 ml, $^{45}Ca^{+2} + CRP_N$ in TBS) was loaded on top of the column equilibrated with TBS, centrifuged at 1200 rpm for 2 min placed inside a centrifuge tube, the eluted solution was collected in a microfuge tube and counted in scintillation fluid in a LKB counter. The spun column works as a miniature gel filtration column in which $^{45}Ca^{+2}$ bound to the protein is eluted leaving behind the free $^{45}Ca^{+2}$. Bound count (CPM) was plotted against $-\log$ of Ca^{+2} concentration (M). The concentration of Ca^{+2} at half-maximal binding was calculated from the graph.

Hemagglutination Assay

The erythrocytes from human healthy donors and other vertebrates were washed with 0.85% saline and 2% (v/v) suspension was used for all the experiments. Hemagglutination assay (HA) [17] was performed by the serial dilution technique in microtiter U plates (Tarson, India). The reciprocal of the highest dilution of the agglutinin that produced visible agglutination after 1 hr was taken as the titre (units) and the specific activity is defined as the units/mg of agglutinin.

The temperature sensitivity was determined by performing the HA at 4–75°C. The effect of pH on the stability of purified CRP_N was studied by performing HA at 25°C in presence of Ca^{+2}. Initially, CRP was incubated separately in the following buffers made in saline: 0.05 M glycine-HCl (pH 3.5); 0.01 M sodium acetate/acetic acid (pH 3.7–5.5); 0.01 M sodium cacodylate/HCl (pH 6.0–7.0); 0.01 M phosphate buffer (pH 7.2–7.4); 0.01M Tris/HCl (pH 7.5–8.8), 0.01M glycine/NaOH (pH 9.0–10.6) overnight at 4°C.

The HA was also studied by incubating CRP_N (5μg in 20μl) with urea (6 M) and guanidine (2–6M), 2-β-mercaptoethanol (0.2 M), NaOH (0.1 N) and HCl (0.1 N) separately at 4–8°C overnight. After incubation the protein was dialyzed against Buffer A and assayed. Similar concentrations of denaturing agents were used separately as controls.

The effect of varying $CaCl_2$ concentrations (0–25 mM) on HA was studied at 25°C (pH 7.8). HA was also scored in the presence of chelating agents like EDTA and EGTA.

HA with enzyme treated erythrocytes

Erythrocytes from human A, B, O and rabbit (0.1 ml) were washed in 0.02 M PB, pH 7.4 and incubated separately with *Clostridium perfringens* neuraminidase (type VI, 1.8 U/mg of protein) or bovine trypsin (0.25 mg/ml) at 37°C for 1 hr [18]. The enzyme treated cell suspensions were washed and used for HA in presence of Ca^{+2}.

Inhibition of HA by sugars

Aliquots of various sugars (25 μl) were serially diluted with 25 μl of saline in microtiter plates. CRP_N, CRP_{Cd} and CRP_{Hg} (25 μl with 10 mM Ca^{+2}, 16 HA units were added to each well separately and incubated for 30 min at 25°C. Rabbit erythrocytes (25 μl) were added and examined after 1 hr. Results were expressed as I_{50} value, the concentration (mM) of the inhibitor required for 50% inhibition of 16 agglutination units [17]. For comparison of inhibitory potencies of different sugars, relative inhibitory factors (RIF_{ref}) were calculated according to the following relations: $RIF_{ref} = (I_{50}$ of the reference sugar/I_{50} of the sample sugar). As galactose has been used as the reference sugar the factor is RIF_{gal}. If the value of RIF_{gal} of a sugar is greater than 1.0, it is a stronger inhibitor than galactose by that factor but if RIF_{gal} is less than 1.0 the sugar is a weaker inhibitor than galactose.

Determination of binding constants by fluorescence spectroscopy

Fluorescence spectra of CRPs (70 μg/ml in TBS) were obtained in a spectrofluorimeter (Hitachi model F-4010) with 10 nm slit width at 25°C using 1 ml quartz cuvette of path length 1 cm. Excitation was at 280 nm and emission spectra were recorded in the range of 300–400 nm. Titrations were carried out by successive addition of concentrated solution of the ligands to the protein solution in presence of Ca^{+2}(10 mM) followed by mixing and recording. Control titrations of protein with buffer A alone were also done. The values of corrected quenching (Q_c) were obtained from the relative intensities at 338 nm of sample and control titrations. The maximum extent of quenching (Q_{max}) was obtained from the extrapolation of the double reciprocal plot ($1/Q_c$ versus $1/[L]_T$), $[L]_T$ being the total concentration of ligand [19]. The fraction of sites occupied (α) was calculated at each titration point. The binding constants were obtained from the intercept of the plot of $\log [\alpha/(1-\alpha)]$ *versus* $\log [L]_T$ according to the following relation [19]: $\log [\alpha/(1-\alpha)] = \log [L]_T + \log K_a$. The free energy change of binding for each ligand was calculated by using the equation: $\Delta G = -RT\ln K_a$, where K_a is the association constant of the protein-ligand binding; $R = 2 \times 4.18$ kJ and T is the absolute temperature.

Competitive inhibition of phosphorylcholine binding with sugars

All three CRPs (8 μg each) were iodinated separately with $Na^{125}I$ using chloramine T method [20]. Aliquots of equal volume (20 μl) of Sepharose-PC matrix were incubated overnight with ^{125}I CRPs (8.5×10^4 CPM) in buffer A at 4°C. The supernatant was removed and matrix washed extensively with buffer A till constant readings. The slurry,

[^{125}I]-CRP bound Sepharose-PC, in buffer A was divided into equal aliquots. To each of these aliquots increasing concentrations (0–50 mM) of different sugars and PC were added, incubated at 25°C for 4 hr with stirring, centrifuged and washed thoroughly with buffer A containing 1% BSA to remove unbound material. Pellets were counted in a gamma counter (Electronix Corporation of India, Ltd.). Experiments were done in quadruplet. Pellet count with sugar was taken as 100% binding. Percentages of [^{125}I]-CRP bound were plotted against sugar concentration for the calculation of half-maximal binding.

Analysis of secondary structures by circular dichroism measurements

Circular dichroic spectra of CRPs (0.4 mg/ml in 20 mM TBS with 10 mM Ca^{+2}) in the presence and absence PC were recorded over a range 200–300 nm in a JASCO J720 recording spectropolarimeter at 25°C with a 3 ml cuvette of path length 1.0 cm. Average of 5 scans were used for the calculation of mean residue ellipticity [θ] in degree cm^2 dmol^{-1} taking 110 as the mean residue molecular weight. The percentage of α-helix, β-sheet and random coil were calculated by using a computer program of curve fitting based on minimum standard deviation developed in our laboratory [21].

Results

Purification of *L. rohita CRPs*

CRPs from the sera of fishes confined in fresh and polluted water were purified by combination of ammonium sulphate precipitation, gel filtration (Fig. 1A) and affinity chromatography of peak 2 using PC-Sepharose affinity matrix. Pure protein eluted as a single peak by 0.1 M EDTA (Fig. 1B), which led to 150 fold purification (Table 1). The pure CRP appeared as a single peak in HPLC (Fig. 1C). Native gel electrophoresis showed a single band (Fig. 2) as shown in lane 4. Lanes 1, 2 and 3 contained crude serum, ammonium sulphate precipitate and peak 2 of Figure 1A from gel filtration respectively. Strong precipitin band with CPS (center well, Fig. 3A) confirmed the C-reactive nature of purified CRP (well 1) and precipitin lines produced by CRPs from *L. polyphemus* (well 2) confirming the quality of CPS. Well 3 (rat CRP, negative control) and well 4 (buffer control) showed no band (Fig. 3A).

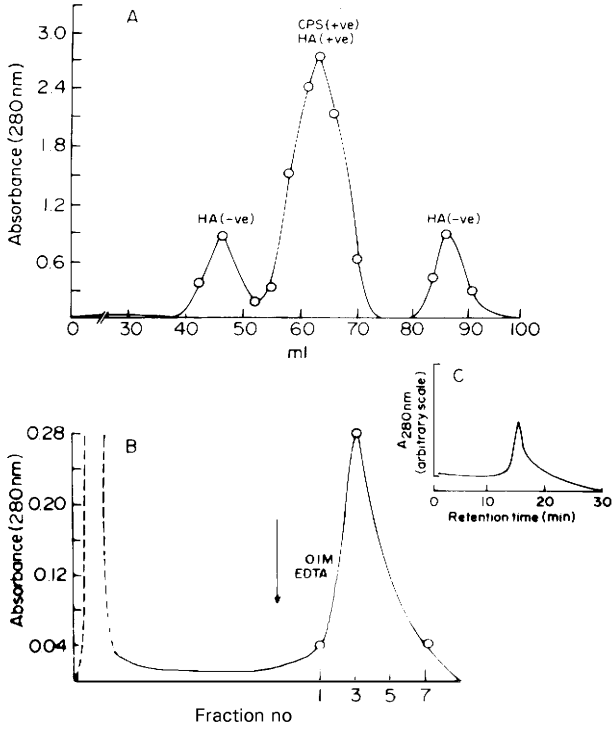

Figure 1. Purification of CRP from the sera of *Labeo. rohita*. Panel A. Elution profile on a Sephadex G-200 column (80 × 1.5 Cm): Ammonium sulphate (70%) precipitated serum was fractionated. Each fraction was monitored at 280 nm and by hemagglutination assay. Panel B. Elution profile on a Sepharose-PC affinity column: The CRP was eluted from the affinity column by 0.1 M EDTA in TBS and analyzed at 280 nm. Panel C. HPLC profile of affinity purified CRP_N: Absorbance was monitored at 280 nm with arbitrary units (AUFS, absorbance units full scale).

Immunodiffusion patterns with polyclonal antibodies

Affinity purified *L. rohita* CRP_N gave a characteristic precipitin line (Fig. 3B) with antisera against normal *L. rohita* CRP (anti-CRP_N). Purified CRP (well 1 and 3) and normal crude fish serum (well 2) showed continuous precipitin lines. No precipitin band was obtained with pre-immune rabbit

Table 1. Purification of galactose binding lectin from serum of *L. rohita*

Steps of purification	*Total volume (ml)*	*Total protein (mg)*	*HA titer (Units/ml)*	*Specific activity (Units/mg)*	*Prification fold*
Crude serum	9	900	4608	5.12	1
Saturated ammonium sulphate (70%) fraction	4.5	180	10240	56.80	11
Sephadex G-200 fraction (Peak 2)	20	90	10240	113.70	22
Eluate of PC-Sepharose	1	0.08	96	768	150

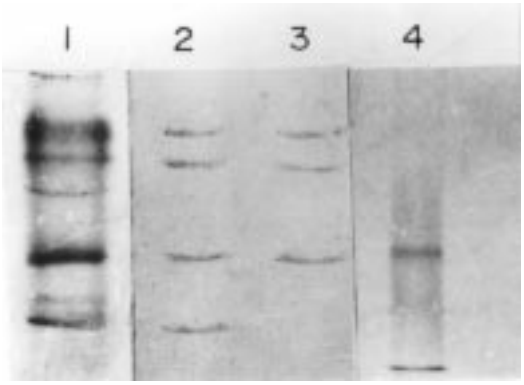

Figure 2. Native PAGE (7.5%) analysis: Lanes 1, 2, 3 & 4 contained crude *L. rohita* serum, 70% ammonium sulphate precipitate, peak 2 from Sephadex G200 column and affinity purified CRP_N respectively.

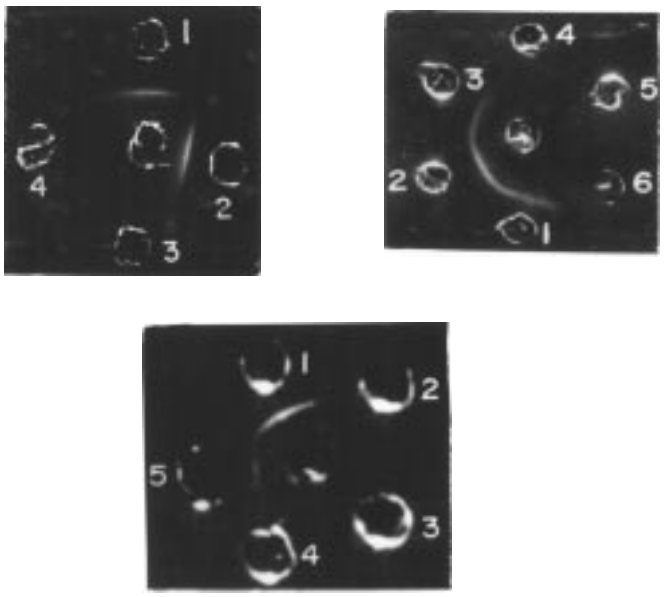

Figure 3. Precipitin reactions. Panel A. Reactivity of CRP with pneumococcal C polysaccharide (CPS): Centre well, 10 µl of CPS (10 mg/ml); wells 1, 2, 3, 4 contained *L. rohita* CRP_N (4 µg); *L. polyphemus* CRP (10 µg); rat CRP (10 µg) and buffer control respectively. **Panel B. Immunodiffusion patterns:** Centre well, anti-serum to CRP_N; wells 1, 2, 3, 4, 5 and 6 contained CRP_N (4 µg); normal crude serum (10 µg); CRP_N (2 µg); CRP depleted serum (10 µg); preimmune rabbit serum and buffer control respectively. **Panel C. Immunological crossreactivity with other CRPs:** Centre well, antiserum to CRP_N; well 1, 2, 3, 4 and 5 contain *L. rohita* CRP_N (4 µg); rat CRP (10 µg); rabbit (10 µg); *L. polyphemus* CRP (10 µg) and *C. catla* CRP (6 µg) respectively.

serum (well 4), carrier buffer (well 5) and CRP depleted *L. rohita* serum (well 6). The anti-CRP_N antibody (center well) gave precipitin bands with *L. rohita* CRP_N (well 1) and another major carp *C. catla* (well 5) but failed to react with rat

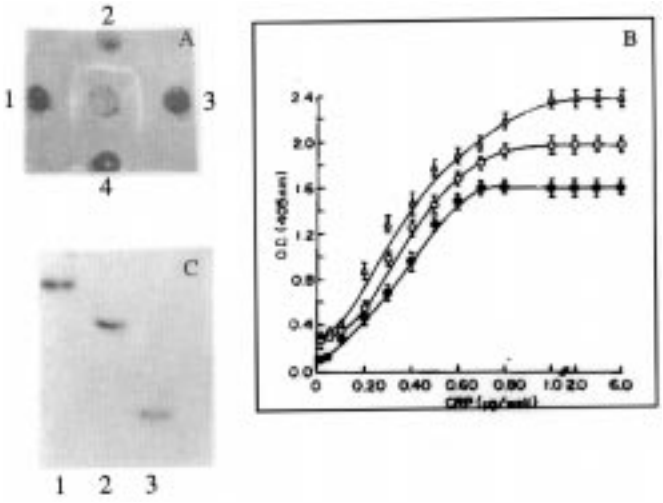

Figure 4. Immunological crossreactivity of normal and induced CRPs Panel A. Immunodiffusion: Centre well, antiserum to CRP_N; well 1, 2, 3 and 4 contain equal amount (4 µg) of CRP_N, CRP_{Cd} CRP_{Hg} and buffer respectively. Panel B. ELISA: Quantities of CRP_N(●–●), CRP_{Hg} (○–○) and CRP_{Cd} (△–△) are plotted against absorbance at 405 nm using HRP conjugated goat anti-rabbit immunoglobulin (1 : 5000). Error bars are mean ± SD. Panel C. Western blot analysis: Equal amount (12 µg) of CRP_{Cd} (lane 1), CRP_{Hg} (lane 2) and CRP_N (lane 3) were blotted onto nitrocellulose after native PAGE analysis. The primary antibody (anti-CRP_N, 1 : 200) and HRP conjugated goat anti-rabbit IgG (1 : 2,000) were used as secondary antibody.

(well 2), rabbit (well 3) and *L. polyphemus* (well 4) CRPs (Fig. 3C).

Immunological relationship among normal and induced CRPs

Purified CRP_N, CRP_{Cd} and CRP_{Hg} produced single continuous precipitin lines against anti-CRP_N antibody (Fig. 4A, wells 1, 2, 3 respectively). A quantitative difference in the reactivity of the antibody towards the three forms of CRP (10 µg each) was evident from ELISA (Fig. 4B). The intensity of colour at saturation levels of CRP_N(●–●), CRP_{Cd} (△–△), and CRP_{Hg} (○–○) were in the ratio 1.00 : 1.50 : 1.25 (Fig. 4B). It may be noted that although the antibody was raised against CRP_N color produced was more in CRP_{Cd} and CRP_{Hg} than CRP_N. Western blot analysis showed the crossreactivity among the different forms of CRP and their differences in electrophoretic mobility as shown in Figure 4C.

L. rohita CRP binds to Ca^{+2}

Titration of CRP_N with $^{45}Ca^{+2}$ was carried out by incubating CRP_N with $^{45}Ca^{+2}$ followed by the separation of bound $^{45}Ca^{+2}$ using spun column (Fig. 5A). The half-maximal binding occurred at 2.85 mM Ca^{+2}. Other CRPs also showed similar binding (data not shown). Specific binding of $^{45}Ca^{+2}$ to CRP_N was also demonstrated by affinity labelling on SDS-PAGE followed by autoradiography (Fig. 5B). It showed a

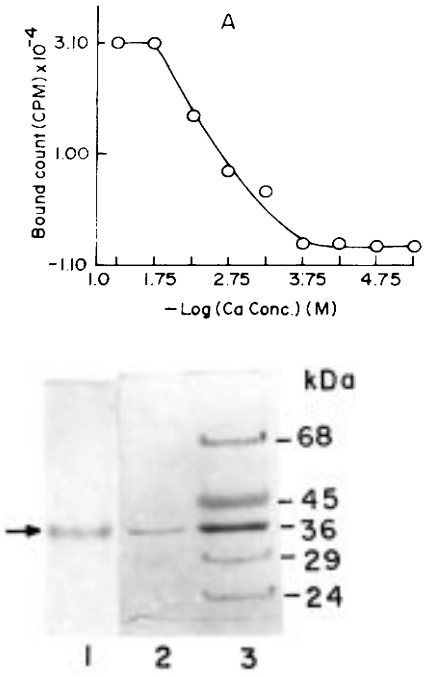

Figure 5. Binding of CRP_N with Ca^{+2}. Panel A. Titration curve of CRP_N with ^{45}Ca: Bound count (CPM) was plotted against − log of Ca^{+2} concentration (M) as described in Material and methods. The concentration of Ca^{+2} at half-maximal binding was calculated from the graph. Panel B. Affinity labeling of CRP by $^{45}Ca^{+2}$ on nitrocellulose blot. Purified CRP_N (8 μg) was transferred on nitrocellulose after SDS-PAGE analysis, blotted with ^{45}Ca and autoradiographed (lane 1). Lanes 2 & 3 were CRP_N (8 μg) and standard SDS-PAGE markers respectively stained with coomassie blue.

single band (Lane 1) which corresponds to a subunit of CRP_N (MW 33 kDa, Lane 2).

Agglutination patterns of CRPs

Agglutination titres of CRPs were different for different erythrocytes (Table 2). HA titres with the erythrocytes of the cattle group, namely, horse, cow and goat were the same as that from rabbit but relatively higher than that of dog, cat and sheep. High titres were also obtained with chicken and human blood group B; other human blood groups showed a little less HA values. CRPs exhibited very low agglutination with rat, mouse and guineapig erythrocytes. No agglutination was observed with hamster erythrocytes. In general, HA titres for most erythrocytes were relatively higher with CRP_{Cd} and CRP_{Hg} as compared to CRP_N.

In general, treatment of erythrocytes with either neuraminidase or trypsin increased HA titres. This may be due to cleavage of terminal sialyl residues or trimming of some peptides. CRP_N mediated agglutination increased linearly in the pH range between 4 and 7 and remained unchanged up to

Table 2. Agglutination profiles of purified CRP_s (80 μg/ml) in presence of Ca^{+2} (10 mM) at pH 7.8.

Erythrocytes	*CRP_N*	*CRP_{Cd}*	*$CRP2_{Hg}$*
Horse	128	512	512
Cow	128	512	512
Goat	128	512	512
Rabbit	128	512	512
Chicken	128	128	128
Human B	128	128	128
Human O	64	64	64
Human A	64	64	64
Dog	64	64	64
Cat	64	64	64
Sheep	32	64	64
Rat	4	16	16
Mouse	2	4	4
Guinea pig	2	4	4
Hamster	NA	NA	NA

NA = no activity.

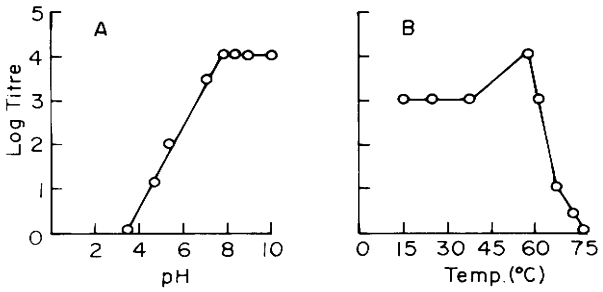

Figure 6. Effect of pH and temperature on HA activity. Agglutination of CRPs (160 μg/ml each) with 2% rabbit erythrocytes suspension was scored in the presence of 0.01 M Ca^{+2} after incubation for 30 min. Logarithm of HA units were plotted against pH (when CRPs were titrated in the different buffers as described in material and methods at 25°C, Fig 6A)/and temperature (when agglutination was scored at different temperature at pH 8.0, Fig. 6B).

pH 10 (Fig. 6A). HA titres remained unaltered up to 37°C then increased up to 56°C followed by a sharp decrease around 75°C (Fig. 6B). Similar patterns were also obtained with CRP_{Cd} and CRP_{Hg}.

All three CRPs required Ca^{+2} (10 mM) for their agglutinating activity; addition of 8.5 mM EDTA and EGTA reversed agglutination and the activity was restored with the addition of 10 mM Ca^{+2}. There was a significant reduction in HA titre (from 128 to 4) in the presence of 6M guanidine-HCl or urea. No HA was observed with 0.1 M NaOH or HCl, but 2-mercaptoethanol had no effect on HA titre.

Carbohydrate Specificity of CRP_N

The inhibitory potencies (I_{50} value) along with relative inhibitory factors (RIF_{gal}), which are directly proportional to the relative inhibitory potency of the inhibitor with reference to D-Gal, have been arranged in order of decreasing RIF_{gal} values from top to bottom for CRP_N mediated agglutination (Table 3). The most potent inhibitor was found to be the disaccharide, Gal $\beta(1 \rightarrow 1)$Gal, with $I_{50} = 0.29$ mM and a RIF_{gal} of 1.9 indicating that it is twice as potent as Gal. Among the disaccharides, the inhibitory potency decreased in the order of melibiose (0.36 mM) > lactosan (0.59 mM) > lactose (1.2 mM) = pNO_2-phenyl β-D-lactopyranoside (1.2 mM). Melibiose is 1.5 times more potent inhibitor than Gal and lactose is half as potent as Gal. Glucose, mannose and arabinose showed no inhibition. Asialofetuin is a more potent inhibitor (3.5 μg) than HGM (31 μg). Fetuin and BSM were completely inactive.

Galactose is a better inhibitor for metals induced CRP mediated agglutination

In general, inhibitory patterns of different sugars of CRP_{Cd} and CRP_{Hg} mediated HA are very similar, but there are quantitative differences with those of CRP_N (Table 3). HA mediated by both these induced CRPs are most strongly inhibited by o-NO_2-phenyl β-Gal ($I_{50} = 0.08$ mM). D-Gal also inhibits CRP_{Cd} and CRP_{Hg} mediated HA ($I_{50} = 0.13$ mM) but the inhibition potency is about 4 fold higher than that of CRP_N mediated HA ($I_{50} = 0.54$ mM). RIF_{gal} values of sugars below D-Gal are less than 1.0, indicating that they are weaker inhibitors of CRP_{Cd} and CRP_{Hg} mediated agglutination. It may be noted that some sugars above D-Gal are also weaker inhibitors than D-Gal. Interestingly, the RIF_{gal} values of CRP_{Cd} and CRP_{Hg} mediated HA decrease up to 0.05 (i.e. 20 times weaker than Gal) for D-Gal-6-phosphate and stachyose while that for CRP_N mediated RIF_{gal} decrease only up to 0.15 (i.e. ~6 times weaker than D-Gal) for Gal NAc.

Binding constant measurements indicate nearly equal affinity for PC and sugars

None of the three CRPs showed any appreciable fluorescence quenching by titrating with Ca^{+2} alone (up to 50 mM). There was also no change in the intrinsic protein fluorescence when CRPs were titrated with mono- and disaccharides in absence of Ca^{+2}, indicating that its presence was a necessary condition for the interaction of CRPs with the sugars. Figure 7 shows the representative plot of titration parameters leading to the determination of Q_{max}, apparent association constants (K_a)

Table 3. Inhibition of purified CRP_s hemagglutination by mono- and oligosaccharides

	CRP_N		CRP_{Cd}		CRP_{Hg}	
Inhibitors	I_{50} (mM)[a]	RIF_{gal}[b]	I_{50} (mM)[a]	RIF_{gal}[b]	I_{50} (mM)[a]	RIF_{gal}[b]
Gal$\beta(1 \rightarrow 1)$Gal	0.29	1.9	0.29	0.45	0.29	0.45
o-NO_2-phenyl β-Gal	0.32	1.7	0.08	1.6	0.08	1.6
4NH_2phenylβ-Gal	0.36	1.5	0.18	0.72	0.18	0.72
Gal$\alpha(1 \rightarrow 6)$Glc	0.36	1.5	0.09	1.4	0.09	1.4
Phenyl β-D Gal	0.38	1.4	0.19	0.68	0.19	0.68
6 thio-Gal	0.46	1.2	0.46	0.28	0.46	0.28
Gal$\beta(1 \rightarrow 1)$S-Gal	0.46	1.2	0.46	0.28	0.46	0.28
2-O-CH_3-Gal	0.50	1.1	0.12	1.08	0.12	1.1
3-O-CH_3-Gal	0.50	1.1	0.25	0.52	1.00	0.13
α-D Gal	0.54	1.0	0.13	1.00	0.13	1.00
α-D-fuc	0.59	0.92	0.28	0.46	0.28	0.46
2-deoxy-D Gal	0.59	0.92	0.15	0.87	0.29	0.45
Lactosan	0.59	0.92	0.59	0.22	0.59	0.22
p-NO_2phenyl-Gal$\beta(1 \rightarrow 4)$Glc	1.2	0.46	1.2	0.11	1.2	0.11
D-Xylose	1.2	0.46	0.59	0.22	0.59	0.22
Gal$\beta(1 \rightarrow 4)$Glc	1.2	0.46	1.2	0.11	1.2	0.11
1,4 D galactonolactone	2.2	0.25	2.2	0.06	2.2	0.06
o-NO_2-phenylGalNAc	2.3	0.23	2.3	0.06	2.3	0.06
6 phosphate-D-Gal	2.5	0.22	2.5	0.05	2.5	0.05
Stachyose	2.5	0.22	2.5	0.05	2.5	0.05
Gal NAc	3.5	0.15	1.8	0.07	1.8	0.07

[a]Figures are minimal inhibitor concentrations required for 50% inhibition of 16 agglutination units.
[b]RIF_{gal} = Relative Inhibitory Factor with respect to α-D Galactose.
Among the glycoproteins asialofetuin (3.5 μg/ml) and HGM (31 μg/ml) showed the same inhibitory activity with all the three CRPs. D-Glc, D GlcNAc, D-man, D-arabinose, fetuin and BSM did not inhibit upto 150 mg/ml.

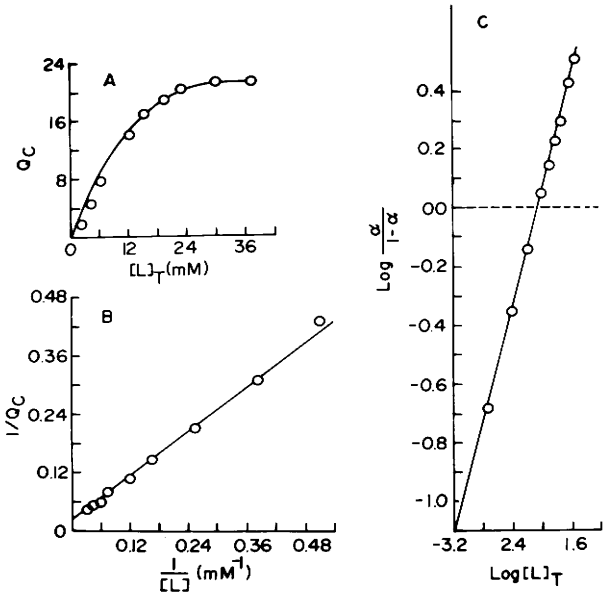

Figure 7. Representative plots for the determination of association constant (K_a) of ligands with CRPs. Fluorescence spectra were recorded as described in materials and methods. Panel A. Plot of percentage quenching (Q_c) Vs ligand concentration [L_T]: Solutions of β-D-galactose were added to 0.6 ml of CRP_N (70 µg/ml) containing 0.01 M Ca^{+2} (Error bars are mean ± SD of triplicate readings). Panel B. Plot of $1/Q_c$ versus $1/[L_T]$: Q_{max} was calculated by extrapolation of the double reciprocal plot. Panel C. Plot of log [$\alpha/(1-\alpha)$] versus log [L_T]: Association constant (K_a) was determined from the intercept of the plot.

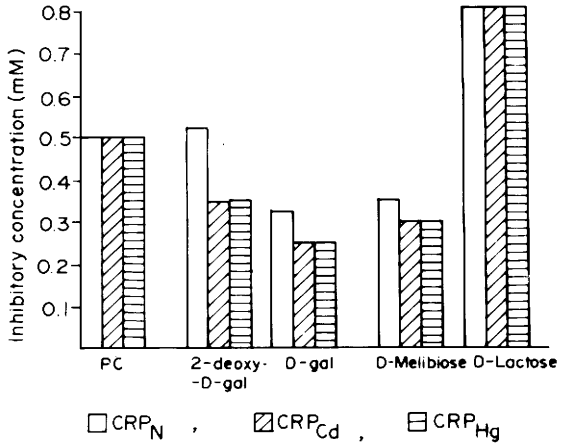

Figure 8. Competitive inhibition of binding of ^{125}I-CRPs to PC-Sepharose matrix by sugars and soluble PC chloride. The inhibitory concentrations of 2-deoxy-D-galacose, D-galactose, D-melibiose, lactose and soluble PC chloride are plotted. Error bars are mean + SD of quadruplet readings.

and free energies (ΔG) of sugar binding. Apparent association constant (K_a) of CRP_N for binding to PC, D-gal and D-meliobiose were 111, 112 and 71 M^{-1} which correspond to dissociation constants of 9.0, 8.9 and 14 millimolar respectively and their respective $-\Delta G$ values were 11.8, 11.7 and 10.6 KJ/mol. The values of K_a and ΔG indicate that the binding with PC and sugars are, in general, weak in nature. Binding of CRP_N with the D-galactose is slightly stronger than with the di-saccharide, D-melibiose, but almost equally strong as PC. Binding affinities are almost the same for all three forms of CRP.

Sugars compete for PC binding site of CRPs

The concentration of a sugar at which the CRP binding was reduced to half (50%) of its maximum value (in absence of the inhibitor) was used as the competitive index (IC_{50} value). IC_{50} values have been plotted in Figure 8 for five inhibitors. The inhibitory concentration for soluble PC chloride is the same (0.50 mM) for all three CRPs. D-Gal is the best inhibitor among the tested sugars ($IC_{50} = 0.25$ mM for CRP_{Cd} and CRP_{Hg} and 0.32 mM for CRP_N). Inhibitory concentrations increase in the order D-gal < D-melibiose < 2-deoxy-D-gal ≪ lactose (Fig. 8).

Variation of secondary structure of CRPs by PC-binding

The analysis of the secondary structures of CRP_N, CRP_{Cd} and CRP_{Hg} by CD spectrometry revealed that α-helix contents are 19.4, 34.9 and 26.9% respectively and β-sheet structures are 19.9, 45.9 and 37.0% respectively in presence of Ca^{+2} (10 mM) alone. In presence of PC chloride (40 mM) and Ca^{+2} (10 mM), CRP_N, CRP_{Cd} and CRP_{Hg} were found to have 19.7, 40.2 and 41.0% α-helix and 31.4, 39.9 and 46.3% β-sheet structures respectively. It may be noted that there are significant changes in secondary structures after PC binding.

Discussion

Different forms of CRPs induced by metals pollutants have been purified to electrophoretic homogeneity using the same PC affinity matrix based on their common property of Ca^{+2}-dependent PC-binding. They exhibited striking differences in their electrophoretic mobility amongst themselves as shown in Native PAGE-Western blot (Fig. 4C) probably due to variation in the carbohydrate moieties [4]. This mobility difference may also arise due to difference in their charged residues namely sialic acid. However, no microheterogeneous change of human CRP was found during various diseases [22].

Immunochemical identity of CRPs from *L. rohita* and *C. catla* indicate antigenic similarity between CRPs from two related fishes suggesting close relations between them but not with CRPs from mammals and lower organisms. CRP_{Cd} and CRP_{Hg} show immunological cross reactivity with CRP_N probably due to the presence of common epitopes in spite of major differences amongst these forms mainly in their glycan moieties [4]. The immunological cross reactivity also implies sequence homology. However, a quantitative difference in the reactivity of the antibody towards the three forms of CRP is

evident from the color intensity at saturation levels (Fig. 4B). Although the polyclonal antibodies detected common epitopes, our findings suggest that there are pollutant specific distinct complimentary surfaces, which can be resolved by developing monoclonal antibodies. This may well pave the way for the development of immunological tools for the detection of CRPs in different heterogeneous forms induced by various toxic pollutants and may be exploited for the differential detection of toxic pollutants in the aquatic environment.

The major finding of this investigation is the demonstration of variable agglutination properties among the induced and normal forms of the *L. rohita* CRP. Induced forms (CRP_{Cd} and CRP_{Hg}) are better agglutinins than normal form (CRP_N) for a number of erythrocytes (Table 2). These findings strongly support our novel observation that the induced CRPs differ considerably from the normal form. Their behaviour like other lectins has been clearly demonstrated by their specificity towards galactosyl groups on blood group antigens. Higher HA titre with blood group B [128] as compared to blood group A [64] may be due to the presence of α D-Gal as terminal nonreducing sugar [23] in Group B and α D-GalNAc in Group A, which is ∼6 times weaker than D-Gal (Table 3). Due to the absence of these terminal sugars on blood group O, HA titre is low [64]. Multiple carbohydrate specificity of these CRPs against ubiquitous blood group substances may be important for innate defensive role against bacteria having these determinants.

Agglutination by all three CRPs were found to be sensitive to temperature, pH and denaturing agents implying that the correct conformations of CRPs were essential for their HA activity. HA activity were greatly effected due to loss of their structural integrity at high temperatures (Fig. 6B), deviation from the native conformation due to protonation of certain residues to produce unfavourable charge–charge interaction at extreme pH (Fig. 6A) and loss of native structures by denaturing agents. All three CRPs, like many invertebrate lectins [2,3,11], required the presence of Ca^{+2}, which is essential for maintaining the active form.

Detailed analysis of carbohydrate specificity of inhibition of agglutination mediated by different forms of the *L. rohita* CRPs supports our observation that there are significant differences between the normal and induced CRPs. Inhibition of agglutination mediated by CRP_N indicates that galactose is a strong inhibitor. C1 position provides an important binding locus and substitution at C1 with another galactose residue as in Gal$\beta(1 \rightarrow 1)$Gal and with a nitrophenyl group as in o-NO_2-phenyl-β-D-Gal increase the binding affinity (∼2 folds) than Gal, probably due to additional interactions like hydrogen bonding and stacking. The α- or β-configurations of the glycosidic linkage seems to be equally acceptable as the inhibitory potencies of Gal$\beta(1 \rightarrow 1)$Gal and Gal$\alpha(1 \rightarrow 6)$Glc are almost equal as long as the linkage to the second residue is $1 \rightarrow 1$ or $1 \rightarrow 6$ but steric hindrance arises if the linkage is $1 \rightarrow 4$.

Small changes at the C2, C3 and C4 positions do not affect the binding affinity to a considerable extent. However, introduction of a bulky group e.g. $NHCOCH_3$, as in GalNAc, greatly destabilize the binding affinity resulting in 6.5 fold reduced inhibitory potency. D-arabinose is a non-inhibitor, in which hydroxyls at C2, C3 and C4 do not have the same configuration as in D-galactose suggesting that proper configurations at these asymmetric carbon atoms are important for lectin like interactions.

Substitution at C6 position by methyl (as in α D-Fuc) or a thio (as in 6-thio-Gal) group showed almost equal degree of inhibition as D-Gal. However, the replacement of thio group by a charged phosphate group, as in D-Gal-6-phosphate, the affinity is markedly reduced (∼5 folds), suggesting a charge impediment. Complete removal of CH_2OH group from D-Gal, as in D-xylose, decreases the binding activity by about 2 folds. Involvement of C6 hydroxyl group is very clearly seen in case of lactosan where the C6 hydroxyl group in one Gal unit is engaged in a galactoside linkage with C1-OH group of the other making only one Gal available for binding as reflected by the reduction of its potency to 50% of the Gal$\beta(1 \rightarrow 1)$Gal. When the ring size is decreased from pyranose to furanose, as in galactonolactone, it becomes 4 fold less potent than galactose, suggesting the importance of the pyranose configuration.

Specificity of CRPs towards α D-Gal is further corroborated by strong inhibition with desialylated fetuin due to the exposure of the most abundant subterminal α D-Gal residues after the removal of terminal sialic acid. Weak inhibition with HGM containing abundant α linked D-GalNAc [23] as compared to desialylated fetuin containing exposed D-Gal may be explained by the preference of D-Gal over D-GalNAc. In native fetuin, negatively charged terminal sialic acid residue may be unfavourable for binding to *L. rohita* CRPs.

Comparative analysis of the carbohydrate interaction of all the three CRPs indicate that in the process of transformation of CRP_N to CRP_{Cd} and CRP_{Hg} under the influence of the toxic metals, both the affinity and specificity of binding are enhanced. The inhibitory potency of D-gal is 4 fold higher in case of CRP_{Cd} and CRP_{Hg} mediated HA as compared to that mediated by CRP_N (Table 3), which is due to increased affinity of the induced CRPs for galactose. Although there is no clear correlation between the affinities of the different derivatives it may be emphasized that the relative order of affinity is very close for the toxic induced forms among themselves. In comparison with CRP_N, o-NO_2-phenyl-Gal remained the strongest inhibitor but the potency of Gal $\beta(1 \rightarrow 1)$ Gal reduced drastically, falling even below that of galactose in case of induced CRPs. Most strikingly, the increase in specificity of the induced CRPs is reflected in the drastic reduction of potency of the weak inhibitors to as low as 15–20 folds. It has been proposed earlier that inflammatory conditions favour the binding of CRP to cell-surface structures and in this immobilized state the lectin-like properties of CRP

be manifested [24]. This could be a possible mechanism for the strictly specific activation of the lectin function of CRP.

Dissociation constant of the CRP_N-PC interaction (9 mM) is of the same order as that of D-Gal (8.9 mM) and D-melibiose (14 mM) indicating weak interactions with both PC and sugars with comparable affinity. Results of inhibition of ^{125}I-labelled CRPs bound to PC-Sepharose reveal that the sugars inhibit the PC-binding with comparable potency as the soluble PC (Fig.-8) indicating that sugar-binding sites are the same as PC-binding sites. Variations in the α and β conformations upon binding to PC show the effect of such interactions on their secondary structures, which might influence their carbohydrate binding properties. Binding affinities of the three forms of the CRP do not differ significantly but from the inhibition studies with four simple sugars there are slight differences (Fig 8). However, sugar specificity become prominent in the HA inhibition experiments (Table 3) probably because of multiple interactions in case of agglutination.

Our results demonstrate that the *L rohita* CRPs behave as galactose binding lectins. Similar lectin properties were observed in eel CRP that is mannose binding [11] and horseshoe crab CRP that is sialic acid binding [2]. However, the function of human CRP as lectin has been questioned by Soelter and Uhlenbruck showing that its interaction with snail galactans was not due to carbohydrate binding but for binding to phosphate groups that are minor constituents of these polysaccharides [25]; positively charged Lys-57, Arg-58 residues were reported to contribute to the structure of PC-binding site of human CRP [26]. Our findings with *L rohita* CRPs indicate that the presence of a negatively charged group like phosphate or sialic acid hinders sugar binding suggesting some innate structural difference with human CRP.

In conclusion, our results indicate that structural variations observed in *L. rohita* CRP due to exposure to metal pollutants contribute towards the differential sugar binding properties. Fine carbohydrate binding specificity might be a way by which the immune recognition capability is enhanced to reinforce their immune response to toxic pollutants, probably because of delayed specific immune response. Thus, understanding the role of CRPs as agglutinins might provide new insight into their biological role in combating metal toxicity.

Acknowledgements

S. Sinha was a senior Research Fellow of Indian Council of Medical Research, India. We express our thanks to Prof. Sailen Mookerjea of Memorial University of New Foundland, Canada for providing Pneumococcal C-polysaccharide (CPS).

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Received 26 June 1999; revised 21 February 2000; accepted 1 March 2000